The primary cilium as a regulator of cellular senescence in human fibroblasts

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DECLARATION

I, Loretta Breslin, certify that this thesis is my own work and I have not obtained a degree in this university or elsewhere based on any of this work. Figure 3.5A and Figure 3.8, were performed in collaboration with Dr. Suzanna Prosser, which is clearly indicated in the figure legends.

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ABBREVIATIONS

53BP1	p53 binding protein 1
ACTB	Actin B
ACTR	Actin-related protein
AKT1	v-akt murine thymoma viral oncogene homolog 1
ALMS1	Alström Syndrome 1
ALNP	Astrocyte-like neural precursor
APS	Ammonium persulphate
Arl13B	ADP-ribosylation factor-like 13B
ATM	Ataxia Telangiectasia, mutated
ATR	ATM-Rad3 related
ATRX	Alpha Thalassemia/Mental Retardation Syndrome X-Linked
ATRIP	ATR-interacting protein
BB	Basal body
BBS	Bardet-Biedl syndrome
BCC	Basal cell carcinoma
BLAST	Basic local alignment search tool
BOC	Brother of Commodo
Bcl-2	B-cell lymphoma 2
BRAF	v-raf murine sarcoma viral oncogene homolog B
BRCA1	Breast cancer associated gene1
BRCA2	Breast cancer associated gene 2
BTRC	β-transducin repeat containing
bp	Base pair(s)
DABCO	1,4-diazabicyclo[2.2.2]octane
DEPC	Diethyl pyrocarbonate
Dvl	Dishevelled
CCDC	Coiled-coil domain containing
CCND1	Cyclin D1
CDC	Cell division cycle
CDK	Cyclin-dependent kinase
CDK5RAP2	CDK5 regulatory subunit associated protein 2
CDKN1A	Cyclin-dependent kinase inhibitor 1A, or p21
cDNA	Complementary DNA
CDO	Commodo
C/EBP-β	CCAAT/enhancer-binding protein β
CENP	Centromere protein
CEP	Centrosomal protein
Chk	Checkpoint kinase
CHO	Chinese hamster ovary
CMA	Chaperone-mediated autophagy
C-NAP1	Centrosomal NEK2-associated protein 1
CPAP	Centrosomal P4.1-associated Protein
DC	Doublecortin
DDR	DNA damage response
DHC	Dynein heavy chain
DHh	Desert hedgehog
DISP	Dispatched
DNA-SCARS	DNA Segments with Chromatin Alterations Reinforcing Senescence
dNTP	Deoxyribonucleotide-5‟-triphosphate
DSB	Double-strand break

Dvl2	Dishevelled 2
E2F	Adenovirus E2 promoter binding factor
ERK	Extracellular signal-regulated kinase
ETS	E-twenty six transcription factors
FAM	Carboxyfluorescein
Fas	TNF receptor superfamily, member 6
FUZ	Fuzzy
Fz	Frizzled
GAS1	Growth arrest-specific gene
GLI	Glioma
GMP	Cerebellar granule precursor
GPC3	Glypican 3
GRO	Growth-regulated oncogenes
GSK3	Glycogen synthase kinase 3
H3K9me3	Histone H3 trimethylated at lysine 9
HDAC	Histone deacetylase
HEF1	Human enhancer of filamentation 1
Hh	Hedgehog
HHIP	Hh-interacting protein
HMEC	Human mammary epithelial cell
HMGA2	High mobility group protein A2
HP-1γ	Heterochromatin protein-1γ
HPV	Human papillomavirus
HR	Homologous recombination
H-RAS	Harvey rat sarcoma viral oncogene homolog
hTERT-RPE1	Human telomerase reverse transcriptase- retinal pigment epithelial
HTLV-1	Human T-cell leukaemia virus type-1
IB	Immunoblot
IF	Immunofluorescence microscopy
IFT	Intraflagellar transport
IGF	Insulin-like growth factor
Hh	Indian hedgehog
IL	Interleukin
IMCD	Immortalised kidney collecting duct
INTU	Inturned
Invs	Inversin
IR	Ionizing radiation
IRIF	IR-induced foci
KAP	Kinesin-associated protein
KIF	Kinesin family member
kb	Kilobase pair(s)
KSHV	Kaposi sarcoma herpes virus
LB	Luria-Bertani medium
LDL	Low-density Lipoprotein
LRP2	Low density lipoprotein receptor-related protein
MAP	Microtubule associated protein
MAPK1	Mitogen-activated protein kinase 1
MCP	Monocyte chemoattractant protein
MCPH	Microcephalin
MDC1	Mediator of DNA damage checkpoint protein 1
MDM2	Mouse double minute 2
MEF	Mouse embryonic fibroblasts
MIP	Macrophage inflammatory protein

MKK	MAP kinase kinase
MKS	Meckel-Gruber syndrome
MRE11	Meiotic recombination 11
MT	Microtubule
mTOR	Mammalian target of rapamycin
MTOC	Microtubule-organising centre
NCS	Newborn calf serum
Nde1	Nuclear distribution homologue E 1
NEDD1	Neural precursor cell expressed, developmentally down-regulated 1
NEK/NRK	NIMA-related kinase
NHDF	Normal Human Dermal Fibroblasts
NHEJ	Non-homologous end joining
NICD	Notch intracellular domain
NIMA	Never-in-mitosis A
NPHP-RC	Nephronophthisis-related ciliopathies
NPM/B23	Nucleophosmin
NF-κB	Nuclear factor κB
OA	Osteoarthritis
ODF2	Outer dense fibre protein 2
OFD1	Oral-facial-digital syndrome 1
OIS	Oncogene-induced senescence
PCM	Pericentriolar material
PCNA	Proliferating Cell Nuclear Antigen
PCP	Planar cell polarity
Pen/Strep	Penicillin/streptomycin
PFA	Paraformaldehyde
Pifo	Pitchfork
PKD	Polycystic kidney disease
PLK	Polo-like kinase
PML	Promyelocytic leukaemia protein
POC	Protein of Centriole
PPARγ	Proliferator-activated receptor γ
PTCH	Patched
PTM	Post-translational modification
qPCR	Quantitative Real-Time PCR
Rad	Radiation sensitive
Rb	Retinoblastoma
RhoA	Ras homolog family member A
RIPA	Radio-immunoprecipitation assay
SA-β-Gal	Senescence-associated β-galactosidase
SAC	Spindle assembly checkpoint
SAHF	Senescence-associated heterochromatic foci
SAK	Snk/Plk-akin kinase
SAP	Shrimp alkaline phosphatase
SAS	Spindle assembly abnormal protein
SASP	Senescence-associated secretory phenotype
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SEPT	Septin
SF3A1	Splicing factor 3a, subunit 1
SFRP1	Secreted frizzled related protein 1
SHh	Sonic hedgehog
Smo	Smoothened
SPD-2	Spindle defective protein 2

ss	Serum-starved
STIL	SCL/TAL1 interrupting locus
SUFU	Suppressor of fused
TAE	Tris-acetate EDTA
TEMED	N,N,N‘,N‘-tetramethylethylenediamine
TERT	Telomerase reverse transcriptase
Tfb	Transformation buffer
TG	Tris-glycine
TP53	Tumour protein 53, or p53
TSC	Tuberous sclerosis complex
TTBK2	Tau tubulin kinase 2
TZ	Transition zone
VANGL2	Van Gogh-like 2
WDR34	WD repeat domain 34
Wnt	Wingless-type MMTV integration site family
XRCC	X-ray repair complementing defective repair in CHO cells
ZYG-1	Zygote defective protein 1

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ABSTRACT

Somatic cells senesce in culture after a finite number of divisions, indefinitely arresting their proliferation. Among the major causes of senescence is persistent DNA damage signalling. DNA damage and senescence increase the cellular number of centrosomes, the two microtubule organising centres that ensure bipolar mitotic spindles. Centrosomes also provide the basal body, a foundation for the formation of primary cilia, microtubule-based organelles that extend from the surface of most human cell types to sense and transduce various extracellular signals. Primary cilium formation is facilitated by cellular quiescence, a temporary exit from the cell cycle, but the impact of senescence on cilia has not been described.

In this project we show that increased cilium frequency and length accompanies senescence in primary human fibroblasts and that ciliation induced by depletion of the centriolar protein CP110 causes senescence. A higher frequency of senescent BJ, MRC5 and NHDF cells had a primary cilium compared to proliferating controls. Cilia were significantly longer on senescent cells. Senescent BJ fibroblasts have elevated numbers of centrioles and this correlates with an increase in ciliary abnormality. Senescent cells showed reduced expression levels of components of the Hedgehog signalling pathway. Inhibition of Hedgehog signalling with cyclopamine reduced proliferation in young cell populations, with increased cilium length accompanying the induction of cell cycle arrest. Ciliary regrowth experiments demonstrated that cilium length is independent of the growth arrest period and that it is intrinsic to the cell. Senescent cells showed reduced levels of the negative ciliary length regulator, CP110. siRNA-mediated depletion of CP110 in young populations increased ciliation, reduced proliferation and elevated cellular senescence. These data demonstrate that primary cilium length regulation through CP110 is a potential novel determinant of cellular proliferative capacity.

CHAPTER 1 – INTRODUCTION

1.1 Cellular Senescence

Cellular senescence refers to the essentially irreversible arrest of cell proliferation in response to certain forms of stress (reviewed by (Collado et al., 2007)). Replicative senescence was first described by Hayflick and Moorhead nearly 50 years ago, when they demonstrated that normal human cells did not proliferate indefinitely in culture. They discovered that cells undergo senescence after a fixed number of cell divisions, marking the end of the proliferative capacity of a cell. They also found that replicatively senescent cells remain metabolically active and can be maintained in culture for long periods of time (Hayflick and Moorhead, 1961). The number of divisions that a normal cell population completes before reaching the end of its proliferative capacity has been termed the "Hayflick limit". The work by Hayflick and Moorhead was performed using fibroblasts, but replicative senescence has been observed in other cell types including endothelial cells (Erusalimsky and Kurz, 2006), lymphocytes (Vaziri et al., 1993) and chondrocytes (Martin and Buckwalter, 2001). Exceptions exist and certain cell lines can divide indefinitely without reaching replicative senescence. These cells are "immortal" and include embryonic germ cells and cell lines derived from tumours (Wright and Shay, 1992; Chiu and Harley, 1997; Pera et al., 2000).

Shortly after Hayflick and Moorhead's discovery, cellular senescence was linked independently to both tumour suppression and aging. Many cancer cells proliferate indefinitely in culture and so cellular senescence was proposed to be an anti-cancer mechanism. Senescence was thus considered to be beneficial, as it protected organisms from cancer. However, tissue regeneration and repair deteriorate with age and senescence was proposed to contribute to reduced tissue renewal and function (reviewed in (Campisi and d'Adda di Fagagna, 2007; Funayama and Ishikawa, 2007; Adams, 2009; Kuilman et al., 2010; Rodier and Campisi, 2011)). The antagonistic pleiotropy hypothesis was proposed as an evolutionary explanation for senescence. This theory suggests that a process can be beneficial to young organisms but harmful to old organisms and thereby may contribute to aging (reviewed by (Kirkwood and Austad, 2000)). There is now substantial evidence linking cellular senescence to cancer (reviewed in (Dimri, 2005; Deng et al., 2008;

Collado and Serrano, 2010; Hanahan and Weinberg, 2011) and mounting evidence that links cellular senescence to aging (Faragher and Kipling, 1998; Shawi and Autexier, 2008; Campisi, 2013).

1.2 Causes of cellular senescence

Cells respond to various types of cell stress by undergoing senescence. Since cellular senescence was first described nearly 50 years ago, a more complex view of the causes has been established. The stressors that induce cellular senescence include: 1) DNA damage; 2) chromatin perturbation; 3) oncogenic stress; 4) telomere shortening; 5) cell culture stress (reviewed in (Campisi and d'Adda di Fagagna, 2007; Kuilman et al., 2010; Campisi, 2013)). The causes of cellular senescence are discussed in more detail below.

1.2.1 Telomere-induced senescence

One underlying cause of replicative senescence has been attributed to telomere shortening (Counter et al., 1992; Martens et al., 2000; Karlseder et al., 2002; Herbig et al., 2004). Telomeres are repetitive DNA-protein structures that cap the ends of linear chromosomes to protect them from degradation. They shorten each time a cell divides as DNA polymerases cannot completely replicate to the end of a DNA template. This is termed the “end replication problem” (Levy et al., 1992; Allsopp et al., 1995). Telomere shortening does not occur in cells that express telomerase. Telomerase is a multi-subunit enzyme which contains a catalytic protein component (telomerase reverse transcriptase, TERT) and a template RNA component which adds telomeric DNA repeats to chromosome ends (Greider and Blackburn, 1989). Telomerase-positive human cells include most cancer cells, embryonic stem cells, some adult stem cells and some somatic cells (Collins, 2000; McEachern et al., 2000). TERT expression in normal human cells prevents telomere shortening and senescence caused by the end-replication problem (Bodnar et al., 1998). However, telomerase cannot prevent senescence caused by non-telomeric senescence induction (Chen et al., 2001). Repeated cell division in the absence of telomerase eventually causes one or more telomeres to become critically short and dysfunctional. Human telomeres range from a few kilobases to 10-15 kb in length.

Cells lose 50-200 bp of telomeric DNA during each cell division, so many divisions are possible before telomeres are rendered dysfunctional (Harley et al., 1990).

Telomeres are thought to end in a large circular structure, termed the T-loop (Griffith et al., 1999). This prevents the DNA repair machinery from recognising chromosome ends as DNA double-strand breaks (DSBs), to which cells rapidly respond and attempt to repair (Wang et al., 2004; Celli et al., 2006). DNA damage markers such as γH2AX and 53BP1 are found at dysfunctional telomeres which elicit a DNA damage response (DDR) through the p53 and Rb/p16 pathways (Takai et al., 2003). The DDR enables the cells to sense damaged DNA and to respond by arresting cell proliferation (d'Adda di Fagagna et al., 2003; von Zglinicki et al., 2005; Carneiro et al., 2010; Fumagalli et al., 2012).

1.2.2 DNA-Damage induced senescence

The involvement of DNA damage in the induction of replicative senescence by telomere shortening has been well established (Martens et al., 2000; Karlseder et al., 2002; d'Adda di Fagagna et al., 2003; Takai et al., 2003; Herbig et al., 2004). Many cells undergo senescence in response to severely damaged DNA, regardless of the genomic location. DNA damaging agents, such as ionising radiation, can induce senescence (Di Leonardo et al., 1994; Robles and Adami, 1998). Furthermore, DNA damage-inducing drugs can cause senescence in tumour cells *in vitro* or *in vivo*, which is used in the treatment of cancer (Wang et al., 1998; Chang et al., 1999; Berns, 2002; Schmitt et al., 2002; te Poele et al., 2002). Activation of the DNA damage response has been shown to contribute to oncogene-induced senescence *in vitro* (reviewed by (Campisi and d'Adda di Fagagna, 2007; Kuilman et al., 2010)). DNA lesions caused by oxidative stress may also drive cells into senescence (Chen et al., 2001; Parrinello et al., 2003; Nogueira et al., 2008). These events lead to persistent DNA damage signalling. The response to persistent DNA damage differs significantly from the response to mild DNA damage, which causes a transient growth arrest and transient DNA damage signalling. Persistent DNA damage induces the formation of DNA damage foci that contain many activated DDR proteins (Sedelnikova et al., 2004; Rodier et al., 2009; Rodier et al., 2011; Fumagalli et al.,

2012). In conclusion, there are many potential causes of DNA-damage initiated senescence which are not independent of one another or mutually exclusive.

1.2.3 Oncogene-induced senescence (OIS)

Cellular senescence can also be induced by oncogenes. An oncogenic form of H-RAS (H-RAS^{V12}) stimulates the mitogen-activated protein kinase (MAPK) signalling pathway, inducing senescence when introduced into primary cells (Serrano et al., 1997). OIS has been shown to be independent of telomere shortening, as OIS cannot be bypassed by expression of TERT (Wei and Sedivy, 1999). The mechanisms of OIS are not fully understood but do not appear to be common across cell types. In mouse cells, p19 is activated by oncogenic signals and mediates senescence; however, it does not have the same role in human cells (Wei and Sedivy, 1999; Michaloglou et al., 2005). In human cells, OIS is dependent on p16 (Ben-Porath and Weinberg, 2005). In mouse cells, inactivation of p53 or its direct upstream regulator, p19, is sufficient to bypass H-RAS^{V12}-induced senescence (Kamijo et al., 1997; Serrano et al., 1997). In addition, H-RAS^{V12}-induced senescence can be bypassed by inactivation of the p16–RB pathway (Serrano et al., 1997), while BRAF^{E600}-induced senescence cannot (Michaloglou et al., 2005; Haferkamp et al., 2009).

Various oncogenic stimuli have been shown to induce senescence through activation of the DDR (Yang and Hu, 2005; Di Micco et al., 2006; Mallette et al., 2007). Disruption of DDR factors, ATM and CHK2, prevents OIS and promotes cellular transformation *in vitro*, and *Chk2*$^{-/-}$ mice show increased susceptibility to mutagen-induced skin tumours *in vivo* (Hirao et al., 2002; Di Micco et al., 2006). Furthermore, OIS does not occur in the absence of DNA replication, suggesting that OIS is a DDR triggered by activation of DNA hyper-replication (Di Micco et al., 2006).

1.2.4 Senescence caused by chromatin reorganisation

Chromatin exists in two states which reflect the extent to which genes are active (euchromatin) or silent (heterochromatin). The chromatin state depends on histone modifications, for example acetylation or methylation. Histone deacetylase

inhibitors (HDACIs) relax chromatin without physically damaging DNA. HDACIs promote euchromatin formation and induce senescence by a mechanism that is not fully understood, and differs between species and cell type. For example, in human fibroblasts, HDACIs promote euchromatin formation and induce senescence through the pRB pathway (Ogryzko et al., 1996; Munro et al., 2004). However, in mouse fibroblasts, the p53 pathway is induced in response to HDACIs. HDACIs have been shown to activate ATM, and so, HDACIs may induce a DDR response without DNA damage (Bakkenist and Kastan, 2003; Pazolli et al., 2012).

1.2.5 Stress and other inducers of senescence

Stress-induced senescence may be caused by abnormal concentrations of nutrients and growth factors, abnormal levels of ambient O_2 levels, or the absence of surrounding cells and extracellular matrix components (Packer and Fuehr, 1977; Chen et al., 1995; Fusenig and Boukamp, 1998; Ramirez et al., 2001; Bennett and Medrano, 2002; Yaswen and Stampfer, 2002; Parrinello et al., 2003). Stress-induced senescence occurs in the absence of any detectable telomere dysfunction or DDR signalling (Sherr and DePinho, 2000). It also occurs in mouse cells which express telomerase (Prowse and Greider, 1995). Signalling by anti-proliferative cytokines, such as interferon-β, also causes senescence. Transient interferon-β treatment reversibly arrests cell proliferation but sustained interferon-β treatment increases intracellular oxygen radicals and elicits a p53-dependent DDR and senescence (Moiseeva et al., 2006). Similarly, sustained treatment with transforming growth factor β, an inhibitor of epithelial cell proliferation, induces senescence by promoting p16-pRB-dependent heterochromatin formation (Vijayachandra et al., 2003; Zhang and Cohen, 2004). These findings demonstrate that supraphysiological growth conditions as well as inadequate growth conditions can induce senescence.

1.3 Characteristics features of senescent cells

Somatic cells can remain in a reversible (quiescent) or irreversible (senescent) state of growth arrest for long periods of time. Senescence is essentially irreversible, while quiescent cells can be stimulated to resume proliferation by appropriate physiological signals. Senescent cells exhibit characteristic features

which distinguish them from quiescent cells: 1) changes in cell morphology; 2) irreversible growth arrest; 3) resistance to apoptosis; 4) accumulation of autophagosomes 5) DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS); 6) senescence-associated heterochromatic foci (SAHF); 7) senescence-associated β-galactosidase (SA-β-Gal); 8) the senescence-associated secretory phenotype (SASP) (reviewed in (Campisi and d'Adda di Fagagna, 2007; Funayama and Ishikawa, 2007; Adams, 2009; Kuilman et al., 2010; Rodier and Campisi, 2011)). The characteristic features of senescent cells are discussed below.

1.3.1 Changes in cell morphology

Cellular senescence is generally accompanied by changes in cell morphology. Depending on the cause of senescence, cells may increase in size, become flatter, more granular in appearance or multinucleated. Senescent cells often enlarge more than twice the relative size of proliferating cells (Hayflick, 1965). A flat morphology is commonly seen in cells undergoing H-RAS^{V12}-induced senescence (Serrano et al., 1997; Denoyelle et al., 2006), stress induced senescence (Parrinello et al., 2003), or DNA damage-induced senescence (Chen and Ames, 1994). However, cells senescing due to BRAF^{E600} expression or the silencing of p400 acquire a spindle-shaped morphology (Chan et al., 2005; Michaloglou et al., 2005). Senescent cells may also become more granular in appearance (when observed at low magnification under a light microscope). Melanocytes undergoing H-RAS^{V12}-induced senescence acquire more vacuoles as a result of endoplasmic reticulum stress caused by the unfolded protein response (Denoyelle et al., 2006). While a change in cell morphology is associated with senescence, it is dependent on cell type and the cause of senescence.

1.3.2 Irreversible growth arrest

Another characteristic of senescence is an inability to progress through the cell cycle. Senescent cells fail to initiate DNA replication due to the expression of cell cycle inhibitors. As illustrated in Figure 1.1, stimuli that induce senescent proliferation arrest, including dysfunctional telomeres which activate the DDR, engage either or both the p53-p21-pRB and p16-pRB (retinoblastoma) tumour suppressor pathways (reviewed by (Campisi and d'Adda di Fagagna, 2007; Adams,

2009)). Each pathway has multiple upstream regulators, downstream effectors and modifying side branches (reviewed by (Chau and Wang, 2003; Levine and Oren, 2009)). The pathways also cross-regulate each other (Zhang et al., 2006; Yamakoshi et al., 2009; Takeuchi et al., 2010). Both pathways control senescence by implementing changes in gene expression. p53 and pRB are transcriptional regulators. p53 is phosphorylated by upstream kinases including ataxia telangiectasia (ATM) and CHK2 (Serrano et al., 1997; d'Adda di Fagagna et al., 2003; Gire et al., 2004). p21 and p16 are cyclin dependent kinase inhibitors (CDKIs) and negative regulators of cell cycle progression (Sherr and Roberts, 1999). Phosphorylated p53 up-regulates transcription of its downstream effector, p21, which activates pRB through inhibition of a cyclin dependent kinase (CDK) complex, cyclin E/CDK2. Activated pRB inhibits the transcription of E2F target genes including cyclin A and PCNA, which are required for cell cycle progression (Dyson, 1998). p16 is a positive upstream regulator of pRB. p16 activates pRB through inhibition of the cyclin D/CDK4,6 complexes (Hara et al., 1996; Gil and Peters, 2006). p16 accumulates in senescent cells and this accumulation is mediated through transcriptional activation by ETS transcription factors (Ohtani et al., 2001).

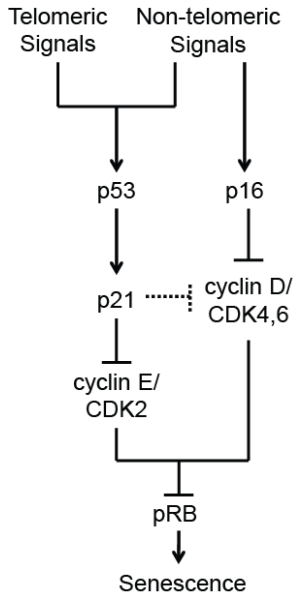

Figure 1.1 Summary of p53-p21 and p16-pRB senescence pathways in human fibroblasts.
Schematic representation of the p53-p21 and p16-pRB senescence pathways in human fibroblasts. Induction of p21 and p16 by senescence-inducing signals results in inhibition of CDK2 and CDK4,6 activity. Down-regulation of these kinases leads to pRB hypophosphorylation, which results in cell cycle arrest during senescence. Diagram adapted from (Dimri, 2005).

Senescent proliferation arrest is essentially permanent, although experimental manipulations can cause some replicatively senescent human cells to proliferate (Gire and Wynford-Thomas, 1998; Beauséjour et al., 2003). In these cases, eliminating p53 function caused senescent cells to resume proliferation, despite having short telomeres, until eventual severe telomere dysfunction drove them into crisis, a state of acute genomic instability. Similarly, inactivation of p21, a p53 target for transactivation and an inhibitor of cell cycle progression, allowed the cells to bypass telomere-induced senescence and enter crisis (Brown et al., 1997). In addition, Rapamycin, an inhibitor of mTOR, decelerates senescence induced by p21, p16 and sodium butyrate. When senescence was induced by these factors in the presence of rapamycin, cells retained the capacity to resume proliferation, once p21, p16 or sodium butyrate were removed (Demidenko et al., 2009).

The features of senescent growth arrest vary depending on the cell type. Senescent cells usually arrest growth with a DNA content that is typical of G1-phase arrest. However, in mouse fibroblasts a defect in the signalling kinase MKK7 can induce a G2-M phase arrest (Wada et al., 2004). Some oncogenes can cause cells to senesce with G2-phase DNA content (Zhu et al., 1998; Olsen et al., 2002; Di Micco et al., 2006). Furthermore, tumour cells can senesce with G2- or S-phase DNA contents. Tumour cells usually proliferate indefinitely in culture, but in response to some anti-cancer therapies they can undergo a senescence-like arrest (Shay and Roninson, 2004).

While a permanent growth arrest is a characteristic feature of senescent cells, their inability to replicate is not. For example, terminal differentiation similarly results in a stable growth arrest. However, differentiation is typically induced by physiological signals and does not involve the activation of tumour-suppressor networks (reviewed in (Buttitta and Edgar, 2007).

1.3.3 Apoptosis resistance

Apoptosis is a process of programmed cell death in which biochemical events lead to the controlled destruction of cellular components and ultimate engulfment by other cells (Ellis et al., 1991). Apoptosis is initiated by two major pathways: intrinsic

or extrinsic. The intrinsic pathway is activated in response to cell stress, for example, DNA damage or endoplasmic reticulum (ER) stress, causing disruption of the outer mitochondrial membrane. Pro-apoptotic proteins are released from mitochondria to activate caspase proteases and trigger apoptosis. The extrinsic pathway is initiated through activation of death receptors, by ligation with pro-apoptotic ligands on the cell surface (reviewed in (Vicencio et al., 2008)). Like senescence, apoptosis is a response to cellular stress, but while senescence prevents the growth of damaged cells, apoptosis eliminates them.

Although most cells have the potential to undergo senescence or apoptosis, these processes are thought to be independent of each other (Campisi and d'Adda di Fagagna, 2007). Not all cell types acquire resistance to apoptotic signals when they become senescent. For example, senescent fibroblasts are resistant to ceramide-induced apoptosis but senescent endothelial cells are not (Hampel et al., 2004). Some senescent cells are only resistant to certain apoptotic signals. For example, senescent fibroblasts are resistant to apoptosis caused by growth factor deprivation and oxidative stress (intrinsic pathway), but are not resistant to apoptosis caused by engagement of the Fas death receptor (extrinsic pathway) (Chen et al., 2000). It is not clear what determines whether a cell undergoes senescence or apoptosis, but cell type seems to be a factor. For example, damaged fibroblasts tend to undergo senescence, while damaged lymphocytes tend to undergo apoptosis. Manipulation of the expression levels of Bcl-2 or caspase inhibition cause cells that normally would have undergone apoptosis to enter senescence (Rebbaa et al., 2003; Nelyudova et al., 2007). Similarly, telomerase overexpression in senescent cells does not prevent cellular senescence but protects cells from apoptosis (Gorbunova et al., 2002; Massard et al., 2006). It is thought that the senescence and apoptosis regulatory systems communicate through their common regulator, p53 (Seluanov et al., 2001). The mechanisms by which senescent cells resist apoptosis are poorly understood. Resistance may be due to changes in protein expression which inhibit, promote or instigate cell death (Marcotte et al., 2004; Murata et al., 2006). Resistance may also be due to preferential activation of genes that arrest proliferation over those which induce apoptosis (Jackson and Pereira-Smith, 2006). While these studies suggest

there is cross-regulation between apoptosis and senescence, further studies are needed to clarify this connection.

1.3.4 Autophagy and senescence

In senescent cells, autophagy serves as a cell survival response to stress conditions. Autophagy is mediated by the formation of vesicles (autophagosomes) which engulf cytoplasmic content and fuse with lysosomes. This ultimately leads to the breakdown of the vesicles and their contents (reviewed by (Kroemer et al., 2010)). Chaperone-mediated autophagy (CMA) has been shown to decline with age in human cells, increasing the risk of neurodegeneration (Kaushik and Cuervo, 2006; Massey et al., 2006; Kaushik et al., 2007). CMA is also correlated with the transient accumulation of autophagosomes (Gerland et al., 2003; Stroikin et al., 2005). More recently, it has been shown that autophagy contributes to the cell cycle arrest and production of senescence-associated interleukins (Young et al., 2009). These findings provide evidence which suggests that cellular senescence and autophagy are linked. However, the exact mechanism by which autophagy reduces aging is not clear. It has been speculated that increased turnover of cytoplasmic structures rejuvenates cells. In addition, autophagy has been shown to have a role in maintaining genomic instability, again through mechanisms which are not known. Therefore, an increase in autophagy may help to avoid the long-term effects of DNA damage (reviewed in (Mathew et al., 2007).

1.3.5 DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS)

Cells that senesce with genomic damage acquire DNA damage foci with persistent DNA damage response (DDR) signalling. Persistent DNA damage foci are found in tissues that have been exposed to genotoxic stress (Rodier et al., 2011) and in aging mice and primates (Sedelnikova et al., 2004; Herbig et al., 2006; Wang et al., 2009). The nuclear foci are termed DNA Segments with Chromatin Alterations Reinforcing Senescence (DNA-SCARS) (Rodier et al., 2011) or telomere dysfunction-induced foci (TIF) when present at telomeres (d'Adda di Fagagna et al.,

2003; Herbig et al., 2004). DNA-SCARS contain activated DDR proteins that are also present in transient DNA damage foci (d'Adda di Fagagna et al., 2003; Herbig et al., 2004; Rodier et al., 2009), but DNA-SCARS are distinguishable from transient DNA damage foci. Unlike transient DNA damage foci, DNA-SCARS associate with PML nuclear bodies, lack the DNA repair proteins RPA and RAD51, lack single-stranded DNA and DNA synthesis and accumulate activated forms of the DDR mediators CHK2 and p53 (Rodier et al., 2011). DNA-SCARS contain the activated DDR proteins required for the senescence-associated secretory phenotype (SASP) (Rodier et al., 2011). DNA-SCARS co-localise with promyelocytic leukaemia protein (PML) nuclear bodies (Rodier et al., 2011). Many, but not all, senescence inducers cause genomic damage, resulting in lasting DNA-SCARS.

1.3.6 Senescence associated heterochromatic foci (SAHF)

Senescence is characterised by the formation of senescence associated heterochromatic foci (SAHF). When senescent cells are stained with 4',6-diamidino-2-phenylindole (DAPI), they display a punctate staining pattern where chromosomes appear to be individually compacted into foci. SAHF are specifically enriched in histone H3 trimethylated at lysine 9 (H3K9me3, a modification catalysed by the methyltransferase Suv39h1), its binding partner heterochromatin protein-1γ (HP-1γ) and the chromatin-bound high mobility group protein A2, HMGA2 (Narita et al., 2006). The linker histone H1 is lost in SAHF (Funayama et al., 2006; Narita et al., 2006). Senescent cells show increased binding of heterochromatin-associated proteins at proliferation promoting gene loci which are resistant to E2F-mediated transcriptional activation (Narita et al., 2003). Therefore, alterations in chromatin structure are believed to contribute to the irreversible nature of senescence by sequestering and silencing genes associated with cell cycle entry and proliferation. SAHF occur in a cell type- and insult-dependent manner (Kosar et al., 2011).

1.3.7 Senescence-associated β-galactosidase (SA- β-Gal)

Senescence-associated β-galactosidase (SA-β-Gal) is a commonly used biomarker for replicative senescence (Dimri et al., 1995; van der Loo et al., 1998; Price et al., 2002; d'Adda di Fagagna et al., 2003; Matthews et al., 2006; Debacq-Chainiaux et al., 2009). β-galactosidase (β-Gal) is a lysosomal hydrolase which can

be detected in most mammalian cells by a cytochemical assay, carried out at pH 4 (Dimri et al., 1995). The β-Gal assay is based on the production of a blue precipitate, resulting from the cleavage of the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). Dimri et al. observed a detectable increase in β-Gal staining in senescent cells at suboptimal pH 6. This was not detectable in pre-senescent, quiescent or immortal cells under identical culture conditions (Dimri et al., 1995). The increase in β-Gal activity in senescent cells is due to an increase in the number and size of lysosomes they contain (Kurz et al., 2000). However, there is no evidence which suggests an actual involvement of this enzyme in the senescence response (Lee et al., 2006a). While SA-β-Gal is a commonly used for the detection of senescent cells, it is not entirely specific to the senescent state. Contact inhibited or serum starved cells may also be positive for β-Gal (Severino et al., 2000; Yang and Hu, 2005).

1.3.8 Senescence-associated secretory phenotype (SASP)

Cells undergoing senescence also exhibit changes in their secretome. This was first reported in fibroblasts undergoing replicative senescence (Shelton et al., 1999). Senescent cells secrete several components including growth factors, cytokines, chemokines and proteases (reviewed in (Coppé et al., 2010a; Freund et al., 2010)). This is termed the senescence-associated secretory phenotype (SASP). The SASP is a characteristic of cells senescing due to DNA damage (Rodier et al., 2011), chromatin perturbation (Pazolli et al., 2012), mitogenic signals (Bavik et al., 2006) and oxidative stress (Coppé et al., 2010b). Cells that senesce due to ectopic overexpression of p21 or p16 do not exhibit the SASP (Coppé et al., 2011).

Persistent DNA damage is required for the induction of some, but not all, SASP components (Rodier et al., 2009). It is not known how DDR signalling promotes the expression of genes that encode SASP components. DNA-SCARS contain the activated DDR proteins that induce the persistent DDR signalling required for the SASP, including ATM, NSB1 and CHK2 (Coppé et al., 2008; Rodier et al., 2009; Rodier et al., 2011). The SASP is expressed after the initial DDR subsides (Coppé et al., 2008; Rodier et al., 2009). p53 negatively regulates the SASP (Coppé et al., 2008; Rodier et al., 2009). Inactivation of p53 in senescent cells which

express the SASP causes an increase in the secretion SASP components (Coppé et al., 2008). In addition, p53 inactivation in cells that do not express p16 causes cells to resume proliferation, although, the SASP is still expressed (Coppé et al., 2008; Rodier et al., 2009).

The SASP is also positively regulated by the transcription factors NF-κB (nuclear factor κB) (Acosta et al., 2008; Freund et al., 2011; Pazolli et al., 2012) and C/EBP-β (CCAAT/enhancer-binding protein β) (Kuilman et al., 2008), which are downstream of signalling cascades that control inflammatory cytokine gene expression. The expression of the plasma membrane associated cytokine, IL-1α (Interleukin-1 α), is increased in senescent cells (Bhaumik et al., 2009; Orjalo et al., 2009). IL-1α binds its plasma membrane receptor IL-1R which initiates a signalling cascade that activates NF-κB (Bhaumik et al., 2009; Orjalo et al., 2009). NF-κB activates the transcription of genes encoding inflammatory mediators, such as IL-6 and IL-8 (Acosta et al., 2008; Kuilman et al., 2008). Signalling through the IL-6 and IL-8 receptors is essential for oncogenic BRAF induced senescence or replicative senescence, respectively (Acosta et al., 2008; Kuilman et al., 2008).

While some SASP components contribute to the deleterious effects of senescence, others have beneficial effects. The SASP component VEGF (vascular endothelial growth factor) contributes to new blood cell formation (Coppé et al., 2006). The SASP components GROs (growth-regulated oncogenes) (Yang et al., 2006) and amphiregulin (a growth factor and mitogen) (Bavik et al., 2006) stimulate cell proliferation. However, proliferation of premalignant cells is also stimulated when they are cultured with (or grown in medium conditioned by) senescent cells (Krtolica et al., 2001; Dilley et al., 2003; Parrinello et al., 2005; Yang et al., 2006). For example, GRO1 induces senescence in ovarian fibroblasts (Yang et al., 2006). The Wnt signalling modulator SFRP1 (secreted frizzled related protein 1) is also a component of the SASP. SFRP1 and IL-6/8 stimulate Wnt signalling, which can drive differentiated cells and stem cells into senescence (Liu et al., 2007a; Acosta et al., 2008; Kuilman et al., 2008; Elzi et al., 2012; Zhang et al., 2013). SASP components, including IL-6, IL-8, MCPs (monocyte chemoattractant proteins) and MIPs (macrophage inflammatory proteins), are thought to promote inflammation (reviewed in (Coppé et al., 2010a; Freund et al., 2010)). As these findings indicate,

the exact function of the SASP has not been elucidated. However, it is thought that one function may be to ensure that damaged cells communicate to neighbouring cells to prepare for repair. Another function may be to stimulate the clearance of damaged cells by the immune system. While the SASP is an important feature of senescent cells, the components of the SASP vary depending on cell type and the senescence-inducing stimulus.

In conclusion, several characteristic features are used to identify senescent cells. However, no single characteristic is exclusive to the senescent state. Likewise, not all senescent cells display all the possible senescence markers. Therefore, senescent cells are usually detected using a combination of biomarkers.

1.4 The centrosome

The centrosome is the main microtubule-organising centre (MTOC) in cells. It has evolved from a basal body/axoneme structure in unicellular organisms. The centrosome has many functions, including organising the spindle pole in mitosis, nucleating microtubules and regulating cell adhesion, motility, polarity and intracellular trafficking (reviewed by (Doxsey, 2001; Rieder et al., 2001; Bettencourt-Dias and Glover, 2007)). Although centrosomes are primarily known for their role in cell division, they have been lost in many eukaryotes, suggesting that centrosomes are not essential for the duplication and survival of cells. It is possible that the essential role of the centrosome is in the formation of cilia, given that cells can still divide without a centriole (Mahoney et al., 2006). *Drosophila melanogaster sas4* (Spindle assembly abnormal 4) mutants, which lack centrioles, develop into morphologically normal adults. However, they die shortly after birth as their sensory neurons lack cilia (Basto et al., 2006).

As illustrated in Figure 1.2, during G1 phase of the cell cycle centrosomes consist of two centrioles, orientated perpendicularly to each other and tethered at their proximal ends by a flexible linker. Centrioles are barrel shaped, microtubule-containing structures that show nine-fold radial symmetry. The centrosome is surrounded by the pericentriolar material (PCM), a scaffolding of coiled-coil

proteins. The centrosome duplicates only once per cell cycle. This generates two centrioles at different stages of maturity, the older "mother" centriole and the younger "daughter" centriole. The mother centriole can be distinguished by two sets of appendages at the distal ends, termed the distal and sub-distal appendages. These appendages anchor the centriole at the plasma membrane during ciliogenesis (reviewed by (Tassin and Bornens, 1999; Salisbury, 2003; Bettencourt-Dias and Glover, 2007; Nigg and Stearns, 2011)).

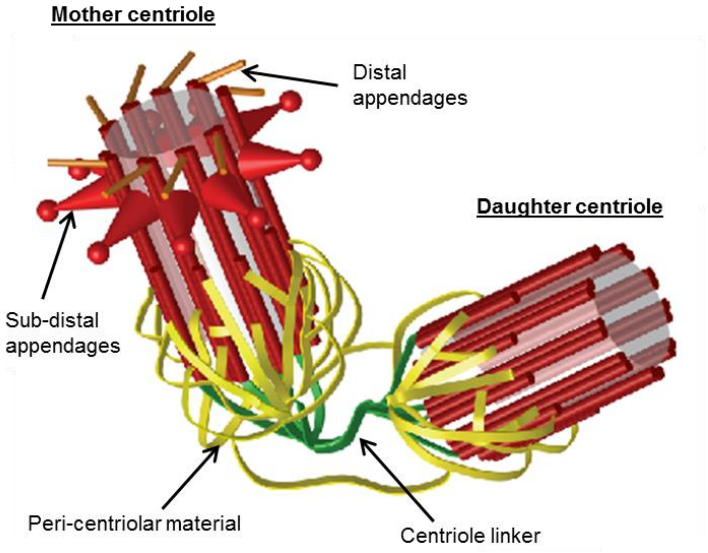

Figure 1.2 The structure of the centrosome.
Schematic representation of the basic structure of the centrosome. The centrosome consists of a mother and daughter centriole. The mother centriole can be distinguished by the distal (orange sticks) and sub-distal (red cones) appendages. The centrioles are linked at their distal ends by a centriole linker (green).The proximal end of each centriole is surrounded by the pericentriolar material (yellow). Diagram adapted from (Sillibourne and Bornens, 2010).

1.5 The primary cilium

Cilia and flagella are highly conserved organelles which project from the cell surface. Cilia exist as motile cilia or immotile primary cilia and are found on most cells in the human body. The immotile primary cilium is a sensory organelle that receives chemical and mechanical signals from other cell and the external environment and transmits them to the nucleus to elicit a cellular response. As illustrated in Figure 1.3, primary cilia consist of a microtubule-based axoneme which is surrounded by a phospholipid membrane. The axoneme emerges from the basal body, which is derived from the mother centriole. The cilium is separated from the

cytosol by the transition zone (reviewed by (Singla and Reiter, 2006; Berbari et al., 2009)). The ciliary axoneme, basal body, ciliary membrane and transition zone are discussed in more detail below.

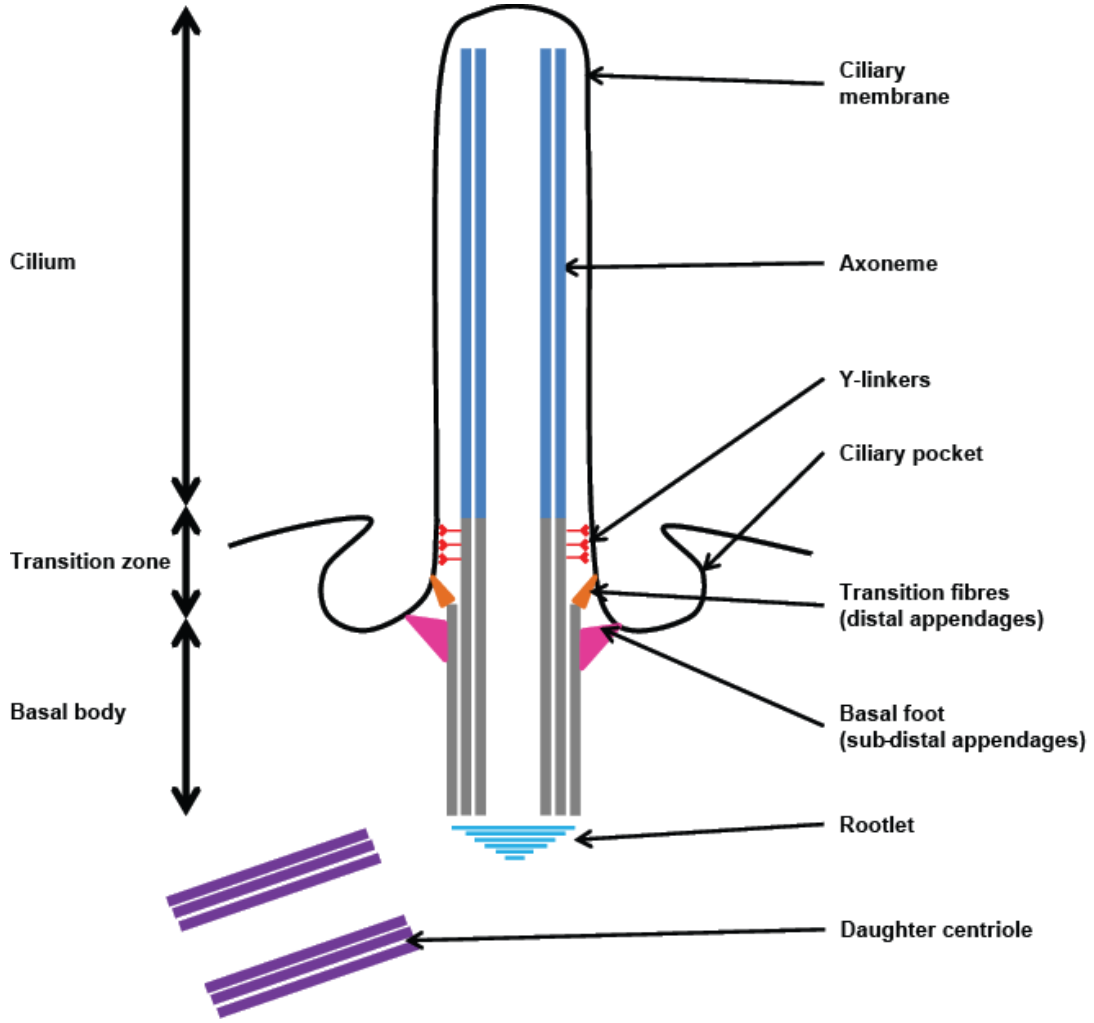

Figure 1.3 The structure of the primary cilium.
Schematic representation of the basic structure of the primary cilium. The mother centriole-derived basal body nucleates the axoneme of the cilium, which is surrounded by the ciliary membrane. The basal body ends with the transition zone, where the transition fibre Y-linkers are located. The cilium is anchored by the basal foot and rootlet. Diagram adapted from (Seeley et al., 2009). Diagram not to scale.

1.5.1 The ciliary axoneme

The ciliary axoneme contains a ring of nine microtubule doublets, which are extensions of the basal body triplets. The axoneme may have a 9+0 or 9+2 microtubule arrangement. Motile cilia have a 9+2 arrangement, in which nine doublet microtubules surround a central pair of singlet microtubules. Primary cilia

have a 9+0 arrangement, in which the central pair of microtubules is missing. 9+0 cilia also lack the molecular motors responsible for ciliary movement, axonemal dyneins; primary cilia are therefore immotile. A single cell may possess several hundred 9+2 motile cilia, whereas it will usually possess only one 9+0 primary cilium (reviewed by (Singla and Reiter, 2006; Satir and Christensen, 2008)).

The tubulin contained in the cilium is derived from the tubulin pool in the cell body. A large number of post-translational modifications (PTMs) have been discovered on tubulin (Westermann and Weber, 2003; Verhey and Gaertig, 2007), particularly ciliary tubulin. Ciliary tubulin has been shown to be acetylated (L'Hernault and Rosenbaum, 1985), detyrosinated (Sherwin et al., 1987), polyglutamylated (Fouquet et al., 1994), and polyglycylated (Rüdiger et al., 1995). Loss-of-function experiments with microtubule modifying enzymes suggest that PTMs are also important for the function, stability, and assembly of the cilium (Pugacheva et al., 2007; Ikegami et al., 2010; Shida et al., 2010; O'Hagan et al., 2011).

1.5.2 The basal body

The basal body is derived from the mother centriole (Gerdes et al., 1983; Goetz and Anderson, 2010). During the conversion of the centriole to the basal body, transition fibres, basal feet and ciliary rootlets are generated. The transition fibres are derived from the distal appendages of the mother centriole. They anchor the basal body to the ciliary membrane at the ciliary pocket (Anderson, 1972). The ciliary rootlets extend from the basal body into the cytoplasm. It was long thought that the ciliary rootlet anchors the basal body. However, depletion of rootletin, a major component of the ciliary rootlet, ablates the rootlet but does not affect cilium assembly (Yang et al., 2005). The basal feet are derived from the subdistal appendages of the mother centriole. They emerge laterally from the basal body, where they stabilise microtubules (Anderson, 1974). Two basal body microtubules extend to form the axoneme; the third microtubule is terminated at the transition zone (Ringo, 1967). The basal body is comprised of a large number of proteins of which can positively or negatively affect cilium formation. ODF2 (Ishikawa et al., 2005) and Cep164 (Graser et al., 2007) are essential for cilium formation, while

Cep97 and CP110 actively suppress ciliogenesis (Spektor et al., 2007). The basal body, therefore, functions in both the assembly and disassembly of the primary cilium, which are discussed in more detail in section 1.8.

1.5.3 The ciliary membrane

The ciliary membrane is a continuation of the plasma membrane, although their composition is different. The ciliary membrane is enriched with sterols (Souto-Padrón and de Souza, 1983), glycolipids (Bloodgood and Salomonsky, 1995) and sphingolipids (Kaneshiro et al., 1984). Proteins and lipids that enter the cilium must pass through two distinct regions near the base of the cilium, termed the ciliary necklace and the ciliary pocket. By electron microscopy, the ciliary necklace is visualised as multiple strands of intramembranous particles that connect to the basal body (Gilula and Satir, 1972), while the ciliary pocket is visualised as an invagination of the plasma membrane (Anderson, 1972). These regions constitute a barrier which separates the ciliary membrane from the plasma membrane (reviewed in (Rohatgi and Snell, 2010)). The presence of a diffusion barrier was demonstrated through expression of an apical GPI-anchored protein in cells, which showed fluorescence along the entire apical membrane, except in an area that corresponded to the site of growth of the primary cilium (Vieira et al., 2006). This diffusion barrier maintains a cilium-specific distribution of proteins within this membrane subregion (Hu et al., 2010) which is central to the sensory function of the cilium. For instance, the initiating event in Hedgehog (Hh) signalling involves the movement of two transmembrane proteins, Patched 1 (PTCH1) and Smoothened (SMO) to the cilium (described in more detail in section 1.12.1). It is also thought that the ciliary pocket is a rate-limiting structure for the trafficking of ciliary proteins (Mahjoub and Stearns, 2012).

1.5.4 The transition zone

The transition zone is the region where the basal body triplet microtubule structure converts into the axoneme doublet microtubule structure. The transition zone contains Y-links that connect the axoneme microtubules to the ciliary membrane (Muresan and Besharse, 1994). Proteins that are associated with ciliopathies, Nephronophthisis (NPHP) and Meckel syndrome (MKS), localise to the

transition zone. Loss of CEP290 (also known as nephrocystin-6/NPHP6) in *Chlamydomonas reinhardtii* alters the protein composition of the flagellum (Craige et al., 2010). Loss of NPHP1 or NPHP4 in *Caenorhabditis elegans*, results in mislocalisation of proteins involved in intraflagellar transport (Jauregui and Barr, 2005). Furthermore, ciliary proteins fail to localise to the cilium in the absence of a number of transition zone proteins (Garcia-Gonzalo et al., 2011). These results suggest that transition zone proteins are required for entry of ciliary proteins into the cilium.

1.6 Intraflagellar transport

Since cilia lack the machinery necessary for protein synthesis, the materials required for ciliary axoneme extension must be transported from the cell body (reviewed by (Pedersen and Rosenbaum, 2008; Satir and Christensen, 2008; Kobayashi and Dynlacht, 2011)). Transport within the cilium is mediated by intraflagellar transport (IFT) (Kozminski et al., 1993), a schematic representation of which is shown in Figure 1.4. Many of the genes involved in IFT have been identified and comparative genomics has shown that these genes are conserved in ciliated organisms such as *Chlamydomonas* (Kozminski et al., 1993), *C. elegans* (Orozco et al., 1999) and humans (Follit et al., 2006). IFT genes are not present in non-ciliated organisms like plants and fungi (Avidor-Reiss et al., 2004; Li et al., 2004). The IFT particle is made up of three complexes, which are discussed in more detail below: 1) ciliary motor proteins; 2) IFT complex A and B; 3) the BBSome.

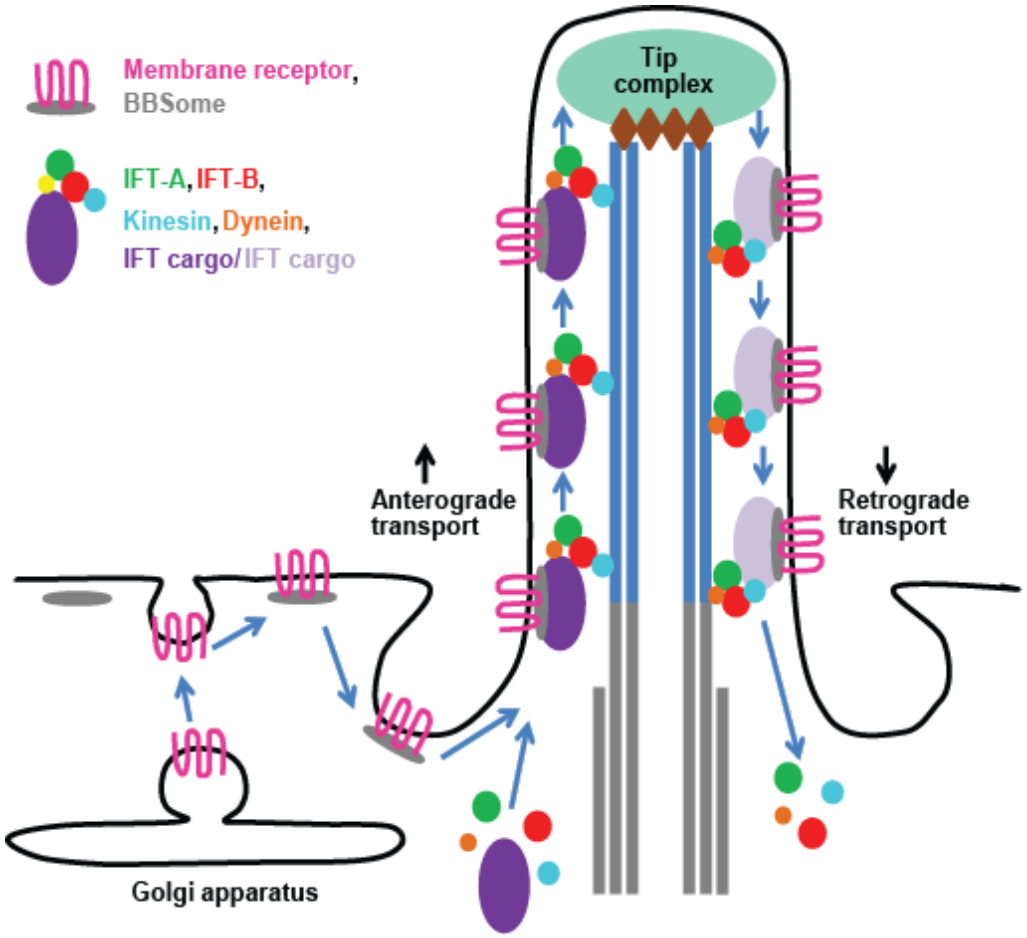

Figure 1.4 Intraflagellar transport (IFT).
Schematic representation of the model for IFT. Vesicles (containing ciliary proteins) are derived from the Golgi apparatus and are targeted to the base of the cilium. The vesicles fuse with the ciliary membrane. Kinesin-II moves the IFT particles (composed of IFT-A, IFT-B, the BBSome, ciliary precursor proteins, signalling proteins, and dynein 2) along the cilium to the cilium tip, where cargo protein unloading/loading and motor protein regulation occur. Dynein 2 transports the IFT particles (now containing kinesin and the turnover products as cargo) back to the cell body, where the IFT components are recycled or degraded. Diagram adapted from (Hildebrandt et al., 2009).

1.6.1 Ciliary motor proteins

Protein complexes are transported from the base of the cilium to the distal tip by anterograde transport (Cole et al., 1998). In vertebrates, the motor protein responsible for anterograde IFT is the heterotrimeric complex, kinesin-II. This complex consists of two motor subunits, KIF3A and KIF3B, and a non-motor subunit called KAP3 (kinesin-associated protein). The *Chlamydomonas* orthologues of these subunits are termed FLA10 (KIF3A orthologue), FLA8 (KIF3B orthologue), and FLA3 (KAP orthologue) (Cole et al., 1993). Kinesin-II is essential for cilium assembly and function. Mutations in the components of kinesin-II in *Chlamydomonas* results in short flagella with no IFT (Kozminski et al., 1995).

Protein complexes are transported from the distal tip back to the cell body by retrograde transport (Pazour et al., 1998). The motor protein responsible for retrograde IFT is cytoplasmic dynein 2 (Pfister et al., 2005). Dynein 2 consists of four homodimeric subunits: a heavy chain, DYNC2H1 (Pazour et al., 1999), a light intermediate chain, DYNC2LI1 (Hou et al., 2004), an intermediate chain, WDR34 (Rompolas et al., 2007) and a light chain, DYNLL1 (Pazour et al., 1998). These proteins have been shown to be part of the same complex (Perrone et al., 2003; Rompolas et al., 2007). Mutations in the components of dynein in *Chlamydomonas, C. elegans* and mouse result in short cilia with accumulated IFT particles, demonstrating its requirement in retrograde IFT (Pazour et al., 1998; Orozco et al., 1999; Pazour et al., 1999; Porter et al., 1999; Wicks et al., 2000; Schafer et al., 2003; Huangfu and Anderson, 2005).

Anterograde and retrograde IFT are required for the assembly and disassembly of the primary cilium, respectively (reviewed in (Avasthi and Marshall, 2012)). However, while anterograde IFT is required for the maintenance of cilium length, retrograde IFT is dispensable for this process (Engel et al., 2012).

1.6.2 IFT complexes A and B

IFT particles are organised into two complexes, called complex A (IFT-A) and complex B (IFT-B) (Piperno and Mead, 1997; Cole et al., 1998), which have different functions. Mutations in IFT-B proteins result in very short or no cilia which lack IFT, similar to mutations in the anterograde kinesin-II motor. Therefore, IFT-B has been implicated in anterograde transport (Pazour et al., 2000; Brazelton et al., 2001; Deane et al., 2001; Haycraft et al., 2003; Follit et al., 2006; Beales et al., 2007; Kobayashi et al., 2007; Absalon et al., 2008; Tsao and Gorovsky, 2008). In contrast to complex B, complex A polypeptides are not always essential for cilia assembly. Mutations in IFT-A proteins result in shortened or swollen cilia with an accumulation of IFT particles, similar to mutations in the dynein retrograde motor. Therefore, the IFT-A complex has been implicated in retrograde transport (Piperno et al., 1998; Iomini et al., 2001; Blacque et al., 2006; Iomini et al., 2009). The binding of IFT particles to IFT motors, axonemal precursors and themselves

suggests that IFT-A and B link the cargo proteins of BBSome to the motor proteins (Kardon and Vale, 2009).

1.6.3 The BBSome

The human ciliopathy Bardet-Biedl syndrome (BBS) is characterised by obesity, mental retardation, retinal degeneration, and cystic kidneys (Zaghloul and Katsanis, 2009). Seven BBS proteins (BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, and BBS9) have been shown form a complex, termed the BBSome (Nachury et al., 2007). The BBSome is thought to function as an IFT cargo, transporting specific proteins to the cilium (Ou et al., 2005; Nachury et al., 2007; Berbari et al., 2008; Lechtreck et al., 2009; Jin et al., 2010). Proteins that comprise the BBSome have been shown to traffic inside the cilium at the same velocity as IFT particles, suggesting that the BBSome associates with a subset of IFT proteins (Blacque et al., 2004; Nachury et al., 2007; Lechtreck et al., 2009).

1.6.4 Model for intraflagellar transport

The following model for IFT was proposed by Pedersen (Pedersen et al., 2008). IFT complexes A and B, kinesin-II, cytoplasmic dynein 2 and axonemal precursor proteins are localised at the base of the flagella (Cole et al., 1998; Pazour et al., 1999) near where the transition fibres attach to the flagellar membrane (Deane et al., 2001). It has been proposed that ciliary proteins are transported in Golgi-derived vesicles to the base of the cilium (Mazelova et al., 2009). Once the post-Golgi vesicles arrive at the ciliary base they fuse with the ciliary membrane. This process is thought to be facilitated by the BBSome (Nachury et al., 2007). At the cilium base, the membrane receptors are recognized by IFT particles and IFT begins.

Active kinesin-II is attached to IFT-A, which is associated with IFT-B (Ou et al., 2005). Axonemal precursors are bound to IFT-B. It is not known where cytoplasmic dynein 2 is attached during anterograde transport. Kinesin-II moves along the microtubule until it reaches the ciliary tip complex, where cargo protein unloading/loading and motor protein regulation occur (reviewed in (Sloboda, 2005)). It has been proposed that IFT-A and IFT-B dissociate from each other and this is essential for the reorganisation of the IFT particle (Iomini et al., 2001; Pedersen et

al., 2003; Perrone et al., 2003; Pedersen et al., 2005). It is then proposed that cytoplasmic dynein 2 is released into the ciliary tip complex, while kinesin-II becomes inactive. It is then suggested that IFT-A binds (directly or indirectly) to active cytoplasmic dynein, and IFT- B then binds to IFT-A. It is not known how cytoplasmic dynein 2 is activated. It is then proposed that kinesin-II then exits the cilium, while active cytoplasmic dynein 2 transports everything back from the tip to the cell body. The IFT cycle is completed when IFT components are returned to the cytoplasm for recycling or degradation (Pedersen et al., 2008).

1.7 Primary cilium biogenesis

Our current model for primary cilium biogenesis, as illustrated in Figure 1.5, is based on electron microscopic studies of tissues and organ cultures derived from rat lungs. Cilium biogenesis begins when the distal end of the mother centriole docks to a Golgi-derived vesicle (Sorokin, 1968) and the mother centriole converts to a basal body. Vesicle docking is mediated by the transition fibres, which are derived from the distal appendages of the mother centriole (Anderson, 1972; Deane et al., 2001). A bud emerges from the basal body, which bends the membrane (Sorokin, 1968). The bud elongates from the tip, allowing the axoneme to form. The base of the bud becomes the transition zone, which remains structurally intact throughout cilium biogenesis (Rosenbaum and Child, 1967; Boisvieux-Ulrich et al., 1989). The transition zone starts where the nine microtubule triplets become doublets, and contains Y-links which connect each doublet to the ciliary membrane (Muresan and Besharse, 1994). The axoneme reaches the cell surface and the ciliary membrane fuses with the plasma membrane, forming the ciliary necklace (Gilula and Satir, 1972). The nascent axoneme then elongates until it reaches a stable length (Ishikawa and Marshall, 2011). As no protein synthesis occurs within the cilium, the IFT machinery transports the components of the axoneme to the ciliary tip for assembly (Rosenbaum and Child, 1967).

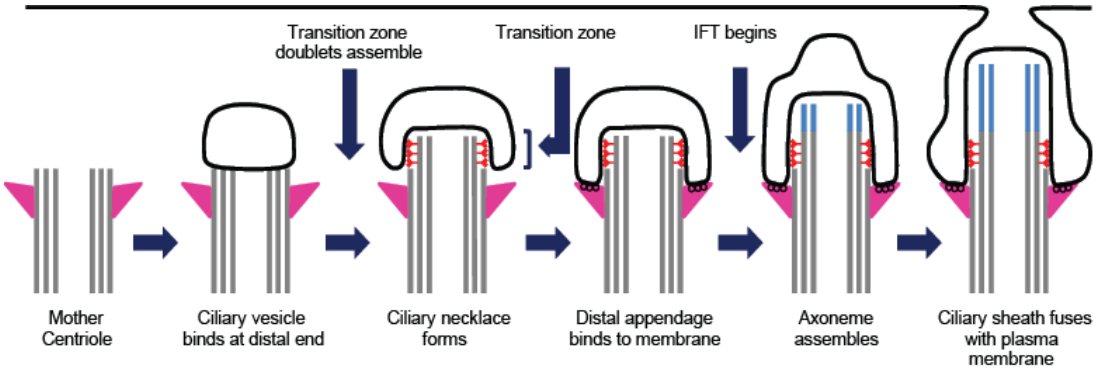

Figure 1.5 Primary cilium biogenesis.
A schematic representation of primary cilium biogenesis. Cilium biogenesis begins when the mother centriole attaches to a vesicle. The axoneme elongates from the tip, until it reaches the cell surface. The ciliary membrane fuses with the plasma membrane, externalising the primary cilium. Diagram adapted from (Rohatgi and Snell, 2010).

1.8 Regulation of ciliogenesis

Cilium assembly and disassembly are linked to cell cycle progression. Cilia are assembled in G1/G0 and are disassembled prior to mitosis. This model is based on experiments in mouse cells (Tucker et al., 1979). Different cell types have cilia of different length, suggesting that cilium length is subject to cell type-specific regulation (reviewed in (Satir and Christensen, 2008)). The factors which regulate primary cilium assembly and disassembly are summarised in Figure 1.6, and these processes are described in more detail below.

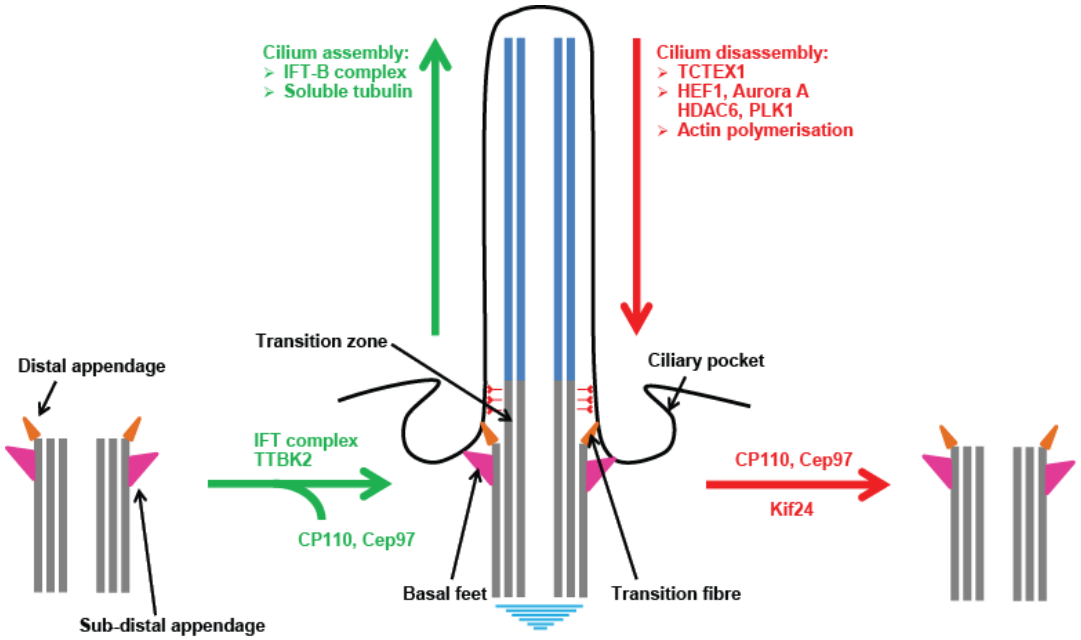

Figure 1.6 Summary of factors that regulate cilium assembly and disassembly.
A schematic representation of factors that regulate cilium assembly and disassembly. To assemble the cilium (green arrow) IFT components and TTBK2 are recruited to the basal body, and the ciliary inhibitors CP110, Cep97 and Kif24 are removed. Ciliary extension is promoted by the presence of IFT components and the availability of soluble tubulin. During cilium formation, several structural modifications occur. The transition zone and ciliary pocket are formed, and the distal and sub-distal appendages become transition fibres and basal feet, respectively. To disassemble the cilium (red arrow), CP110, Cep97 and Kif24 are recruited to the basal body. Several factors involved in IFT, axonemal deacetylation, axonemal microtubule polymerisation and actin polymerisation negatively regulate cilium assembly. Diagram adapted from (Kim and Dynlacht, 2013).

1.8.1 Assembly of the cilium

The exact molecular mechanisms which control primary cilium assembly are unclear, but there is some knowledge of the early stages of ciliogenesis. The capping of the distal end of the mother centriole with a ciliary vesicle and the docking of the basal body to the plasma membrane are mediated by the appendages. The distal appendage protein Cep123 (also known as Cep89 or CCDC123) is required for the assembly of primary cilia. In the absence of Cep123 ciliary vesicle formation fails. Cep123 interacts with PCM1 (pericentriolar protein 1), Cep290 and OFD1 (Oral-facial-digital syndrome 1), all of which play a role in primary ciliogenesis (Sillibourne et al., 2013). Pericentrin interacts with BBS4, which has been implicated in the tethering of IFT components to the basal body (Jurczyk et al., 2004; Nachury et al., 2007). CEP290 binds to PCM1 and the depletion of CEP290 disrupts the subcellular distribution and protein complex formation of PCM1. CEP290 and PCM1 are required for ciliogenesis and are involved in the targeting to the cilium of

Rab8, a small GTPase shown to associate with BBSome to promote ciliogenesis (Nachury et al., 2007; Kim et al., 2008). CEP290 and CEP72 regulate the localisation of BBS4 to the cilium which in turn affects the assembly and recruitment of the BBSome. CEP72 is a PCM1-interacting partner, required for the recruitment of CEP290 to the centriolar satellites. Depletion of PCM1 results in relocalisation of CEP72 and CEP290 to the centrosome and overexpression of CEP72 results in the aggregation of PCM1, CEP290 and BBS4. Localisation of BBS4 to the cilium is impaired by depletion of CEP72, resulting in the defective recruitment of BBS8 (Stowe et al., 2012).

ODF2 (Outer dense fibre 2, also known as cenexin) is localised to the distal and sub-distal appendages of the mother centriole (Lange and Gull, 1995; Nakagawa et al., 2001). In Odf2 knockout mouse cells, the distal appendages on the mother centriole are absent and the cells fail to form cilia (Ishikawa et al., 2005). Similarly, depletion of the distal appendage protein CEP164 (centrosomal protein of 164 kDa) results in failure to assemble cilia (Graser et al., 2007). CEP164 also interacts with the GTPase Rab8 and is essential for the docking of vesicles at the mother centriole (Schmidt et al., 2012).

CP110 (centriolar coiled-coil protein of 110 kDa) has been described as a negative regulator of ciliogenesis as its depletion promotes cilium formation in growing cells and in quiescent cells its overexpression inhibits their ability to form cilia (Spektor et al., 2007; Tsang et al., 2008). CP110 localises to the distal ends of both the mother and daughter centrioles (Chen et al., 2002b). During ciliogenesis CP110 is specifically eliminated from the mother centriole. The localisation of CEP97 has been shown to be strongly dependent on CP110, suggesting that CP110 recruits CEP97 to the centrosome. Depletion of CEP97 results in the loss of CP110 at the centrosome (Spektor et al., 2007). Kobayashi et al. identified the kinesin KIF24 as a CP110-CEP97 interaction partner. KIF24 depolymerises centriolar microtubules to prevent premature cilia assembly and the loss of KIF24 promotes ciliogenesis (Kobayashi et al., 2011). In addition, CP110 has also been shown to interact with CEP290 to prevent NPHP6 and Rab8a from initiating ciliogenesis (Tsang et al., 2008). Goetz et al. have shown that Tau tubulin kinase 2 (TTBK2) is essential for mouse SHh signalling and ciliogenesis. TTBK2 removes CP110 and

promotes the recruitment of IFT proteins, which build the ciliary axoneme (Goetz et al., 2012). Loss of the distal appendage protein CEP83 blocks centriole to membrane docking and that undocked centrioles fail to recruit TTBK2 or release CP110 (Tanos et al., 2013). The microRNA miR-129-3p has been shown to control cilia assembly by down-regulating CP110 and repressing branched F-actin formation. Blocking miR-129-3p inhibits serum starvation-induced ciliogenesis, while its overexpression induces ciliation in proliferating cells and promotes ciliary elongation (Cao et al., 2012).

CPAP (centrosomal P4.1-associated protein) has also been shown to be essential for primary cilium formation in neuronal cells. shRNA depletion of CPAP prevents ciliation and overexpression caused increased levels of primary cilium formation (Wu and Tang, 2012). ALMS1 (Alström syndrome 1) localises to the proximal end of centrioles and plays a role in ciliogenesis. siRNA depletion of ALMS1 leads to the formation of stunted primary cilia (Hearn et al., 2002; Collin et al., 2005; Li et al., 2007). Poc1 (Protein of Centriole 1) proteins are highly conserved and well characterised in *Chlamydomonas, Tetrahymena, Drosophila* and *Xenopus*. Poc1 localises to the proximal ends of centrioles and has two human paralogues, POC1A and POC1B. Depletion of POC1A does not affect primary cilium formation, while depletion of POC1B leads to reduced levels of primary cilia and shorter primary cilia. Truncation of *POC1A* causes primordial dwarfism, a ciliopathy. Fibroblasts from an individual with primordial dwarfism were shown to have reduced levels of ciliation and cilia were shorter than in control fibroblasts (Pearson et al., 2009; Shaheen et al., 2012).

1.8.2 Disassembly of the cilium

Mitogenic stimulation of quiescent cells promotes cell cycle re-entry and primary cilium disassembly in two waves. The first wave of disassembly occurs shortly after mitogenic stimulation in G1 phase and the second wave occurs 18–24 h after mitogenic stimulation (Pugacheva et al., 2007). PDGF (platelet-derived growth factor) induces cilium disassembly without DNA synthesis, and Ca^{2+} or FGF (fibroblast growth factor) can substitute for PDGF in 3T3 cells (Tucker et al., 1979). A PDGF receptor, PDGFαα, localises to the cilium in mouse cells. Ligand-dependent

activation of PDGFαα is followed by activation of Akt and Mek1/2–Erk1/2 pathways (Schneider et al., 2005). It was proposed that these pathways mediate the signal to induce primary cilium disassembly.

Intraflagellar transport has also been implicated in cilium disassembly. Flagellum resorption in *Chlamydomonas* is accompanied by an increase in the number of empty IFT particles moving in the anterograde direction, while IFT particles moving in the retrograde direction continue to return ciliary proteins to the cell body (Pan and Snell, 2005). IFT proteins are also required for the disassembly of the primary cilium in mammalian cells (Pugacheva et al., 2007), suggesting that IFT-mediated cilium resorption is conserved.

Several proteins have been shown to promote primary cilium disassembly. Stabilisation of HEF1 (Human enhancer of filamentation 1) by growth factors activates Aurora A kinase. Aurora A activates the tubulin deacetylase HDAC6 (histone deacetylase 6), which is localised to the basal body and ciliary axoneme, resulting in destabilisation of axonemal microtubules (Pugacheva et al., 2007). Pifo (Pitchfork), a protein which associates with ciliary targeting complexes and accumulates at the basal body during cilia disassembly, interacts with Aurora A and facilitates its activation (Kinzel et al., 2010). It has also been shown that PLK1 (polo-like kinase 1), which stabilises HEF1, may activate HDAC6 (Lee et al., 2012). A light chain subunit of cytoplasmic dynein Tctex-1 is recruited to the transition zone before S phase, where it controls cilia disassembly (Li et al., 2011). In addition, flagellar proteins have been found to be labelled with ubiquitin during flagellar resorption, suggesting that the ubiquitination system also plays a role in cilia/flagella resorption (Huang et al., 2009).

1.9 The primary cilium and the centrosome cycle

The centrosome cycle is composed of five main events (as shown in Figure 1.7): centriole disengagement, procentriole biogenesis, procentriole elongation, centrosome maturation and centrosome separation. The centrosome cycle begins in late mitosis or early G1 phase. The centrioles lose their orthogonal arrangement and the two centrioles become disengaged, but remain tethered by an intercentriole link.

Duplication begins in late G1-S phase where the new centrioles, termed procentrioles, assemble adjacent to the proximal end of each mother centriole. The procentrioles elongate and mature throughout S and G2 phase. The two newly duplicated centrosomes separate, with the PCM, during the G2-M transition. Each centrosome moves to opposite poles of the cell to organise the mitotic spindle (reviewed by (Meraldi and Nigg, 2002; Mattison and Winey, 2006; Nigg and Stearns, 2011)). Because the centrosome is involved in both cell division and ciliogenesis, these processes are mutually exclusive and tightly regulated. Cilia provide a block for cell cycle progression. Disassembly of the cilium is required to free the centrosome, allowing duplication during S-phase and subsequent formation of the mitotic spindle during chromosomal segregation (reviewed by (Pan and Snell, 2007; Nigg and Stearns, 2011; Basten and Giles, 2013)).

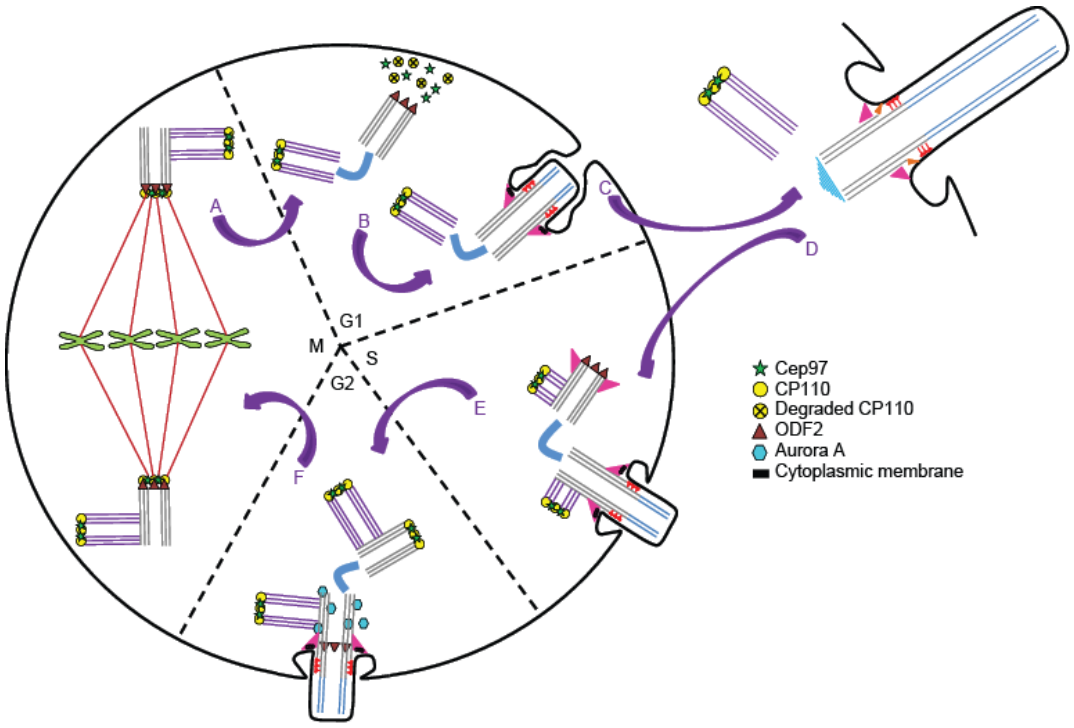

Figure 1.7 The centrosome cycle and cilium formation.
A. Upon entry into G1 phase, the cell has one centrosome composed of a mother and daughter centriole. **B.** In G1, in the absence of CP110 and the presence of ODF2, the mother centriole converts to a basal body. The basal body tethers to the membrane where it forms the axoneme. **C.** The cell exits the cell cycle, becomes quiescent and the cilium grows further. **D.** The cell re-enters the cell cycle. **E.** The centrioles duplicate in S phase. **F.** The centrosome matures at the G2/M transition, the primary cilium is resorbed and the centrioles move to the cell centre to participate in mitotic spindle assembly. Diagram adapted from (Bettencourt-Dias and Carvalho-Santos, 2008).

1.10 Centrosome duplication

Centriole duplication occurs by two mechanisms: semi-conservative duplication or *de novo* assembly. *De novo* centriole assembly typically occurs in specialised cells during ciliogenesis or in proliferating cells after removal of centrioles by laser ablation or microsurgery (reviewed by (Dawe et al., 2007; Vladar and Stearns, 2007)). Following laser ablation, new centrioles are assembled in electron-dense areas of pericentriolar-like material, suggesting that the PCM contains the precursors needed for centriole assembly. These centrioles are capable of duplicating in the next cell cycle. However, following the ablation of a single centriole, no extra centrioles were formed, indicating that the centrioles which are already present suppress the formation of *de novo* centrioles (Khodjakov et al., 2002; La Terra et al., 2005). In most ciliated and non-ciliated cells, centriole duplication occurs in a semi-conservative fashion. Centrosome duplication begins after the disengagement of centrioles in mitosis, a key licencing step for centrosome duplication, with procentrioles being formed next to pre-existing centrioles (reviewed by (Tsou and Stearns, 2006b)). For some time it was considered that these two mechanisms were fundamentally different. However, it was shown that procentrioles are not attached to the wall of the parental centriole, but, similar to *de novo* assembly, are generated in the PCM (reviewed by (Loncarek et al., 2007)). Both pathways are possible in somatic cells but semi-conservative duplication is more tightly regulated and is more efficient in ensuring correct centrosomal assembly. The organisation of the PCM, associated with the mother centriole, limits the number of procentrioles forming to one per cell cycle (Loncarek et al., 2008). Furthermore, procentrioles that are formed *de novo* after laser ablation exhibit structural aberrations (Khodjakov et al., 2002).

The components and mechanisms of centriole duplication were first identified in *Caenorhabditis elegans* (reviewed by (Leidel and Gönczy, 2005)). In *C. elegans*, the cartwheel provides a scaffold for procentriolar assembly. The cartwheel is a tubular structure consisting of nine symmetrical spokes in the centre, which is assembled near the base of each pre-existing centriole. The procentriole is elongated by the addition of microtubule triplets at the spokes. The spokes are necessary for ensuring the nine-fold symmetry of centrioles (reviewed by (Leidel and Gönczy,

2005)). RNA interference studies in *C. elegans* identified proteins which are essential for centriole duplication (reviewed by (Leidel and Gönczy, 2005)). SPD2 (Spindle defective protein 2) is recruited to the centrosome by a CDK2-dependent mechanism, before disengagement of the parental centrioles initiates procentriole assembly. The recruitment of SPD2 is responsible for the recruitment of ZYG1 (Zygote defective protein 1), which in turn permits the recruitment of the SAS5-SAS6 (Spindle assembly abnormal protein 5 and 6) complex (reviewed by (Delattre et al., 2006; Pelletier et al., 2006; Azimzadeh and Bornens, 2007)). SAS5 is required for the recruitment of SAS6 to procentrioles in *C. elegans* and both are essential for formation and elongation of procentrioles (Delattre et al., 2006). The assembly of microtubules to the spokes and the recruitment of the PCM are dependent on SAS4 (Kirkham et al., 2003; Leidel and Gönczy, 2003).

Homologues of these proteins have been identified in other species. There are conflicting reports as to whether SPD2 is essential for centriole duplication as the human orthologue of SPD2 (Cep192) has been shown to be essential for centriole duplication (Zhu et al., 2008), while the *Drosophila* orthologue (DSpd2) has been shown to be dispensable for this process (Dix and Raff, 2007). The human and *Drosophila* orthologues of ZYG1, PLK4 (Polo-like kinase 4) and SAK (Snk/Plk-akin kinase) respectively, are essential for centriole duplication (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). In human cells, SAS6 ensures that a single procentriole is formed adjacent to each mother centriole (Strnad et al., 2007). The human orthologue of SAS5, STIL/SIL (SCL/TAL1 interrupting locus), is recruited to procentrioles at the onset on centriole duplication (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). Both *de novo* and semi-conservative centriole duplication require PLK4, SAS6 and SAS5, therefore, these proteins may be indispensable for both mechanisms of centriole duplication (reviewed by (Rodrigues-Martins et al., 2008)).

1.11 Centrosome amplification

The control of centrosome structure and number is essential for the formation of a bipolar mitotic spindle. However, under certain conditions, dysregulation of this control results in the reduplication of centrosomes and subsequent centrosome

amplification. This can occur through several mechanisms: 1) DNA damage; 2) overexpression of centrosome duplication proteins; 3) viruses; 4) cytokinesis failure; 5) centrosome fragmentation or premature splitting. The mechanisms of centrosome amplification are described in more detail below.

1.11.1 DNA-damage induced centrosome amplification

Centrosome duplication and DNA replication are tightly coordinated cycles (reviewed by (Tsou and Stearns, 2006a; Strnad and Gönczy, 2008)). However, the centrosome cycle can become uncoupled from DNA replication in the presence of DNA damage. Following DNA damage, ATM/ATR activation leads to a G2/M checkpoint arrest and activation of the DNA damage response (DDR) (reviewed in (Sancar et al., 2004)). This results in activation of Chk1/Chk2 and inhibition of Plk1. This leads to Cdc25 inhibition which prevents dephosphorylation and activation of Cdk1. Cdk1 is required for mitotic entry and so DNA replication is stalled to allow for DNA damage repair, the centrosome cycle continues and this leads to centrosome amplification (Hochegger et al., 2007; Steere et al., 2011). Several DNA damage checkpoint proteins localise to the centrosome during the cell cycle, for example, ATM (Oricchio et al., 2006), ATR (Zhang et al., 2007), ATRIP (Zhang et al., 2007), CHK1 (Krämer et al., 2004), CHK2 (Takada et al., 2003), CDC25B (Dutertre et al., 2004), cyclin B/CDK1 (Jackman et al., 2003) and MCPH1 (Microcephalin 1) (Jeffers et al., 2008). Dysfunction of these proteins can lead to centrosome amplification (reviewed in (Shimada and Komatsu, 2009)). Centrosome amplification has also been observed in cell lines deficient in the DNA damage proteins BRCA1 (Xu et al., 1999), BRCA2 (Tutt et al., 1999), RAD51 (Dodson et al., 2004), XRCC2 and XRCC3 (Griffin et al., 2000).

p53 has been proposed to coordinate centrosome duplication and DNA replication, and prevent centrosome reduplication. Loss/mutation of p53 and centrosome amplification has been observed in various types of cancers (reviewed in (Feng et al., 2008)). However, it has also been shown that inactivation of p53 in some cell lines does not induce centrosome amplification (Bunz et al., 2002). Centrosome amplification was observed in mouse embryonic fibroblasts with inactive p53 but constitutively active CDK2/cyclin E, suggesting that control of

CDK2/cyclin E may also restrict centrosome amplification in human cells (Mussman et al., 2000). IR-induced centrosome amplification is reduced in CHK1-deficient cells compared to wild-type cells, indicating that CHK1 activity is needed for centrosome amplification (Bourke et al., 2007). In addition, following IR, CDK2 activity is up-regulated in wild-type but not *CHK1*$^{-/-}$ DT40 cells, indicating that CHK1 signalling can cause centrosome amplification by up-regulating CDK2 activity (Bourke et al., 2010).

1.11.2 Overexpression of centrosome duplication proteins and centrosome amplification

The number of procentrioles formed is limited by a process termed copy number control, which ensures that each mother centriole can only nucleate one procentriole. However, this control may be bypassed by overexpression of proteins involved in procentriole formation. Overexpression of PLK4, SAS5 or SAS6 leads to the formation of a single mother centriole surrounded by several procentrioles. Inhibition of PLK4, SAS5 or SAS6 prevents centriole duplication and leads to abnormal spindle formation (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Strnad et al., 2007; Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012).

1.11.3 Viruses and centrosome amplification

Centrosome amplification can also occur following viral infection. The human papillomavirus Type 16 (HPV-16) E6 and E7 oncoproteins can induce centrosome amplification through uncoupling centrosome duplication from the cell cycle. E6 oncoprotein causes the accumulation of centrosomes through proteasomal degradation of p53. E7 oncoprotein induces centrosome amplification through binding and degradation of Rb, inactivation of cyclin-dependent inhibitors such as p21, which causes dysregulated expression of CDK2/cyclin (reviewed by (Duensing et al., 2009)). The Kaposi sarcoma herpes virus (KSHV) encodes K-cyclin, a homologue of cyclin D. K-cyclins phosphorylate nucleophosmin (NPM1), which is required for the initiation of centrosome duplication. Expression of K-cyclin is associated with failure in cytokinesis, multinucleation and centrosome amplification (Verschuren et al., 2002; Cuomo et al., 2008).

1.11.4 Cytokinesis failure and centrosome amplification

Cytokinesis failure leads to the formation of cells with double the correct chromosome content and double the correct number of centrosomes. Unrepaired DNA damage, mitotic slippage, aberrant spindle assembly checkpoint (SAC) or the overexpression of oncoproteins can lead to division failure. Cells with p53 activate a checkpoint response if cytokinesis fails and will eventually undergo cell death (reviewed by (Fukasawa, 2008)). However, in the absence of p53, cells may continue to cycle and undergo repeated cytokinesis failures. Several oncogenes and tumour suppressor genes have been shown to induce tetraploidization and centrosome amplification. These include Aurora A (Meraldi et al., 2002), PLK1(Liu and Erikson, 2002), SAC components (Anand et al., 2003), survivin (Saito et al., 2008) and DNA response genes such as BRCA1, BRCA2, ATR, Rad51 and MDM2 (reviewed by (Fukasawa, 2008)).

1.11.5 Centrosome fragmentation and premature splitting

Centriole splitting occurs during G1 phase. If premature centrosome splitting occurs, each of the split centrioles may form individual centrosomes, which are capable of generating multipolar spindles (Hut et al., 2003). Human T cell leukaemia virus type-1 (HTLV-1) is an oncogenic virus which induces centrosome fragmentation in mitosis, creating centrosomal abnormalities (Peloponese et al., 2005). Depletion of kizuna, a centrosomal substrate of Plk1 and a structural component of centrosomes, may cause fragmentation and dissociation of the pericentriolar material from centrioles at prometaphase, resulting in multiple spindle poles (Oshimori et al., 2006).

In conclusion, centrosome amplification can arise through various mechanisms. In general, supernumerary centrosomes can result from either dysregulation of the centriole duplication cycle or failure of cytokinesis (reviewed by (Nigg and Stearns, 2011)).

1.12 The primary cilium as a sensory organelle

The major function of the primary cilium is to sense and transduce extracellular signals (reviewed in (Michaud and Yoder, 2006; Singla and Reiter, 2006; Berbari et al., 2009)). Appropriate activation of signalling molecules and receptors are important for controlling various cellular processes. Therefore, mutations in genes involved in signalling through the primary cilium result in a broad range of disorders such as developmental defects, polycystic kidneys, skeletal malformations and obesity (reviewed in (Baker and Beales, 2009)). Cilium-based signal transduction pathways that have essential roles in tissue development and homeostasis in adults are: 1) Sonic Hedgehog; 2) Wnt; 3) PDGF (platelet-derived growth factor); 4) Notch (reviewed in (Seeger-Nukpezah and Golemis, 2012)).

1.12.1 Sonic Hedgehog signalling

Hh signalling has diverse roles in animal development, tissue homeostasis, pain perception, metabolism and interacts with other signalling pathways (Babcock et al., 2011; Polizio et al., 2011; Büller et al., 2012; Chen et al., 2012; Wang et al., 2012; Briscoe and Thérond, 2013). There are three ligands in the Hh signalling family: Sonic hedgehog (SHh), Indian Hedgehog (IHh) and Desert Hedgehog (DHh) (Echelard et al., 1993). They are synthesised as precursor proteins that are processed into two fragments, an amino-terminal (HhN) and a carboxyterminal (HhC) polypeptide. HhN mediates Hh signalling; the function of HhC is not yet known (Porter et al., 1995). Release of Hh requires DISP (Dispatched), a large transmembrane protein which transports Hh across the plasma membrane (Burke et al., 1999).

The cellular receptor of Hh is PTCH, a large transmembrane protein. The amount of SHh available for binding to PTCH is regulated by Hh binding proteins, such as the positive regulator HHIP (Hh-interacting protein) and the negative regulator GAS1 (Growth arrest-specific gene 1) (Beachy et al., 2010). CDO (Commodo) and BOC (Brother of Commodo) facilitate the calcium dependant binding of Hh to PTCH1 (McLellan et al., 2008). GPC3 (Glypican 3) interacts with Hh ligand and has been implicated in inhibiting the Hh pathway by competing with PTCH for Hh binding (Capurro et al., 2008). SUFU (Suppressor of fused) sequesters

the inhibitory Glioma (GLI) protein complex to the axoneme microtubules and negatively regulates GLI transcription factor activity (Stone et al., 1999).

A schematic representation of Hh signal transduction through the primary cilium is presented in Figure 1.8. Signalling through the Hh pathway requires the controlled access of the Hh signal transducer SMO (Smoothened) to the primary cilium (Corbit et al., 2005). In the absence of Hh ligand, PTCH localises to the base of the cilium. PTCH inhibits the activity of SMO and prevents its trafficking to the cilium. In the presence of Hh signalling, Hh binds to PTCH1 and is internalised, relieving the inhibition of SMO and allowing it to move from intracellular vesicles. Activated SMO translocates to the cilium and decouples GLI proteins from the inhibitory complex, leading to their activation. GLI proteins translocate to the nucleus where they activate transcription of Hh target genes. There are three GLI proteins which belong to the Kruppel family of zinc finger transcription factors: GLI1, GLI2 and GLI3. GLI1 acts as a transcriptional activator, while GLI3 acts mainly as a repressor. GLI2 acts both as an activator and repressor, depending on the cellular level of SMO-regulated proteins (Stecca and Ruiz I Altaba, 2010). The balance of the activator and repressor functions of the three GLI factors determines the cellular response. Genomic analysis has identified several hundred GLI target genes (Harris et al., 2011).

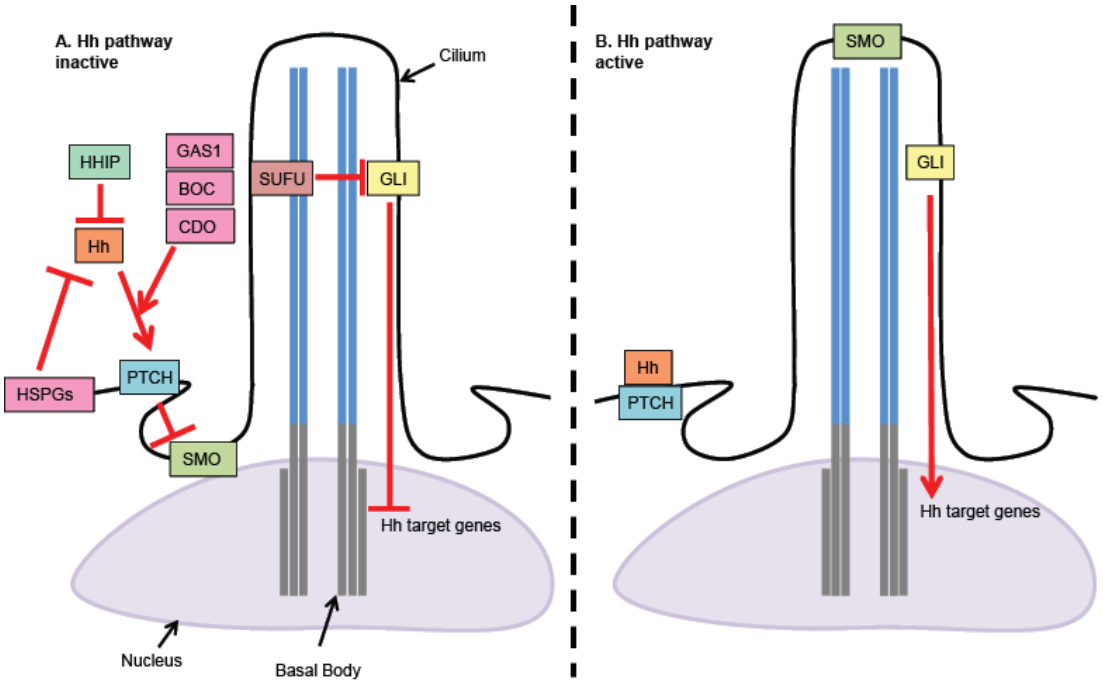

Figure 1.8 Hedgehog signal transduction through the primary cilium.
A. In the absence of Hedgehog (Hh) ligand, Patched (PTCH) inhibits Smoothened (SMO) activation and trafficking to the cilium. Suppressor of fused (SUFU) inhibits Glioma (GLI) proteins from entering the nucleus, repressing transcription. Hh binding is regulated by cell surface proteins. Hh-interacting protein (HHIP) and heparan sulfate proteoglycans (HSPGs) compete with the Hh-binding while GAS1 (Growth arrest-specific gene), CDO (Commodo) and BOC (Brother of Commodo) proteins facilitate Hh-binding to PTCH. B. Binding of Hh to PTCH releases the inhibition of SMO. SMO moves to the tip of the cilium. GLI proteins then translocate to the nucleus to activate target genes.

1.12.2 Wnt signalling

Intracellular signalling of the Wnt pathway is classified into two categories: the non-canonical pathway and the canonical/β-catenin pathway (Komiya and Habas, 2008). The canonical Wnt pathway is involved in cell proliferation, differentiation, adhesion and survival (Dravid et al., 2005; Kirstetter et al., 2006; Lee et al., 2006b; Schlange et al., 2007). This pathway is activated when Wnt ligands bind to Fz (Frizzled) or the LDL (Low-density lipoprotein) receptor-related proteins 5/6, which in turn, activate Dvl (Dishevelled) (Cong et al., 2004; González-Sancho et al., 2004; Zeng et al., 2008). Dvl inhibits GSK3 (Glycogen synthase kinase 3) and prevents phosphorylation and destruction of β-catenin (van Noort et al., 2002). This allows β-catenin to accumulate in the cytoplasm before translocating to the nucleus where it activates TCF/LEF transcription factors (Behrens et al., 1996; Molenaar et al., 1996). This leads to the transcription of Wnt target genes such as *c-Myc*, *Tcf1*

and *CyclinD1* (He et al., 1998; Roose et al., 1999; Tetsu and McCormick, 1999). In the absence of Wnt ligands, β-catenin is phosphorylated and destroyed (Ha et al., 2004). Non-canonical Wnt signalling diversifies into several pathways including the Wnt-calcium pathway and the planar cell polarity (PCP) pathway (Komiya and Habas, 2008). The Wnt-calcium pathway is involved in embryonic dorsal-ventral patterning, regulating cell migration and development (Westfall et al., 2003; Garriock et al., 2005; Garriock and Krieg, 2007; Cheng et al., 2008). PCP is involved in cell polarity, migration and orientation during embryogenesis. Recent reports suggest that the primary cilium can act as a switch between canonical and non-canonical signalling, with overexpression of the ciliary protein, Invs (Inversin) inhibiting canonical Wnt signalling and inhibition of Invs resulting in loss of PCP signalling (Simons et al., 2005).

1.12.3 PDGF signalling

Platelet-derived growth factor (PDGF) signalling is required for embryogenesis (Schatteman et al., 1992), inflammation (Tak and Firestein, 2001) and wound healing (Chung et al., 2009). Aberrant PDGF signalling has been implicated in cancer. The PDGF pathway consists of four ligands (PDGF-A, B, C, D) and two receptors (PDGFα and PDGFβ). PDGFα localises to the primary cilium in mouse embryonic fibroblasts (MEFs). The ligands form homodimers (and AB heterodimers) and bind to two receptors simultaneously, resulting in activation of the receptors. This activation allows the binding of intracellular signalling molecules to autophosphorylated tyrosine residues on the receptors and initiates signalling cascades, such as the Akt and ERK (extracellular signal-regulated kinase) pathways (reviewed by (Michaud and Yoder, 2006; Zafiropoulos et al., 2008)). The primary cilium plays a critical role in growth control via PDGFRα, which localizes to the cilium during growth arrest in NIH-3T3 cells and MEFs (Schneider et al., 2005).

1.12.4 Notch signalling

Notch signalling is required for development, stem cell maintenance and proliferation (reviewed in (Hori et al., 2013)). The heterodimeric Notch receptor and its ligands are transmembrane proteins with large extracellular domains that consist primarily of epidermal growth factor (EGF)-like repeats. The Notch receptor on the

responding cell binds to ligands from the signalling cell (Bray, 2006; Kopan and Ilagan, 2009). Ligand binding promotes two proteolytic cleavage events in the Notch receptor that promote transcription of Notch target genes (Bray, 2006; Kopan and Ilagan, 2009). The primary cilium is involved in Notch signalling and epidermal differentiation during skin development (Ezratty et al., 2011). Ablation of cilia in mice, by shRNA-mediated knockdown of intraflagellar transport proteins (IFTs) and conditional knockout of *Ift88* and *Kif3a* during embryogenesis, resulted in cell hyperproliferation and defects in Notch signalling (Ezratty et al., 2011). In addition, hyperactivation of Notch signalling results in the elongation of cilia (Lopes et al., 2010).

In conclusion, the primary cilium acts as a signalling centre that mediates cell-to-cell communication by sensing extracellular signals during development. However, as discussed in more detail below, dysregulation of these pathways with advanced aging may contribute to age-related diseases.

1.13 The primary cilium and senescence

Cilium formation is closely regulated by and linked to the cell cycle, as cilia must be resorbed to allow the mitotic functioning of centrosomes in bipolar spindle formation. Cellular quiescence, a temporary exit from the cell cycle that can be induced by the removal of growth factors, facilitates ciliogenesis (Seeley and Nachury, 2010; Kobayashi and Dynlacht, 2011). However, the impact of cilia on replicative senescence, an essentially permanent exit from the cell cycle, and whether there is any relationship between the cilium, senescence and degenerative diseases have not been extensively studied. However, there is accumulating evidence which suggests that signalling through the primary cilium has an anti-aging effect and that dysfunctional signalling with advanced age may be a potential trigger for senescence.

C. elegans sense environmental signals through cilia located at the dendritic endings of sensory neurons. Mutations affecting components of sensory cilia or sensory signal-transduction pathways have been shown to extend the mean lifespan of *C. elegans*. These worms feed and reproduce normally and have normal rates of

development (Apfeld and Kenyon, 1999). Genetic epistasis experiments indicate that sensory neurons influence lifespan, to an extent, by regulating the insulin/IGF-1 (Insulin-like growth factor 1) signalling pathway. The proposed model is that an environmental signal triggers the sensory neurons to secrete an insulin/IGF-1-like hormone that binds to the DAF-2 receptor and accelerates the ageing process. When the environmental signal is absent, the hormone is not secreted, the level of DAF-2 activity is decreased and lifespan is extended (Kenyon et al., 1993; Kimura et al., 1997; Guarente and Kenyon, 2000).

Recent studies have shown that Hh down-regulation is associated with senescence (Bishop et al., 2010) and age-related diseases such as type 2 diabetes, neurodegeneration, atherosclerosis and osteoporosis (Thomas et al., 2000; Hurtado-Lorenzo et al., 2004; Beckers et al., 2007). Although aging is a multifactorial process, a reduced propensity to regenerate tissues contributes to the process and this appears to be a consequence of reduced integrity of stem cell compartments in the body (Rando and Chang, 2012). Their maintenance requires the continuous replenishment of stem cells (Weissman, 2000). Hh signalling has been shown to be essential for stem cell maintenance and senescence has been associated with reduced stem cell function (Liu et al., 2006; Han et al., 2008; Gao et al., 2009; Michel et al., 2012). Hh signalling promotes mitogenesis by suppression of the growth-inhibitory p16, which is a well-established mediator of senescence (Alcorta et al., 1996). A fragment of GLI2 (Gli family zinc finger 2) was found to directly bind and inhibit the *p16* promoter and loss of GLI2 was associated with the induction of stem cell senescence (Bishop et al., 2010). The influence of Hh signalling on stem cells may also suggest why activation of the pathway has been found in many human tumours. The primary cilium can either activate or repress tumourigenesis depending on the nature of the oncogenic initiating event, playing a dual role in activating and repressing Hh signalling (Han et al., 2009; Wong et al., 2009).

The Wnt signalling pathway has also been shown to be involved in maintenance of stem cells in various tissues and organs during development and adult life (Wang and Wynshaw-Boris, 2004; Duncan et al., 2005; Miki et al., 2011). Recent results have shown that the Wnt signalling pathway is involved in regeneration (Kawakami et al., 2006; Stoick-Cooper et al., 2007) and the aging

process. Muscle stem cells from aged mice convert from a myogenic to a fibrogenic lineage as they begin to proliferate. This conversion is associated with the activation of the Wnt signalling pathway in aged myogenic progenitors and can be suppressed by Wnt inhibitors (Brack et al., 2007). Furthermore, analysis of tissues and organs from young *Klotho*-deficient mice, a mouse model of accelerated aging, showed a decrease in stem cell number, an increase in progenitor cell senescence and an increase in Wnt signalling. Ectopic expression of Klotho antagonised Wnt activity and accelerated cellular senescence (Liu et al., 2007a).

Notch signalling has also been shown to be involved in senescence. An increase in senescent human dental pulp cell numbers was observed after Notch inhibition (Zou et al., 2010). Expression of Notch3 was elevated in senescent cells and this up-regulation was required for the induction of *p21* expression in senescent cells. Down-regulation of Notch3 leads to delayed onset of senescence and extended replicative lifespan. Deletion of *p21* in cells decreased Notch3-induced senescence. A decrease in Notch3 expression was observed in tumour cells and restoration of Notch3 expression in these cells resulted in inhibition of cell proliferation and activation of senescence (Cui et al., 2013).

The SASP is involved in a complex signalling network in which the secreted factors affect not only the cells producing them (autocrine effects), but also the microenvironment and hence neighbouring cells (paracrine effects) (Kuilman and Peeper, 2009). Senescent cells have been postulated to contribute to degenerative changes through the SASP (Campisi et al., 2011). For example, senescence and associated SASP of astrocytes can promote the age-related neurodegeneration that results in Alzheimer's and Parkinson's diseases (Bitto et al., 2010; Salminen et al., 2011). Furthermore, the presence and SASP of senescent chondrocytes, which are abundant in age-related osteoarthritis (OA), are thought to contribute to the pathogenesis of this disease (Roberts et al., 2006; Shane Anderson and Loeser, 2010). Interestingly, an increase in primary cilium length was observed in primary chondrocytes post treatment with IL-1, which is up-regulated in OA (Wann and Knight, 2012). These findings provide a correlative link between the primary cilium and the SASP. It is therefore possible that the primary cilium is involved in this complex signalling network.

Taken together, these results suggest that the primary cilium may have a role in the ageing process and a better understanding of the cilium structure and function in senescent cells may lead to the prevention of age related diseases.

1.14 Aims of this project

In this project we aimed to study the impact of replicative senescence on centrosome/cilium structure and formation in primary human fibroblasts, or conversely, the impact of the centrosome/cilium structure and formation on replicative senescence. Previously-published observations in other human fibroblast cell lines have shown that there is an increase in the number of amplified centrosomes in senescent cells. The primary cilium is derived from the basal body, a mother centriole-derived structure and cilium formation is facilitated by cellular quiescence, a temporary exit from the cell cycle. However, the impact of senescence on cilia has not been described.

Given the relationship between the centrioles and the primary cilium, we wished to investigate whether amplified centrosomes gave rise to multiple cilia in senescent cells. We wished to explore if ciliary regulators were differentially expressed in BJ cells as they become senescent. The primary cilium is essential for Hh signalling and Hh down-regulation has been associated with the induction of senescence. We investigated whether inhibition of Hh would have an effect on cilia in senescent cells. We also aimed to test if manipulation of the cilium had any effect on cellular senescence.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical reagents

Chemicals used in this project were of analytical grade and were purchased from Sigma-Aldrich (Arklow, Ireland), BDH (Hertfordshire, UK), Fisher (Leicestershire, UK) or GE Healthcare Life Sciences (Buckinghamshire, UK). All solutions were prepared using ddH_2O or Milli-Q purified water, Millipore (Billerica, USA) and where appropriate, were autoclaved or filtered before use. Organic solvents, alcohols and acids were supplied by Sigma-Aldrich (Arklow, Ireland), VWR (Bridgeport, USA) or Fisher (Leicestershire, UK). Oligodeoxynucleotide primers were purchased from Sigma-Aldrich. All reagents and buffers used in this project are listed in Table 2.1.

Table 2.1 Reagents and buffers

Name	Composition	Purpose
3 X SDS-PAGE sample buffer	150mM Tris pH 6.8, 45% sucrose, 6mM K-EDTA (ethylenediaminetetraacetic acid) pH 7.4, 9% SDS (sodium dodecyl sulphate), 0.03% bromophenol blue 10 % β-mercaptoethanol	Denaturation and loading of proteins for SDS-PAGE
6 X DNA loading dye	20% sucrose, 0.1 M EDTA pH 8.0, 1% SDS, 0.25% bromophenol blue, 0.25% xylene cyanol.	DNA sample loading for running agarose gels
Blocking solution 1	1 X phosphate buffered saline (PBS), 0.05% Tween-20, 5% skimmed milk	Reduction of non-specific antibody binding in immunoblotting
Blocking solution 2	1 X PBS, 1% bovine serum albumin (BSA)	Reduction of non-specific antibody binding in immunofluorescence
DABCO	2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO), 50 mM Tris base pH 8, 90% Glycerol	Mounting slides
DEPC water	0.1% diethyl pyrocarbonate (DEPC) in ddH_2O	RNA work
Fixing solution	Chilled methanol, 5 mM ethylene glycol tetraacetic acid (EGTA)	Fixation of cells for immunofluorescence microscopy

Incubation buffer 1	1X PBS, 0.05% Tween-20, 1% skimmed milk	Dilution of antibodies in immunoblotting
Incubation buffer 2	1X PBS, 1% BSA	Dilution of antibodies in immunofluorescence
LB (Luria-Bertani Medium)	1% tryptone, 0.5% yeast extract, 1% NaCl, pH adjusted to 7.0 with 4 M NaOH	Bacterial culture
PBS	2.68 mM KCl, 1.47 mM KH_2PO_4, 136.9 mM NaCl, 8.1 mM Na_2HPO_4	General buffer
PBS-Tween	1x PBS with 0.1% Tween-20	Washing western blots
Phosphatase inhibitors (50x)	2.5 mM NaF, 1.8 mM β-glycerophosphate, 0.5 mM Na_3VO_4, 2.4 mM EGTA, 12.5 mM sodium pyrophosphate	Inhibition of phosphatase enzymes in immunoblotting
Ponceau S. solution	0.5% Ponceau S, 5% acetic acid	Staining of proteins on nitrocellulose membrane
Protease inhibitors (100x)	3 μM leupeptin, 10 μM pepstatin A, 4.9 mM PMSF (phenylmethylsulfonyl fluoride), 10.5 mM benzamidine, 10 μM antipain, 6.6 μM chymostatin (dissolved in dimethyl sulfoxide, DMSO) in ethanol	Inhibition of protease enzymes during sample preparation for immunoblot
Radio-immunoprecipitation assay (RIPA) buffer	50 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 0.25% Na-Deoxycholate, 1 mM EDTA (protease and phosphatase inhibitors added before use)	Lysis of cells and extraction of proteins from total cell extracts
Running buffer	25 mM Tris, 250 mM glycine, 0.1% SDS	Running SDS-PAGE gels
Semi-dry transfer buffer	25 mM Tris pH 8.5, 0.2 M glycine, 20% methanol	Semi dry transfer
Senescence associated (SA)-β-Gal staining solution	40 mM citric acid/sodium phosphate buffer, 5 mM $K_4[Fe(CN)_6]$ $3H_2O$, $K_3[Fe(CN)_6]$, 150 mM NaCl, 2 mM $MgCl_2$, 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	Detection of senescent cells
TAE (Tris-acetate EDTA)	40 mM Tris-acetate pH8.0, 1 mM EDTA	Running agarose gels
TG (Tris-glycine)	25 mM Tris base, 192 mM Glycine, pH 8.3	Making running/transfer buffer
Tfb I (Transformation buffer I)	30 mM CH_3CO_2K, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol; pH adjusted to 5.8 with 50% HCl, filter sterilised and stored at 4°C.	Preparation of competent *E. coli*
Tfb II (Transformation buffer II)	10 mM 3-propanesulfonic acid (MOPS), 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol, pH adjusted to 6.5 with KOH, filter sterilised and stored at 4°C.	Preparation of competent *E. coli*

2.1.2 Molecular biology reagents

Unless otherwise stated, the biological reagents used for DNA digestion and cloning reactions, such as restriction enzymes, DNA polymerase (Klenow Fragment I) and DNA ligase, were obtained from New England Biolabs (Ipswich, USA). The DNA polymerases TaKaRa LA Taq and KOD, used in PCR, were purchased from Takara Shizo Co. Ltd. (Osaka, Japan) and Novagen (Darmstadt, Germany). Deoxyribonucleoside tri-phosphates (dNTPs) used in cloning were purchased from Sigma-Aldrich (St. Louis, USA). Shrimp Alkaline Phosphatase (SAP) was from USB (Cleveland, USA). DNA 1kb ladder was supplied by Invitrogen (Carlsbad, USA) and PageRuler Plus protein ladder was supplied by Thermo Scientific (Waltham, USA). Molecular biology kits used throughout this project are listed in Table 2.2.

Table 2.2 Molecular biology kits

Name	Use	Source
GenElute Plasmid Miniprep Kit	Small scale plasmid DNA isolation	Sigma (Arklow, Ireland)
Midi/Maxi Prep Kit (Endotoxin-free)	Large scale plasmid DNA isolation	Macherey-Nagel (Dueren, Germany)
QIAquick Gel Extraction Kit	Extraction and purification of DNA Fragments from the agarose gel	Qiagen (Crawley, UK)
SigmaSpin sequencing reaction clean-up	Purification of DNA fragments	Sigma
High Capacity RNA to cDNA Kit	cDNA synthesis	Applied Biosystems (Foster City, USA)
RT^2 First Strand Kit	For cDNA synthesis and genomic DNA elimination in RNA samples	Qiagen
RNeasy Mini Kit	For purification of total RNA from cells	Qiagen

DNA transformation was performed on competent *Escherichia coli* Top10 cultures. The strain used in this research project has the following genotype: F*mcrAΔ(mrr-hsdRNS-mcrBC) φ80lacZΔM15 ΔlacX74deoR recA1 araD139 Δ(araleu)7697 galU galK rpsL(StrR) endA1 nupG. E. coli* clones were selected using ampicillin or kanamycin antibiotics (Sigma) at the final concentrations of 50μg/ml or 30μg/ml, respectively. The cloning and expression plasmids used in this project are shown in Table 2.3.

Table 2.3 Commercial plasmids

Plasmid Name	Use	Source
pGEMT-Easy	General cloning	Promega (Southampton, UK)
pEGFP-C1/N1	Expression in mammalian cells	Clontech (Palo Alto, USA)

All reagents and kits used in qRT-PCR such as RNAse/DNAse free water, Human Primary Cilia RT² Profiler PCR Arrays, Human RT² Primer Assays and RT² SYBR Green qPCR Mastermix were obtained from Qiagen. The geNorm kit was purchased from Primer Design (Southampton, UK). Oligodeoxynucleotide primers for CP110 were also obtained from Primer Design. Real-time PCR was performed using the ABI 7500 Fast system controlled by an Applied Biosystems 7500 Real Time PCR System.

2.1.3 Antibodies

Primary and secondary antibodies (Table 2.4 and 2.5) were used in immunoblotting (IB) detection and immunofluorescence (IF) microscopy. Tables 2.4 and 2.5 show the clone/reference number, host species, working dilutions and source of these antibodies.

Table 2.4 Primary antibodies used in this study

Antigen	Clone/ Reference number	Host Species	Working dilution for IB	Working dilution for IF	Source
Actin	A2066	Rabbit polyclonal	1:5000	-	Abcam (Cambridge, UK)
Acetylated-tubulin	T6793	Mouse monoclonal	-	1:2000	Sigma Aldrich
Arl13b	17711-1-AP	Rabbit polyclonal		1:500	Proteintech (Chicago, USA)
α-tubulin	B512	Mouse monoclonal	1:10000	1:2000	Sigma Aldrich
Centrin2	poly6288	Rabbit polyclonal	-	1:500	Biolegend (San Diego, USA)
Centrin3	3E6	Mouse monoclonal	-	1:1000	Abnova (Taipei, Taiwan)
Cep135	ab75005	Rabbit polyclonal	-	1:1000	Abcam
Cep170	-	Rabbit polyclonal	-	1:500	Giulia Guarguaglini (Guarguaglini *et al*., 2005)

CP110	Q-12	Rabbit monoclonal	1:100	-	Santa Cruz (Dallas, USA)
Gli1	L42B10	Mouse monoclonal	1:100	-	Cell Signalling (Danvers, USA)
Gli2	C-10	Mouse monoclonal	1:200	-	Santa Cruz
Kizuna		Rabbit polyclonal	-	1:500	Naoki Oshimori (Oshimori et al., 2006)
Pericentrin	ab4448	Rabbit polyclonal	-	1:1000	Abcam
Rad51	PC130	Rabbit polyclonal	-	1:100	Calbiochem (San Diego, USA)
Rootletin	Q15	Goat polyclonal	-	1:250	Santa Cruz
Smo	1D9	Mouse monoclonal	1:100	-	Cell Signalling
Tri-Methyl-Histone H3 Lys9	#9754	Rabbit polyclonal	-	1:800	Cell signalling
VangL2	PA5-18654	Goat polyclonal	1:1000	-	Thermo Scientific
γ-H2AX	JBW301	Mouse monoclonal	1:1000	1:1000	Millipore
γ-tubulin	T3559	Rabbit polyclonal	-	1:500	Sigma Aldrich

Table 2.5 Secondary antibodies used in this study

Conjugation/ Serial Number	**Reactivity**	**Host Species**	**Working dilution for IB**	**Working dilution for IF**	**Source**
HRP (horseradish peroxidase) 705-035-003	Goat IgG (H & L)	Donkey	1:5000	-	Jackson Labs (West Grove, USA)
HRP 111-035-003	Rabbit IgG (H & L)	Goat	1:5000	-	Jackson Labs
HRP 115-035-003	Mouse IgG (H & L)	Goat	1:5000	-	Jackson Labs
FITC (fluorescein isothiocyanate) 705-095-003	Goat IgG (H & L)	Donkey	-	1:200	Jackson Labs
FITC 711-095-152	Rabbit IgG (H & L)	Donkey	-	1:200	Jackson Labs
Alexa 594 715-585-150	Mouse IgG (H & L)	Donkey	-	1:1000	Jackson Labs
Cy5	Rabbit IgG (H & L)	Goat	-	1:50	Abcam

2.1.4 Tissue culture reagents and cell lines

All sterile plasticware used for tissue culture was obtained from Sarstedt (Numbrecht, Germany) and Sigma. Dulbecco's modified eagle medium (DMEM) was used in cell culture. Tissue culture reagents such as trypsin, newborn calf serum (NCS), penicillin and streptomycin (pen/strep), serum-free OptiMEM and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Foetal bovine serum (FBS) was purchased from Sigma-Aldrich or Gibco (Carlsbad, USA). Cells were frozen down for both -80^{o}C and liquid nitrogen storage in FBS with 10% DMSO. For DNA and siRNA transfection, Lipofectamine was purchased from Invitrogen. Table 2.6 shows the cell lines used during this project, along with their culture medium and growth conditions.

Table 2.6 Cell lines and growth conditions used during this project

Cell Line	Description	Source	Culture Medium	Growth Conditions
BJ	Primary normal human foreskin fibroblast cells	ATCC (Middlesex, UK)	DMEM, 10% FBS, 1% Pen/Strep	37°C, 5% CO_2
NHDF	Primary normal human dermal fibroblast cells	ATCC	DMEM, 10% FBS, 1% Pen/Strep	37°C, 5% CO_2
MRC5	Primary normal human lung fibroblast cells	ATCC	DMEM, 10% FBS, 1% Pen/Strep	37°C, 5% CO_2

Drugs used for pharmacological treatment of cells can be found in Table 2.7.

Table 2.7 Drugs used in this project

Drug	Solvent	Purpose	Stock Conc.	Final Conc.	Source
Chloral hydrate	H_2O	Removal of cilia	1 M	4 mM	Fisher Scientific
Cyclopamine	Ethanol	Inhibition of sonic hedgehog signalling	1 mM	20 μM	Santa Cruz

siRNA was used to deplete expression of genes of interest. The genes targeted with siRNA and the target gene sequences are shown in table 2.8.

Table 2.8 siRNA sequences used during this project

Target Gene	siRNA sequence	Final Conc.	Source
***CP110* #1**	Sense 5' GCAAAACCAGAAUACGAGATT 3' Antisense 5' UCUCGUAUUCUGGUUUUGCAT 3'	50 nmol	Ambion (Carlsbad, USA)
***CP110* #2**	Sense 5' CAAGCGGACUCACUCCAUATT 3' Antisense 5' UAUGGAGUGAGUCCGCUUGAG 3'	50 nmol	Ambion
GAPDH	Sense 5' UGGUUUACAUGUUCCAAUATT 3' Antisense 5' UAUUGGAACAUGUAAACCATG 3'	50 nmol	Ambion

2.1.5 Computer Software

DNA plasmid maps were created using pDRAW32 software (Acaclone, http://www.acaclone.com). Sequenced DNA samples were viewed using Chromas software (version 2.1.1, Technelysium Pty Ltd., Brisbane, Australia). For bioinformatic analyses, BlastN or BlastP (http://www.ncbi.nlm.nih.gov/BLAST) and ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) were used. Microscopy imaging was performed using an Olympus BX-51 microscope, driven by Volocity software (version 6.2.1, Improvision, PerkinElmer, Waltham, USA). Deconvolved images were saved as Adobe Photoshop images (version 10, San Jose, USA). Statistical analysis of microscopy data was carried out on Prism 5 (Graphpad, La Jolla, USA).

2.2 Nucleic Acid Methods

The nucleic acid methods and techniques used in this project are described in (Sambrook and Russell, 2001).

2.2.1 RNA preparation

RNA was isolated from 10 x 10^6 adherent cells. Cells were pelleted at 250 *g* for 5 minutes and resuspended in 1 ml of TRIzol (Total RNA Isolation Reagent, Invitrogen). Using filter tips and DEPC treated reagents, total RNA was extracted after cell lysis according to the manufacturer's instructions. The RNA pellets were air-dried for 5 minutes, resuspended in 20 µl of 0.1% DEPC-treated water and

incubated at 55°C for 10 minutes for good re-suspension. RNA was quantified using a NanoDrop 2000c spectrophotometer and stored at -80°C.

2.2.2 Reverse Transcriptase-PCR (RT-PCR)

cDNA synthesis was performed using the High Capacity RNA to cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. The first-strand cDNA was generated using 1µg of total RNA (extracted in section 2.5.1), Oligo(dT) primers and synthesised according to the manufacturer's instructions. PCRs were carried out as described in section 2.2.3.

2.2.3 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was carried out using either KOD or Takara LA Taq polymerases depending on the experiment performed. PCR experiments were carried out on a TGradient (Biometra, Göttingen, Germany). The sequences of all the primers used in this project are shown in table 2.9. Table 2.10 gives an example of the PCR conditions and programmes used for sequence amplification.

Table 2.9 Primers used for PCR-based cloning and Real-Time PCR

	Primer Name	**Sequence (5' to 3')**
Primers used in the cloning of *CP110 isoform 1* cDNA	*CP110* Iso1 F	CCGTCGACATGGAGGAGTATGAGAAG
	CP110 Iso1 R	CCGGATCCAATTGTCGCAACATTGG
Real-Time PCR primers for *CP110*	CP110	GGACCAAGTGCTCTCAAAGG
		TCTGAAAGCTGCCGTTTAGTT

Table 2.10 Typical PCR reaction and conditions

Reagents	**TaKaRa La Taq**	**KOD**	**PCR steps**	**TaKaRa La Taq**	**KOD**
Buffer (10x)	1x	1x	**'Hot Start'**	94°C – 1 min	94°C – 2 min
Primers	0.2 µM	0.2 µM	**Denaturation**	98°C – 10 sec	94°C – 1 min
dNTPs	200 µM	200 µM	**Annealing**	58-68°C – 30 sec	58 – 68°C 30 sec
$MgCl_2$	2.5 mM	2.5 mM	**Extension**	68°C – 3 min	72°C – 2 min
Enzyme	0.5 µl (5 U/ µl)	0.5 µl (5 U/ µl)	**Final Extension**	72°C - 10 min	72°C - 10 min
			No. of cycles	30	30

2.2.4 Plasmid DNA preparation

Mini and midi plasmid DNA isolation was carried out using GeneElute Plasmid MiniPrep Kit (Sigma) and NucleoBond Xtra Midi Kit Endotoxin free (Macherey-Nagel), respectively. In both procedures, plasmid DNA was prepared according to the manufacturer's instructions. Briefly, bacterial cell cultures were grown overnight at 37°C with shaking, in the presence of selective antibiotics (referred to in section 2.2). For mini plasmid preparations 2 ml of an overnight *E. coli* culture were used and the DNA pellet was resuspended in 30-100 µl MilliQ water. For midi plasmid preparations 50 ml of the overnight *E. coli* culture were used and the DNA pellet was resuspended in 100-300 µl MilliQ H_2O. DNA was quantified using a NanoDrop 2000c spectrophotometer and stored at -20°C.

2.2.5 Restriction digest of DNA

All restriction enzymes used for digestion of DNA were supplied by New England Biolabs (NEB). The reactions were performed using the 10 x buffer provided and bovine serum albumin (BSA, 0.1 mg/ml) where required. Digestions were performed at the manufacturer's indicated temperatures on a thermo-stable heat block for 2-16 hours depending on the amount of DNA being digested. Where appropriate, the enzyme was inactivated by incubation at 65°C for 20 minutes.

2.2.6 Preparation of DNA for cloning

Digested DNA used for cloning was purified with SigmaSpin^{TM} Sequencing Reaction Clean-Up columns (Sigma) to remove restriction enzyme(s) and traces of buffer. To reduce self-ligation, digested DNA was dephosphorylated on the 5'ends with shrimp alkaline phosphatase (SAP, 1U / pmol of DNA ends) at 37°C for 1 hour, followed by a 20 minute heat inactivation step at 65^{o}C. Ligations were performed using T4 DNA ligase in 1X T4 DNA ligase buffer at 4°C overnight or at room temperature for 4 hours. An excess of insert over plasmid was generally used (1:3 to 1:10, depending on the DNA concentration, as estimated by agarose gel electrophoresis). Reactions were then transformed into competent *E. coli* cells.

2.2.7 Preparation of competent *E. coli* and transformation

To prepare competent *E. coli* cells (see section 2.1.2), a 5 ml culture was grown overnight with shaking in LB broth at 37°C. This culture was then added to 500 ml of LB broth and shaken at 37°C until the culture reached an OD_{600} of 0.35-0.4. In order to obtain highly efficient competent cells, all subsequent steps were performed in a cold room with all reagents and equipment chilled to 4°C. The *E. coli* cells were incubated on ice for 5 minutes and pelleted by centrifugation at 5000 *g* for 15 minutes at 4°C and the supernatant was decanted. The pellet was resuspended in ice cold Tfb I (see Table 2.1, 40 ml per 100 ml culture). The cells were spun and re-suspended in ice cold Tfb II (see Table 2.1, 4 ml per initial 100 ml culture). The cells were incubated on ice for 15 minutes, snap frozen in liquid nitrogen and stored at -80°C.

2.2.8 *E. coli* transformation

For transformations, 50 µl of competent *E. coli* cells were thawed on ice, mixed with DNA/ligation mix and incubated on ice for 20 minutes. Cells were then heat shocked at 42°C for 90 seconds and then allowed to recover on ice for 90 seconds. 1 ml of LB broth was added to the cells and gently shaken for 30 minutes at 37°C. For ligations, cells were pelleted by centrifugation at 16,000 *g* for 1 minute and the entire culture plated onto agar plates containing appropriate antibiotics. In the case of plasmid DNA transformations, 100 µl of such culture was plated onto agar plates. Plates were incubated at 37°C overnight. Colonies were picked and grown overnight in LB broth cultures with antibiotics at 37°C and used for plasmid DNA preparation the next day (see section 2.2.4).

2.2.9 Agarose gel electrophoresis and purification of DNA

0.7-1.0% agarose gels were prepared using Sigma electrophoresis grade agarose in 1 x TAE buffer containing 0.5 µg/ml ethidium bromide (see table 2.1). Gels were run in 1 x TAE buffer in Hoefer HE33 tanks (Mini Horizontal Submarine Unit, GE Healthcare Life Sciences). DNA was visualised using a Multi Image Light Cabinet (ChemiImager 5500, Genetic Technologies Inc., Miami, USA) and images were taken using a digital camera. For DNA extraction, bands were cut out of the agarose gel with a scalpel blade. DNA was purified using the Qiagen QIAquick Gel

Extraction Kit according to the manufacturer's instructions. DNA was eluted in 20-50 µl MilliQ water.

2.2.10 Sequencing

DNA samples were sent to Source Bioscience (Dublin, Ireland) for commercial sequencing. In general, 250 ng of DNA (mini or midi prepped) and 5 – 10 pM primers were used per reaction. Analysed sequences were used to construct correct vector maps with the pDRAW32 (Acaclone, www.acaclone.com) software.

2.2.11 Quantitative Real Time PCR

RNA was extracted by direct addition of lysis buffer to cultured cells using a Qiagen RNeasy mini kit. 1 µg of RNA was converted to cDNA using the Qiagen RT² First Strand Kit. Real-time PCR was performed using Qiagen Human Primary Cilia RT² Profiler PCR Arrays on an ABI 7500 fast system following the standard Qiagen PCR array SYBR Green protocol. The amplification conditions used were 15 s at 95°C and 60 s at 60°C for 40 cycles. C_T values for the housekeeping genes and for the genes of interest were measured in proliferating and senescent samples. Relative gene expression was analysed as outlined in (Livak and Schmittgen, 2001). The C_T values for each gene of interest were normalised to the housekeeping genes:

ΔC_T Proliferating = [C_T(Proliferating) - Average C_T(Housekeeping Genes)]

ΔC_T Senescent = [C_T(Senescent) - Average C_T(Housekeeping Genes)]

The fold change was calculated using the formula:

$$\frac{2^{(-\Delta C_T)}\ \text{Senescent}}{2^{(-\Delta C_T)}\ \text{Proliferating}}$$

Where the fold change is greater than 1, the result represents a fold up-regulation in gene expression. Where the fold change is less than 1, the negative

inverse of the result represents a fold down-regulation. The standard deviations were also calculated in the same way. Subsequent qPCR analysis was carried out using individual Qiagen RT² qPCR primers for *ACTB*, *SF3A1*, *GLI2*, *VANGL2*, *SMO* and *LRP2* or primers for *CP110* (Primer Design).

2.2.12 geNorm kit

Relative gene expression for each gene of interest was obtained by normalising their expression to a stably expressed control gene. The most stable endogenous controls for our experiments were chosen by using the Primer Design geNorm kit. This provides 11 housekeeping genes that are normally stably expressed (*GADD45A, EIF4A2, CYC1, UBC, SDHA, YWHAZ, GAPDH, ATP5B, 18SrRNA, ACTB, SF3A1*). The stability of expression of these genes was tested by standard real-time PCR and analysis with the geNorm software. C_T values were converted into relative quantification (RQ) values by subtracting the highest C_T value from all other C_T values for each gene and applying the formula: Relative expression = $2^{-\Delta CT}$. All data were expressed relative to the least stable gene. The relative expression data are then input into the geNorm software to determine the general stability of the genes analysed (Vandesompele et al., 2002).

2.3 Protein Methods

The protein methods and techniques used in this project are described in (Simpson et al., 2008).

2.3.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were prepared for SDS-PAGE as follows. Adherent cells were detached from the surface of the tissue culture dish by trypsinisation and scraping with a cells scraper. The cells were centrifuged at 160 *g* for 5 minutes, washed once in 1 X PBS and centrifuged again at 160 *g* for 5 minutes. The PBS was removed and the cells were resuspended in 20-50 µl lysis buffer containing protease and phosphatase inhibitors. The cells were lysed for 60 minutes on ice before centrifugation at 16,000 *g* for 10 minutes at 4°C. The supernatant was removed and transferred to a fresh eppendorf tube. The protein concentration was determined

using the Bradford assay (see section 2.3.2). 3X sample buffer supplemented with 10% β-mercaptoethanol was added to the samples and boiled for 5 minutes at 95°C. The samples were either stored at -20°C or loaded on a gel. In general, 20-40 μg of protein was loaded per lane. The different percentages of gel mixes used are shown in Table 2.11.

Table 2.11 Upper gel mixes and stacking gel for SDS-PAGE

Gel Percentage	**8%**	**10%**	**Stack**
Acrylamide/bis-acrylamide ratio	37.5:1	37.5:1	37.5:1
Acrylamide	7.8%	9.73%	5%
Bis-acrylamide	0.2%	0.27%	0.13%
Tris-HCl pH 8.8	375 mM	375 mM	-
Tris-HCl pH 6.8	-	-	125 mM
Sodium dodecyl sulphate (SDS)	0.1%	0.1%	0.1%
Ammonium persulphate (APS)	0.1%	0.1%	0.05%
Tetramethylethyenediamine (TEMED)	0.6%	0.4%	1%

2.3.2 Bradford Protein Assay

To determine protein concentration, the Bradford dye-binding protein assay was employed as described by (Bradford, 1976). 1 μl of protein extract was added to a 1:1 dilution of Bradford : MilliQ water in a plastic cuvette. The absorbance at 595 nm was measured with a spectrophotometer (Eppendorf, Hamburg, Germany). The protein concentration was calculated based on a BSA (bovine serum albumin) standard curve, in which absorbance was plotted *vs.* varying concentrations of the BSA protein.

2.3.3 Semi-dry transfer

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare) by semi-dry transfer. Transfer of gels was performed using a Semi Dry Transfer Unit (GE Healthcare, Bucks, UK) according to the manufacturer's instructions. Briefly, the SDS-PAGE gel was placed on a nitrocellulose membrane between 3 sheets of Whatman paper, which had been soaked in transfer buffer. The semi-dry transfer was carried out at room temperature for 1-2 hours at amperage that depended on the size of the gel (1 mA per cm^2 of gel).

2.3.3 Western blotting

After transfer, the membrane was rinsed three times in dH_2O and stained with Ponceau S solution to visualise the quality of protein transfer and protein loading. The stain was removed by washing for 5 minutes in dH_2O. To decrease non-specific binding of antibodies, the membrane was blocked with 5% milk in 1 X PBS-Tween for 30 minutes at room temperature with gentle rocking. The membrane was then incubated with gentle rolling at 4°C overnight in primary antibody at the concentrations shown in Table 2.4. The membrane was then washed three times for 5 minutes in 1 X PBS-Tween and incubated with secondary antibody (see Table 2.5) in 3% milk for 45 minutes at room temperature with gentle rocking. 3 X 5 minute washes in 1 X PBS-Tween were performed. The proteins were detected with an ECL detection kit (GE Healthcare or Millipore) according to the manufacturer's instructions. This was followed by autoradiograph film exposure (Hartenstein, Germany) and development using a developing machine (CP 1000, AGFA, Brentford, UK).

2.4 Cell Biology Methods

2.4.1 Tissue culture techniques

The cells used for this study were cultured as described in Table 2.6. Adherent human cells reached confluency at a cell density of 7 X 10^6 cells/75 cm^2 flask (80% confluency). Cells were passaged by washing in 1 X PBS and trypsinising in 2X trypsin for 5 minutes in the 37°C incubator. Once cells were detached, the trypsin was inactivated by adding pre-warmed medium to return the culture to its original volume. In general, a 1:5 dilution of the cell suspension was made for continued cultivation of the cells. For the freezing of cell stocks, 2 X 10^6 cells/vial were harvested and resuspended in 1 ml of freezing medium (90% complete medium, 10% DMSO), before transferring to cryo-vials. These were stored at -80°C for a week before being transferred to liquid nitrogen for long-term storage. Cells were irradiated using a ^{137}Cs irradiator (Mainance Engineering, Hampshire, UK), at a dose rate of 23.5 Gy/minute. Cells were serum starved by washing twice in warm 1 X PBS before the addition of medium containing 0.1% newborn calf serum (NCS).

2.4.2 Transient transfection

Cells were split the day before transfection and plated at an appropriate density to yield 70% confluent cells the next day. On the day of transfection, two mixtures were made for each reaction. 4 µg of plasmid DNA was mixed with 250 µl serum-free OptiMEM. In a separate tube, 4 µl Lipofectamine 2000 was mixed with 250 µl serum-free OptiMEM. The two reactions were incubated at room temperature for 5 minutes, mixed together and incubated for a further 20 minutes to allow for the formation of lipid-DNA complexes. The cells were washed three times with 1 X PBS and the media replaced with serum-free OptiMEM, as serum can inhibit the formation of lipid-DNA complexes. The lipid-DNA mixture was added drop-wise to the cells, rocked gently and returned to the 37°C incubator. 6 hours post transfection, media containing 10% FBS and 1% Pen/Strep was added to the cells. Transfected cells were analysed 48 hours post transfection.

2.4.3 RNA mediated interference

RNA mediated interference was carried out using Silencer Select siRNAs (Ambion) to knock down messenger RNA transcripts for the proteins listed in table 2.8. *GAPDH* was used as a positive control. As a negative control, a non-targeting short interfering pool of RNA duplexes were used (Dharmacon, Lafayette, CA). Cells were seeded the day prior to transfection so that a density of 50-60% would be obtained at the time of transfection. siRNAs were resuspended in RNA-free water to yield a concentration of 20 µM. The siRNAs were transfected using Lipofectamine 2000 and as outlined in section 2.4.2.

2.5 Microscopy Methods

The microscopy methods and techniques used in this project are described in (Harlow and Lane, 1999). Adherent cells were plated and grown on UV-sterilised coverslips for 24 hours before performing immunofluorescence microscopy. In order to visualise the primary cilia using an acetylated tubulin antibody, the cells must be incubated on ice for 30 minutes prior to fixation and staining. This deacetylates the microtubules and allows for the visualisation of the highly acetylated primary cilia (Piperno et al., 1987).

2.5.1 Methanol fixation

Medium was removed and the cells were fixed (and permeabilised) for 10 minutes in 95% methanol supplemented with 5 mM EGTA at -20°C. The cells were then washed three times for 5 minutes in 1 X PBS before proceeding to immunofluorescence microscopy.

2.5.2 Paraformaldehyde fixation

Media was removed and the cells were fixed for 10 minutes at room temperature in 4% paraformaldehyde (PFA) in 1 X PBS. Cells were washed three times for 5 minutes in 1 X PBS and permeabilised at room temperature in 0.15% Triton X-100 in 1 X PBS for 2 minutes. The cells were then washed three times in 1 X PBS before proceeding to immunofluorescence microscopy.

2.5.3 Immunofluorescence microscopy

Cells were fixed in methanol or PFA as described above before staining with the antibodies listed in table 2.4. To decrease non-specific binding of antibodies, the cells were blocked in 1% BSA in PBS and incubated with primary antibodies for 1 hour at 37°C followed by a 45 minute incubation at 37°C with secondary antibodies. Coverslips were mounted in DABCO supplemented with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI), sealed with nail varnish and stored at 4°C in the dark. Counting/imaging were performed using an Olympus BX51 microscope, using a 100 X oil (NA 1.35) objective, using Velocity software. Serial Z-sections (0.2 µm) were taken, deconvolved, merged and saved as Photoshop TIFF files. Cilia length was measured manually using the "Line Tool" in Volocity.

2.5.6 Senescence associated β-galactosidase (SA-β-Gal) staining

This procedure was described by (Dimri et al., 1995). Medium was removed, the cells were fixed for 5 minutes at room temperature in PFA and washed three times for 5 minutes in 1 X PBS. Where immunofluorescence microscopy was to be performed immediately after the SA-β-Gal staining, the cells were permeabilised at room temperature in 0.15% Triton X-100 in 1 X PBS for 2 minutes. The cells were then washed three times for 5 minutes in 1 X PBS. The SA-β-Gal staining solution

(see table 2.1) was added to the cells and incubated in a CO_2-free incubator at 37°C for 12-16 hours. The cells were then washed three times for 5 minutes in 1 X PBS before either washing the cells once with methanol and allowing to air dry or proceeding with immunofluorescence microscopy. The coverslips were sealed with nail varnish and stored in the dark at -20°C.

CHAPTER 3 – THE PRIMARY CILIUM AS A REGULATOR OF SENESCENCE IN HUMAN FIBROBLASTS

3.1 Introduction

In response to certain forms of stress, cells can enter an irreversible state of growth arrest, termed senescence (Collado et al., 2007). Senescence was first described by Leonard Hayflick nearly 50 years ago and occurs after a fixed number of cell divisions in culture, marking the end of the proliferative capacity of a cell. Senescent cells remain metabolically active and can be maintained in culture for long periods of time (Hayflick and Moorhead, 1961). Senescent cells accumulate in the body with age (Jeyapalan et al., 2007). Senescence can arise through persistent DNA damage signalling, through telomeric sequences that can become exposed after multiple cell divisions or in response to strong mitogenic signalling induced by oncogenes (d'Adda di Fagagna et al., 2003; Herbig et al., 2004; Campisi and d'Adda di Fagagna, 2007; Rodier and Campisi, 2011; Fumagalli et al., 2012).

Cancer development requires cell proliferation (Hanahan and Weinberg, 2000) and so senescence is beneficial in early age as it prevents tumourigenesis. However, failure to senesce is usually insufficient for malignant transformation. For example, telomerase prevents telomere-induced senescence but does not cause cells to become malignant (Morales et al., 1999). On the other hand, senescence can become detrimental to aging cells. Senescent cells secrete common proteins that can alter or inhibit the normal function of neighbouring cells (Acosta et al., 2013). They can also stimulate the proliferation and malignant transformation of nearby premalignant cells (Parrinello et al., 2005). Therefore, senescence can both supress cancer and contribute to aging.

Cancer is a disease of aging and centrosome abnormalities and aneuploidy play an important role in cancer development and progression. Boveri proposed that centrosome overduplication and subsequent malformation of mitotic spindles may result in asymmetric cell division, leading to chromosome instability in cancer cells (Boveri, 2008). Previously-published observations in other human fibroblast cell lines have shown that there is an increase in the number of amplified centrosomes in senescent cells (Ohshima and Seyama, 2010). Mitotic centrosomes are composed of

two barrel-shaped centrioles linked together at their proximal ends and embedded in a pericentriolar matrix. Centrioles contain a nine-fold arrangement of triplet microtubules that taper to doublets at their distal end. After cell division, the individual centrioles dissociate to serve as templates on which new centrioles will form during the next S phase (Nigg and Stearns, 2011). This generates a new daughter centriole which is located adjacent to each of the pre-existing mother centrioles. The mother centriole can be distinguished by its distal and sub-distal appendages which are required for primary cilium formation and anchoring (Bornens, 2002; Graser et al., 2007). Most cells have a type of cilium known as a primary cilium (Pazour and Witman, 2003). Primary cilia are highly conserved, non-motile organelles. They extend from the basal body, which is derived from the mother centriole (Gerdes et al., 2009; Goetz and Anderson, 2010; Kobayashi and Dynlacht, 2011). The cilium core, the axoneme, consists of 9 microtubule doublets that extend from the basal body. The axoneme is surrounded by phospholipid membrane and a diffusion barrier maintains a cilium-specific distribution of proteins within this membrane subregion (Hu et al., 2010). Most primary cilia are formed during G0/G1 and are resorbed prior to mitosis (Tucker et al., 1979). Cells must exit the mitotic cycle to allow the centrioles to migrate and dock to the cell surface for axoneme nucleation (Tanos et al., 2013). Extension of the ciliary axoneme and membrane is mediated by a process called intraflagellar transport. This moves structural components from the cell body to the cilium tip (Pedersen and Rosenbaum, 2008; Silverman and Leroux, 2009).

Primary cilia sense and transduce various extracellular signals, such as Wnt and Hedgehog (Hh) (Pazour and Witman, 2003). Cell signalling through the primary cilium is essential for normal cell proliferation. Ablation of cilia in mice, by shRNA-mediated knockdown of intraflagellar transport proteins (IFTs) and conditional knockout of *Ift88* and *Kif3a* during embryogenesis, results in cell hyperproliferation and defects in Notch and Sonic Hedgehog signalling (SHh) signalling (Ezratty et al., 2011). SHh signalling is important for the proliferation and development of postnatal hippocampal progenitors (Han et al., 2008). Stumpy is a protein which localises to the primary cilium and is required for ciliogenesis. Mutant mice conditionally homozygous for *Stumpy* in astrocyte-like neural precursor (ALNP) cells have

decreased numbers of cilia. This led to abrogated SHh activity and increased cell cycle exit (Breunig et al., 2008). While these studies provide evidence that signalling is required for normal cell proliferation, the impact of primary cilium signalling on senescence, or conversely, the primary cilium on senescence has been largely unexplored.

Recently, there has been increased interest in the role of primary cilium signalling in the progression of age-related diseases. Hedgehog (Hh) down-regulation has been associated with senescence (Bishop et al., 2010) and age-related diseases such as type 2 diabetes, neurodegeneration, atherosclerosis and osteoporosis (Thomas et al., 2000; Hurtado-Lorenzo et al., 2004; Beckers et al., 2007). Although aging is a multifactorial process, a reduced propensity to regenerate tissues contributes to the process and this appears to be a consequence of reduced integrity of stem cell compartments in the body (Rando and Chang, 2012). Hh signalling has been shown to be essential for stem cell maintenance (Liu et al., 2006; Han et al., 2008; Gao et al., 2009; Michel et al., 2012). The Wnt signalling pathway is also involved in the aging process. Muscle stem cells from aged mice convert from a myogenic to a fibrogenic lineage as they begin to proliferate. This conversion is associated with the activation of the Wnt signalling pathway in aged myogenic progenitors and can be suppressed by Wnt inhibitors (Brack et al., 2007). Notch signalling has also been shown to be involved in senescence. Expression of Notch3 was elevated in senescence cells and this up-regulation was required for the induction of p21 expression in senescent cells (Cui et al., 2013). These results suggest that signalling through the primary cilium has an anti-aging effect and that dysfunctional signalling with advanced age may be a trigger for senescence.

The impact of senescence on cilia, or vice versa, has not been extensively studied. In this chapter we study the impact of replicative senescence on the formation and structure of the primary cilium in primary human fibroblasts. We investigate the effect of senescence on the expression levels of ciliary components. Finally, we test if manipulation of the cilium has any effect on cellular senescence.

3.2 Results

3.2.1 BJ, MRC5 and NHDF cells enter senescence in culture after extensive passaging

We tested the impact of senescence on cilia. To do this we used three normal primary human fibroblast cell lines which have been shown to senesce in culture - BJ, MRC5 and NHDF (Normal Human Dermal Fibroblast) cells. Replicative senescence was achieved by serial passaging of the cells until they stopped proliferating. Cell morphology changes are associated with senescence. Senescent cells become large, flat and granular (Chen and Ames, 1994). We observed these morphological changes in our cells (data not shown); however, we wanted to confirm that our cells were senescent in more detail. We calculated the population doubling times of the cells at five time-points. As shown in Figure 3.1, over an extended culture period, their doubling times eventually increased to an extent where the bulk of the population were no longer proliferating. This result suggested the cells we were using were senescent.

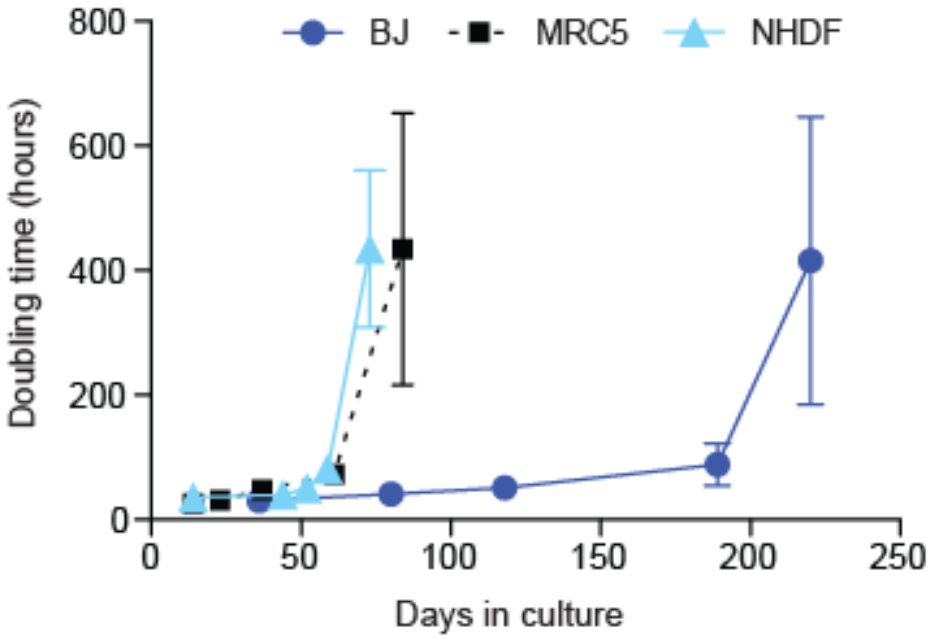

Figure 3.1 Doubling times of BJ, MRC5 and NHDF cells increase over extended culture periods. Cells were plated and counted at 5 time-points. The population doubling time was calculated using the formula Doubling Time = $T*\ln_2/\ln_{(Xe/Xb)}$ where: T is the incubation time in any units, Xb is the cell number at the beginning of the incubation time and Xe is the cell number at the end of the incubation time (Freshney, 2005). Histograms show means $\pm$ s.d. of 3 separate experiments.

To confirm that the growth arrest observed was due to the induction of replicative senescence, we tested for senescence-associated β-Galactosidase (SA-β-Gal) activity. This is a commonly used biomarker for replicative senescence (Dimri et al., 1995; van der Loo et al., 1998; Price et al., 2002; d'Adda di Fagagna et al., 2003; Matthews et al., 2006; Debacq-Chainiaux et al., 2009). β-galactosidase (β-Gal) activity can be detected in most mammalian cells by a cytochemical assay, carried out at pH 4 (Dimri et al., 1995). The β-Gal assay is based on the production of a blue precipitate, resulting from the cleavage of the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). Dimri et al. observed an increase in β-Gal staining in senescent cells at suboptimal pH 6. This was not detectable in presenescent, quiescent or immortal cells under identical culture conditions (Dimri et al., 1995).

BJ, MRC5 and NHDF cells were fixed and stained for 12 hours with X-Gal and, as shown in Figure 3.2, we observed an increase in blue staining in over 80% of cells which were extensively passaged. This confirmed that the cells we were using were senescent.

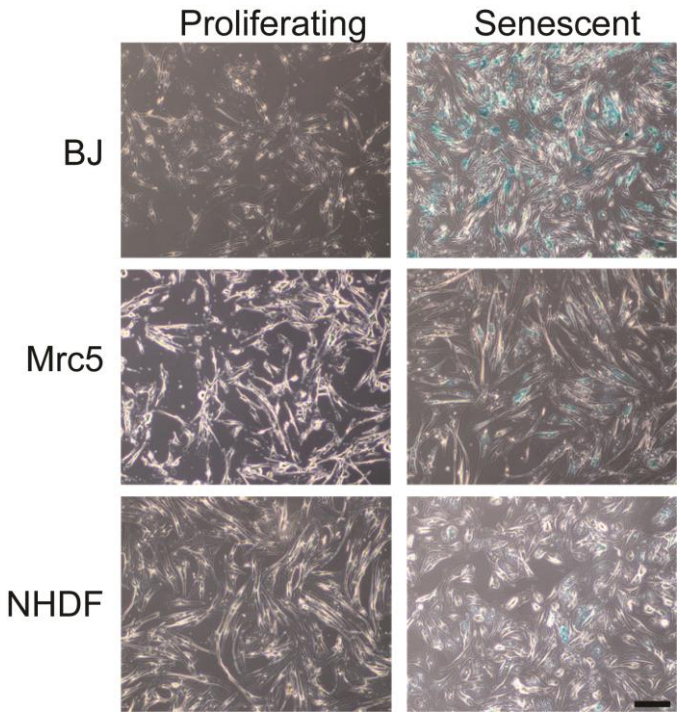

Figure 3.2 Increased SA-β-Gal activity in BJ, MRC5 and NHDF cells after extended passaging. BJ, MRC5 and NHDF cells were fixed and incubated with the chromogenic β-Gal substrate X-Gal in the SA-β-Gal staining solution at pH 6.0 for 12 hours. Scale bar, 100 μm.

Senescence is also characterised by the formation of senescence associated heterochromatic foci (SAHF). When senescent cells are stained with 4',6-diamidino-2-phenylindole (DAPI), they display a punctate staining pattern where chromosomes appear to be individually compacted into foci. SAHF are specifically enriched in histone H3 trimethylated at lysine 9 (H3K9me3, a modification catalysed by the methyltransferase Suv39h1), its binding partner heterochromatin protein-1γ (HP- 1γ) and the chromatin-bound high mobility group protein A2, HMGA2 (Narita et al., 2006). The linker histone H1 is lost in SAHF (Funayama et al., 2006; Narita et al., 2006). Alterations in chromatin structure are believed to contribute to the irreversible nature of senescence by sequestering and silencing genes associated with cell cycle entry and proliferation. Senescent cells show increased binding of heterochromatin-associated proteins in the promoters of some E2F target genes, such as cyclin A (Narita et al., 2003).

BJ, MRC5 and NHDF cells were fixed and stained with an antibody against histone H3 trimethylated at lysine 9 to stain the SAHF. As shown in Figure 3.3, immunofluorescence microscopy showed an increase in H3K9me3 staining in cells which were extensively passaged. This result further confirmed that the cells we were using were indeed senescent.

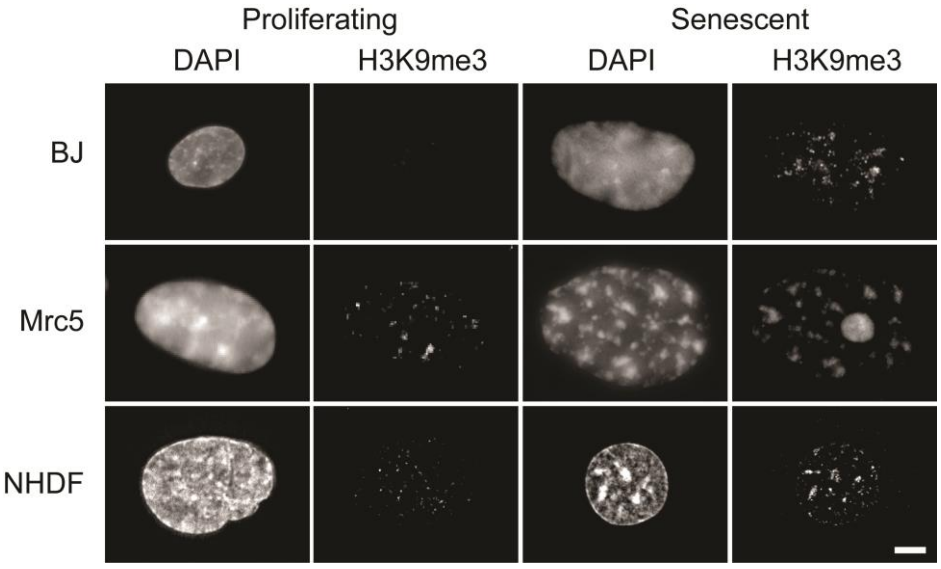

Figure 3.3 Increased H3K9me3 staining in BJ, MRC5 and NHDF cells after extended passaging. Immunofluorescence microscopy of the indicated cell lines stained with an antibody against trimethyl-histone H3 Lys9 (green), used to visualise the senescence-associated heterochromatic foci (SAHF). DNA was visualised with DAPI (blue). Scale bar, 10 μm.

Based on the calculated population doubling time, H3K9me3 and SA-β-Gal staining, throughout the rest of this project we defined the following as senescent: BJ: ≥250 days in culture; MRC5: ≥100 days; NHDF: ≥80 days. Proliferating cells were defined as: BJ: <120 days in culture; MRC5: <30 days; NHDF: <20 days.

3.2.2 Senescent human fibroblasts have an increased frequency of cilia and these cilia are consistently longer than those in proliferating fibroblasts

Primary cilium formation is facilitated by cellular quiescence (Tucker et al., 1979), a temporary exit from the cell cycle, but the impact of senescence on cilia has not been described. We wished to investigate whether senescence might have an impact on centrosome/cilium formation or structure.

To first determine whether senescence has an effect on primary cilium formation, the frequency with which primary cilia occur in proliferating and senescent BJ, MRC5 and NHDF cell populations after no treatment or serum starvation was determined. Proliferating and senescent cells were fixed and stained for acetylated tubulin, which localises to the cilium and centrosomes, to distinguish between ciliated and non-ciliated cells (Poole et al., 2001). We co-stained with γ-tubulin, which localises to the centrosome (Stearns et al., 1991). As shown in Figure 3.4, we found that a significantly higher frequency of senescent BJ, MRC5 and NHDF cells had a primary cilium compared to proliferating controls. 42% of the untreated senescent BJ cell population had a primary cilium, 10% of MRC5 and 15% of NHDF. This is in comparison to the untreated proliferating controls where 13% of the BJ cell population had a primary cilium, 3% of MRC5 and 7% of NHDF. Little additional ciliation occurred when the populations were induced to quiesce by serum starvation. 38% of the serum-starved senescent BJ cell population had a primary cilium, 15% of MRC% and 27% of NHDF. 11% of serum-starved proliferating BJ cells had a primary cilium, 6% of MRC5 and 10% of NHDF. These results suggest that senescence may have an impact on the number of cells which have a primary cilium.

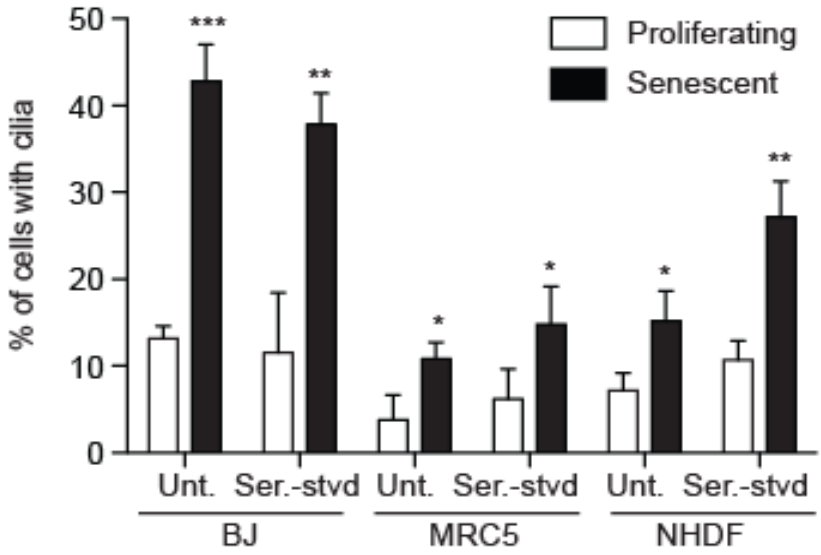

Figure 3.4 Increased frequency of cilia in senescent human fibroblasts.
Quantitation of the ciliation frequency in cells of the indicated genotype, based on imaging of acetylated tubulin. 'Unt', untreated. Serum starvation ('Ser.-stvd') consisted of 24 h culture with 0.5% newborn calf serum. Histograms show means ± s.d. of 3 separate experiments in which at least 200 cells were quantitated. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ by unpaired t-test.

During our immunofluorescence microscopy analysis, we noted that the primary cilia in senescent cells appeared to be longer than those in proliferating cells (Figure 3.5A). We measured the length of the cilium in proliferating and senescent BJ, MRC5 and NHDF cells, from the basal body (as determined by γ-tubulin staining) to the tip of the cilium (as determined by acetylated tubulin staining). We found that cilia were consistently longer in senescent cells. The mean cilium length in BJ cells was 3.2 ± 1 μm in the senescent population as against 1.5 ± 0.7 μm in the proliferating controls. The mean cilium length in MRC5 cells was 3.5 ± 2.2 μm in the senescent population as against 1.2 ± 0.4 μm in the proliferating controls. The mean cilium length in NHDF cells was 3 ± 1.5 μm in the senescent population as against 1.5 ± 0.6 μm in the proliferating controls (Figure 3.5B). These results demonstrate increased primary cilium length in senescent human fibroblasts.

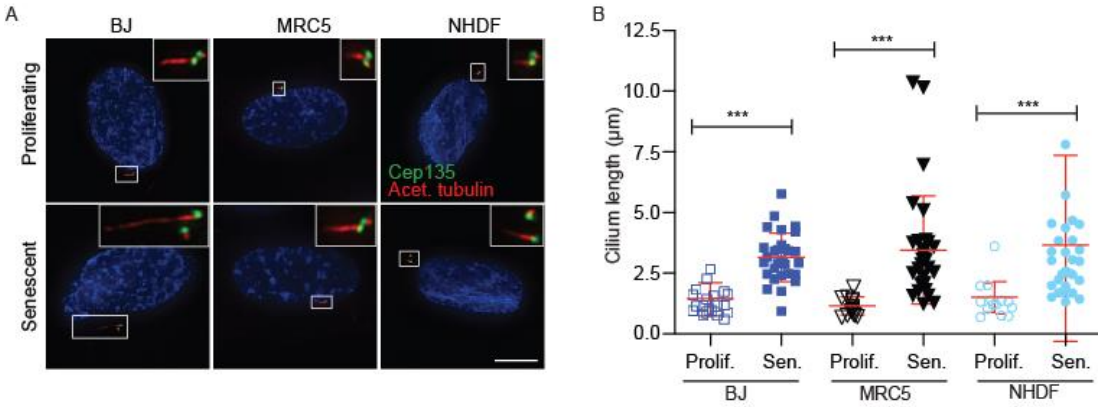

Figure 3.5 Increased primary cilium length in senescent human fibroblasts.
A. Immunofluorescence microscopy of the indicated cells stained with antibodies to Cep135 (green) and acetylated tubulin (red) (Work carried out by Dr. S. Prosser). DNA was visualised with DAPI (blue). Scale bar, 10 μm. B. Quantitation of cilium length in the indicated cell lines. Histograms show means ± s.d. in which the lengths of at least 30 cilia were measured. ***, $P<0.001$ by unpaired t-test.

3.2.3 Microscopy analysis of the centrosome and cilium in proliferating and senescent BJ fibroblasts

Boveri proposed that centrosome overduplication and subsequent malformation of mitotic spindles may result in asymmetric cell division, leading to chromosome instability in cancer cells (Boveri, 2008). Many studies have shown a significant increase in chromosome instability with aging (Fenech, 1998; Bukvic et al., 2001; Livak and Schmittgen, 2001; Erceg et al., 2007; Wojda et al., 2007; Thomas and Fenech, 2008). Previously-published observations in other human fibroblast cell lines have shown that there is an increase in the number of amplified centrosomes in senescent cells (Ohshima and Seyama, 2010). Chromosome instability is one of the most commonly observed genetic changes in cancer cells. Age-associated chromosomal instability is thought to contribute to tumourigenesis, which is also known to increase with age (Storchova and Pellman, 2004; Ganem et al., 2007; Storchova and Kuffer, 2008). It has long been debated whether centrosome amplification is a cause or consequence of cancer, as mechanisms exist to prevent the effects of centrosome amplification resulting in normal cell division. These include a) extrusion: cells remove extra centrosomes by forming cytoplasts containing centrosomes b) inactivation: activity of additional centrosomes is inactivated c) segregation: centrosomes as asymmetrically divided into daughter cells d) clustering: coalescence of centrosomes into two groups (Godinho et al., 2009).

During our immunofluorescence microscopy analysis we observed that senescent BJ fibroblasts had elevated numbers of centrioles (Figure 3.6A). Proliferating and senescent BJ cells were fixed and stained for acetylated tubulin and centrin2, which localises to the distal lumen of centrioles throughout the cell cycle (Paoletti et al., 1996). Centrosome amplification was scored as more than four centrin2 spots in a cell. We counted the frequency with which amplified centrosomes arose and found that 22% of senescent cells had amplified centrosomes (Figure 3.6B). We then examined the composition of the centrioles by staining with various centriolar markers. We stained for Cep170, which localises to the sub-distal appendages (Guarguaglini et al., 2005), Kizuna, which is normally associated with mature pericentriolar material (Oshimori et al., 2006), and Rootletin, which is involved in centrosome cohesion (Bahe et al., 2005). These analyses showed that senescent cells have amplified centrosomes and that the centrosomes appear to be fully intact.

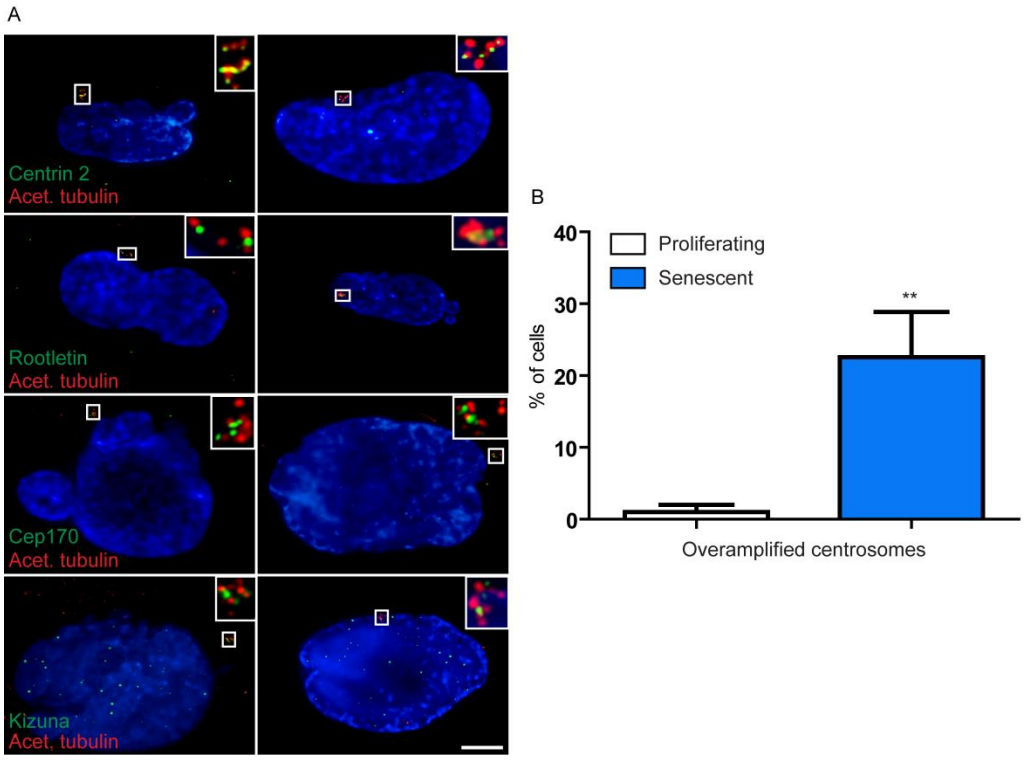

Figure 3.6 Amplified centrosomes in senescent BJ cells.
A. Immunofluorescence microscopy of proliferating and senescent BJ cells stained with antibodies against centriolar markers (green) and acetylated tubulin (red). DNA was visualised with DAPI (blue). Inserts show blow-ups of amplified centrosomes. Scale bar, 10 µm. **B.** Quantitation of the frequency of centrosomal amplification in BJ cells, scored as >4 centrin2 spots in a cell. Histograms show means ± s.d. of 3 separate experiments in which at least 200 cells were quantitated. **, $P<0.05$ by unpaired t-test.

The relationship between the centrioles and the primary cilium means that extra centrioles, such as those induced by DNA damage or overexpression of the centriole-regulatory polo-like kinase 4 (PLK4), can lead to multiple cilia being formed in a single cell (Conroy et al., 2012; Mahjoub and Stearns, 2012). Proliferating and senescent cells were fixed and stained with centrin2 and acetylated tubulin. In our immunofluorescence microscopy analysis we noted the presence of abnormal primary cilia in senescent BJ cells (Figure 3.7A). These primary cilia were normally in close proximity to each other as only the tip of each cilium could be seen separately. Ciliary abnormality was scored as more than one ciliary structure per cell, one cilium emanating from overamplified centrosomes, or both. We counted the frequency with which abnormal cilia arose and found that 25% of senescent cells had abnormal cilia (Figure 3.7B). These results suggest a correlation between centrosome amplification and ciliary abnormality in senescent BJ cells.

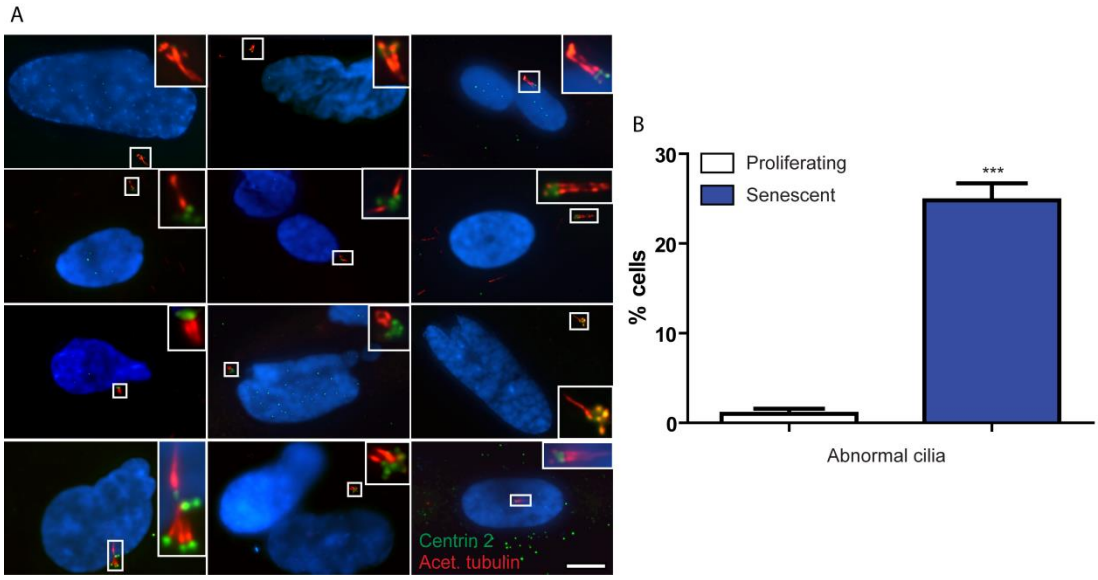

Figure 3.7 Abnormal cilia in senescent BJ cells.
A. Immunofluorescence microscopy of senescent BJ cells stained with antibodies against centrin 2 (green) and acetylated tubulin (red). DNA was visualised with DAPI (blue). Inserts show blow-ups of abnormal cilia, magnified at the side of the micrograph. Scale bar, 10 µm. **B.** Quantitation of the frequency of ciliary abnormality in BJ cells, scored as >1 ciliary structure in a cell. Histograms show means ± s.d. of 3 separate experiments in which at least 200 cells were quantitated. ***, $P<0.01$ by unpaired t-test.

We went on to examine the composition of centrioles and cilia by immunofluorescence microscopy on fixed BJ cells. Proliferating and senescent BJ cells were fixed and stained for the centriolar components centrin3 and γ-tubulin, and pericentrin, which localises to the pericentriolar material (Dictenberg et al.,

1998). We co-stained with antibodies against the ciliary components Arl13b, IFT88 and acetylated tubulin. Despite the increase in centrosome amplification and ciliary abnormality, IF microscopy analysis showed apparently normal centrosome and cilium protein composition in senescent BJ cells, as shown in Figure 3.8.

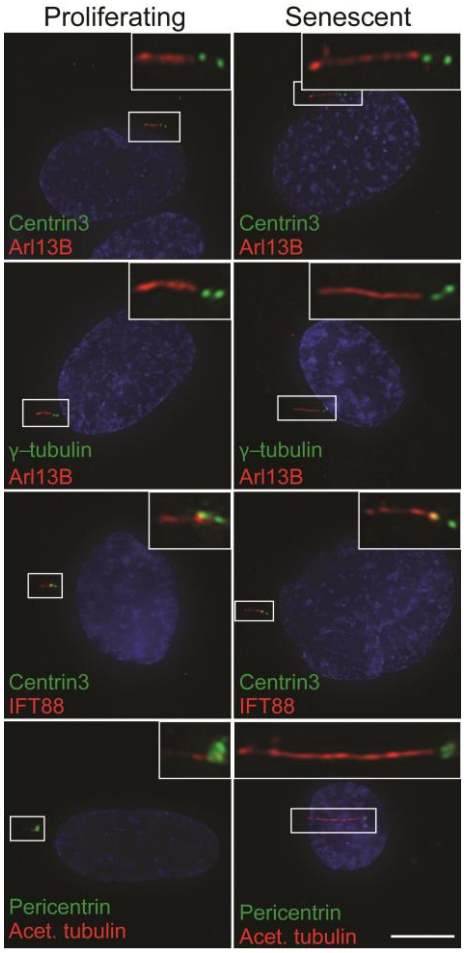

Figure 3.8 Apparently normal cilium and centrosome components in senescent BJ cells. Immunofluorescence microscopy of proliferating and senescent BJ cells stained with antibodies against centriolar (green) and ciliary (red) components as indicated. DNA was visualised with DAPI (blue). Inserts show blow-ups of cilia, magnified at the top of the micrograph. Scale bar, 10 µm. (Work carried out by Dr. S. Prosser)

Previous studies have identified proteins which are involved in both ciliogenesis and DNA damage repair, such as Nek1 and CEP164 (Graser et al., 2007; Shalom et al., 2008; Sivasubramaniam et al., 2008; Pan and Lee, 2009; Pelegrini et al., 2010). This indicates that proteins involved in the control of ciliogenesis may also be involved in the DNA damage response. We wanted to test whether ciliation has an impact on homologous recombination and thus DNA repair as previously-published data from our laboratory have suggested that this is the case in hTERT-

RPE1 cells (Conroy et al., 2012). Proliferating and senescent BJ cells were treated with 5 Gy ionising radiation (IR) and fixed 0, 1, 4 and 8 hours post irradiation. As shown in Figure 3.9A, cells were stained with acetylated tubulin to distinguish between ciliated and non-ciliated cells, and for Rad51 recombinase, which forms IR-induced foci (IRIF) during DNA repair (Haaf et al., 1995). We found comparable levels of Rad51 foci in ciliated and non-ciliated cells. Senescent cells had a slightly increased number of Rad51 foci per cell when compared to proliferating cells (Figure 3.9B). These results show that ciliated and non-ciliated BJ cells respond in the same way to genotoxic stress. This suggests that ciliation has no obvious impact on DNA repair, specifically homologous recombination, as it has no effect on Rad51 foci formation.

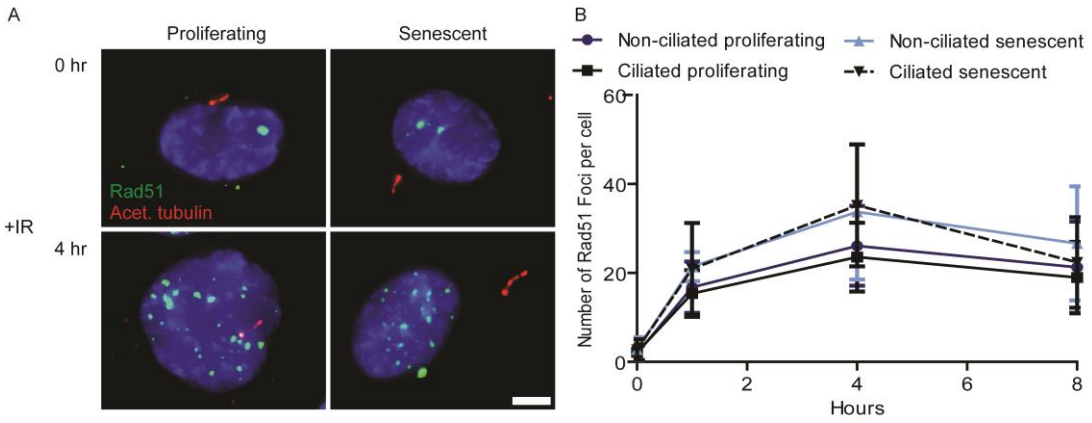

Figure 3.9 Ciliation does not affect Rad51 focus formation in proliferating or senescent BJ cells. **A.** Immunofluorescence microscopy of BJ cells, which were treated with 5 Gy IR, fixed at the indicated time points and stained with antibodies to Rad51 (green) and acetylated tubulin (red). DNA was visualised with DAPI (blue). Scale bar, 10 μm. **B.** Quantitation of the number of foci in the indicated cells over time. Histograms show means ± s.d. of 3 separate experiments in which at least 50 cells were quantitated per condition.

3.2.4 Senescent cells showed reduced expression of components of the Hedgehog signalling pathway

We wished to explore if ciliary regulators were differentially expressed in BJ cells as they become senescent. To date, the impact of senescence on ciliary gene expression has not been studied. The increase in ciliary abnormality we observed by immunofluorescence microscopy suggested that ciliary gene expression might be altered in senescent cells. To identify ciliary genes whose transcription might be affected by senescence, we performed a quantitative real-time PCR screen using a

commercial Primary Cilium PCR array. This is made up of a panel of 96 primer sets: 84 genes involved in ciliary organisation and maintenance, 5 housekeeping genes, 3 reverse transcription controls, 3 positive PCR controls and a genomic DNA control. RNA was purified from proliferating and senescent BJ cells, reverse transcribed into cDNA and analysed by qPCR. Changes in expression levels for each gene were normalised to the housekeeping genes and then compared to the proliferating controls. The fold change in gene expression was calculated using $2^{(-\Delta\Delta CT)}$ (Livak and Schmittgen, 2001). Data from three separate experiments were averaged and plotted on a graph. Where the fold change is greater than 1, the result represents a fold up-regulation in gene expression. Where the fold change is less than 1, the negative inverse of the result represents a fold down-regulation. The standard deviations were calculated in the same way. The data generated in the qPCR screen are presented in Appendix I and are summarised in Table 3.1.

Table 3.1 Altered ciliary gene expression in senescent cells

Up-regulated genes		Down-regulated genes	
Significant *0.1>P*<0.01	**Not Significant** P≥0.1	**Significant** *0.1>P*<0.01	**Not Significant** P≥0.1
Intraflagellar transport			
DYNC2LI1, IFT172, IFT74, IFT88, KIF3A, KIF3B	*IFT20, IFT80,*		
Cilium morphogenesis			
BBS2, VANGL2, IFT172, IFT88, MKKS,	*ARL6, BBS1, BBS4, BBS7, ODF1, PKHD1, RPGRIP1L, WWTR1*		*ALMS1*
Cell cycle			
CDKN1A, MAP2K1	*AKT1, BBS4, CCND1, CDK5RAP2, PKD2, TP53*		*IGF1, PKD1*
Genes mutated in non-motile cilia diseases			
BBS2, MKKS, NPHP1	*AHI1, ARL13B, ARL6, BBS1, BBS4, BBS7, CEP290, INVS, IQCB1, MKS, PKD2, PKHD1, RPGRIP1L, TMEM67, TTC8*		*ALMS1, CC2D2A, GLIS2, NEK8, NPHP3, PKD1*
Cellular signalling			
Hh: *LRP2* **PCP:** *VANGL2* **bRaf/MEK/ERK:** *MAP2K1*	**Hh:** *BTRC, FUZ, GLI3, GSK3β, IHH, INTU, PTCH1, SHH, SMO, SUFU* **cAMP:** *PKD2* **mTOR:** *AKT1, GSK3B MTOR, PIK3CA, PRKCA, TP53, TSC2* **WNT:** *AXIN2, INVS* **PCP:** *FJX1, FUZ, INTU* **PDGFRα/Integrin:** *ITGB1, PDGFRα* **bRaf/MEK/ERK:** *FOS, PRKCA, PTPN5*	**Hh**: *GLI2*	**Hh**: *GLI1, RAB23* **cAMP:** *ADCY3, ADCY7* **mTOR:** *CDC42, IGF1, MAPK1, RHOA, TSC1, TSC2* **PCP:** *DVL1, FAT4, FZD1, RHOA, ROCK2* **bRaf/MEK/ERK:** *KRAS, MAPK1*

Abbreviations: Cyclic adenosine monophosphate (cAMP); bRaf/mitogen-activated ERK kinase/extracellular signal regulated kinase (bRaf/MEK/ERK); Hedgehog signalling (Hh); mammalian target of rapamycin (mTOR); planar cell polarity (PCP); platlet-derived growth factor receptor alpha (PDGFRα); Wnt signalling (Wnt)

As shown in Figure 3.10, of the 84 cilium-specific genes analysed, 18 genes had a statistically significant difference in gene expression, in senescent compared to proliferating cells.

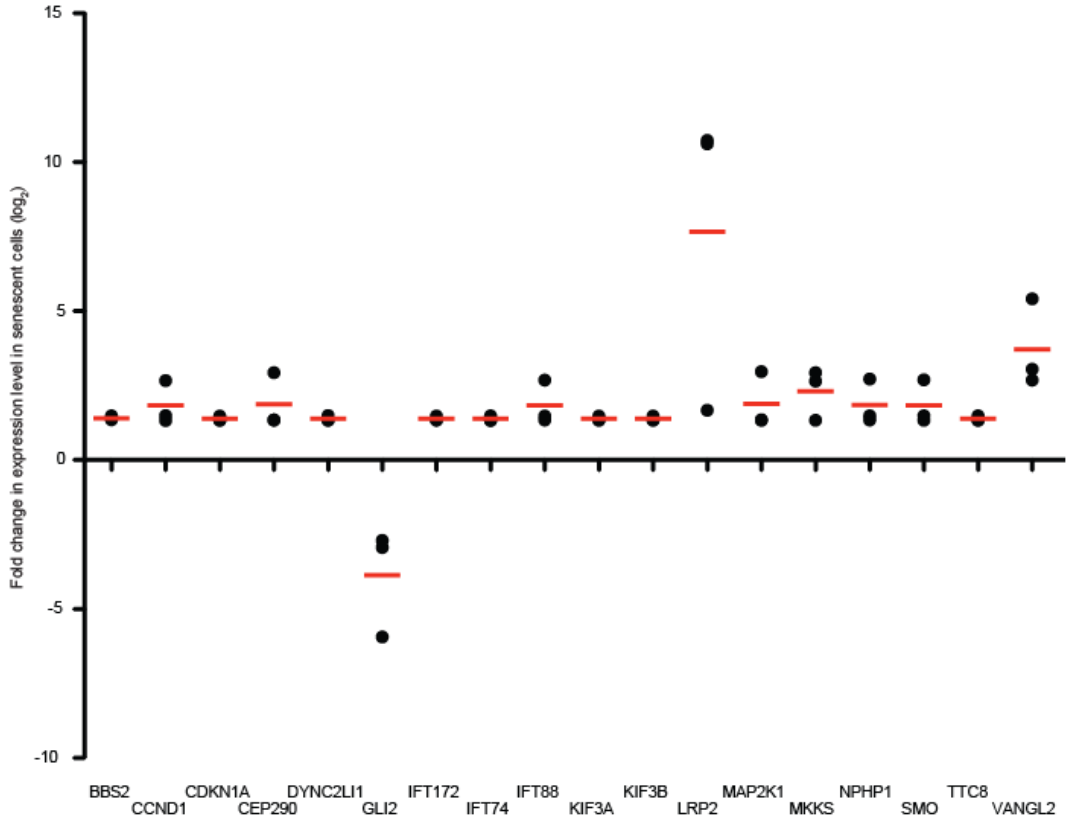

Figure 3.10 The expression of cilium-related genes is altered in senescent human fibroblasts. Quantitation of ciliary gene expression by qPCR showing all significant alterations seen in senescent cells within the set of genes analysed with the Qiagen Human Primary Cilia RT² PCR Array. Changes in the expression levels for each gene were normalised to endogenous controls. Quantification is based on the relative expression of a target gene in the senescent sample versus a reference gene in the proliferating sample. Data shown are individual values and the means of 3 separate experiments.

We observed a trend towards up-regulation of genes involved in intraflagellar transport (IFT) and ciliary morphogenesis. Ciliary proteins are synthesised in the cell body and must be transported to the tip of the axoneme. Transport within the cilium is mediated by IFT (Kozminski et al., 1993). During IFT, protein complexes are transported from the base of the cilium to the distal tip by kinesin motors (anterograde transport) and from the distal tip back to the cell body by dynein motors (retrograde transport). In order for the cilium to increase in length, the IFT machinery has to deliver additional axonemal proteins at the distal tip (Cole et al., 1998; Pazour et al., 1998). IFT contributes to ciliary length control. This can be

achieved by several changes in the IFT machinery such as IFT particle size (Engel et al., 2009), IFT velocity (Besschetnova et al., 2010) and the frequency of IFT events (Marshall and Rosenbaum, 2001). The up-regulation of genes involved in IFT and cilium morphogenesis may be a result of the increased frequency and length of cilia observed in senescing cells. *ALMS1* (Alström syndrome 1) was found to be down-regulated in senescent cells. ALMS1 localises specifically to the proximal ends of centrioles and basal bodies, where it co-localises with the centrosome cohesion protein C-NAP1 (Knorz et al., 2010). ALMS1 is required for ciliogenesis. Truncation mutations in *Alms1*/*ALMS1* do not prevent cilium formation; however, knockdown experiments indicate that the complete loss of ALMS1 impairs cilia formation. Loss of ALMS does not affect transcriptional regulation of other ciliary genes. *BBS4* and *TTC10* (*IFT88/polaris*) mRNA were up-regulated even when ciliogenesis was disrupted with *ALMS1* siRNA (Li et al., 2007). These data indicate that the down-regulation of *ALMS1* would not have an effect on ciliogenesis or transcriptional regulation of other ciliary genes in senescing cells.

AKT1 and *MAP2K1* (also known as *MEK1*) were also up-regulated in senescent cells. AKT1 activation has been shown to induce senescence (Nogueira et al., 2008; Astle et al., 2012). Inhibition of Aurora A causes a decrease in AKT1 phosphorylation (Guan et al., 2007). Aurora A is essential for primary cilium resorption (Pugacheva et al., 2007) and down-regulation of Aurora kinases is sufficient to induce senescence (Huck et al., 2010). The increase in ciliation frequency observed in our senescent cells may be caused by down-regulation of Aurora A, leading to a decrease in the phosphorylation of AKT1, thus an increased level of *AKT1*, resulting in senescence.

Activation of PDGFRα (platelet-derived growth factor receptor α) signalling within the ciliary membrane is followed by activation of AKT and the MEK1/2-ERK1/2 pathways, with MEK1/2 being phosphorylated within the cilium and at the basal body (Schneider et al., 2005). *MEK1* has also been shown to be up-regulated in senescent cells (Boucher et al., 2004). If a threshold length of cilium is still responsive to signalling, a lengthening of the structure may contribute to an effective dilution of the signalling capacity and blunting of the signal (Mahjoub and Stearns, 2012). The lengthening of the cilium we observed in our senescent cells may result

in dilution of the PDGFRα signalling and thus accumulation of AKT, MEK and ERK. In addition, it has been shown that endogenous RAS-MEK and AKT signalling regulate the transcriptional activity of *GLI1* in cancer cells (Stecca et al., 2007), suggesting that the up-regulation of *AKT1* we observed may be associated with the down-regulation of *GLI1* in senescing cells.

CDKN1A (cyclin-dependent kinase inhibitor 1A, or p21) and *TP53* (tumour protein 53, also known as p53) were up-regulated in our screen. As mentioned previously, AKT activity increases along with cellular senescence. Activation of AKT promotes senescence-like arrest of cell growth via increased transcriptional activity of *p53*, resulting in up-regulation of *p21* (Miyauchi et al., 2004). This result suggests that the up-regulation of *AKT1* observed in our senescent cells may be associated with the (non-significant) up-regulation of *p53* and *p21*. Telomeres are repetitive DNA-protein structures that cap the ends of linear chromosomes to protect them from degradation. They shorten each time a cell divides as DNA polymerases cannot completely replicate to the end of a DNA template. This is termed the "end replication problem" (Levy et al., 1992; Allsopp et al., 1995). Telomere shortening triggers senescence through the ATM-p53 pathway, up-regulating p21 (CDKN1A) and causing growth arrest (Herbig et al., 2004). DNA damage has been shown to induce centrosome amplification (Dodson et al., 2004), so that the centrosome amplification observed in our senescing cells is a likely consequence of elevated DNA damage signalling.

Our screen also showed that *PKD1* was down-regulated and *PKD2* was up-regulated. Polycystin-1 (PKD1) and Polycystin-2 (PKD2) localise to the centrosome and cilium (Yoder et al., 2002). Mutations in *PKD1* and *PKD2* cause defects in ciliary signalling and cell division. PKD1 and PKD2 are also important in the maintenance of normal centrosome number. Depletion of PKD1 induces centrosome amplification *in vitro* and *in vivo* (Battini et al., 2008). Centrosome amplification was also observed in *Pkd2* transgenic mice and in *Pkd2* knockout mouse embryos (Burtey et al., 2008). These results suggest that the centrosome amplification we observed in senescent cells may be caused by dysregulation of *PKD1* and *PKD2*.

The level of *IGF1* (insulin-like growth factor 1) has been reported to be lower in senescent than proliferating rat liver cells (Park and Buetow, 1991), which again is in agreement with what was observed in our screen. In *CDK5RAP2* (CDK5 regulatory subunit associated protein 2) mutant MEFs, both the mother and daughter centrioles are amplified, excess mother centrioles template multiple primary cilia and the cells enter premature senescence (Barrera et al., 2010). *CDK5RAP2* also functions in the DNA damage response (Barr et al., 2010). We found that *CDK5RAP2* was up-regulated in senescent cells. Very little data have been reported on the up-regulation of *CDK5RAP2*. However, it is possible that the up-regulation of *CDK5RAP2* is contributing to the centrosome amplification we observed in our senescent cells.

The primary cilium is essential for Hh signal transduction (Goetz and Anderson, 2010). Hh signalling has essential and diverse roles in animal development, tissue homeostasis, pain perception, metabolism and cross-talk with other signalling pathways (Babcock et al., 2011; Polizio et al., 2011; Büller et al., 2012; Chen et al., 2012; Wang et al., 2012; Briscoe and Thérond, 2013). There are three ligands in the Hh signalling family: Sonic hedgehog (SHh), Indian Hedgehog (IHh) and Desert Hedgehog (DHh) (Echelard et al., 1993). Signalling through the Hh pathway requires the controlled access of the Hh signal transducer SMO to the primary cilium (Corbit et al., 2005). In the absence of Hh signalling, the tumour suppressor Patched (PTCH1) inhibits the activity of SMO and prevents its trafficking to the cilium. In the presence of Hh signalling, Hh binds to PTCH1, relieving the inhibition of SMO and allowing it to move to the cilium and activate the Hh pathway through activation of the Glioma (Gli) family (Chen and Struhl, 1996; Taipale et al., 2002; Bijlsma et al., 2006; Rohatgi et al., 2007). Gli proteins translocate to the nucleus where they activate transcription of Hh target genes including *GLI1*, *GLI2*, *PTCH1*, *Cyclin*, *BCL2*, and *NMYC* (Dai et al., 1999; Kenney and Rowitch, 2000; Kenney et al., 2003; Agren et al., 2004; Bigelow et al., 2004; Pan et al., 2006). As discussed below, our results suggest that Hh signalling is down-regulated in senescent cells.

We observed a significant up-regulation in the expression of *LRP2* (low density lipoprotein receptor-related protein 2, also known as megalin). LRP2 has

been identified as a SHh receptor which is found on the surface of cells (Ranganathan et al., 1999; McCarthy et al., 2002). There are several mechanisms that could control LRP2 expression, such as the regulation of its mRNA levels, protein synthesis and its availability at the cell surface. Several of the molecules that regulate LRP2 mRNA and/or protein levels also are ligands of the receptor, and their levels and availability are regulated by LRP2 itself. Clusterin provides an example of regulation of LRP2 by its own ligand. Clusterin can induce or inhibit apoptosis through an LRP2-mediated signalling process involving the activation of PI3K/AKT and it was shown to increase LRP2 mRNA and protein expression (Ammar and Closset, 2008). Various molecules also regulate LRP2 protein expression and availability at the cell surface through modifications in the LRP2 receptor. For example, phosphorylation of the LRP2 cytoplasmic domain by GSK3β decreases its cell surface expression by negative regulation of LRP2, without changing its distribution or endocytosis (Yuseff et al., 2007). LRP2 has been shown to sequester SHh and control internalisation and cellular trafficking of the SHh/PTCH1 complex. Lack of LRP2 in mice results in failure to respond to SHh, despite functional expression of PTCH1 and SMO, whereas overexpression of LRP2 increases SHh signalling capacity (Christ et al., 2012). These results suggest that the up-regulation of *LRP2* we observed in our senescent cells may be associated with the (non-significant) up-regulation of *SHH* and *PTCH1*.

We also observed a significant up-regulation in the expression of *SMO*. SHH signalling up-regulates *PTCH1* expression and so excess PTCH1 protein can act on free SMO to prevent the signalling cascade (Chen and Struhl, 1996; Marigo and Tabin, 1996; Stone et al., 1996). In addition, increasing concentrations of SHh induce a progressive increase in SMO phosphorylation (Chen et al., 2011). Denef et. al. have shown that although levels of *SMO* RNA are uniform across the epidermis of *Drosophila melanogaster*, levels of SMO protein vary. This variation is due to the degradation of SMO by PTCH, but not in cells which receive Hedgehog signalling. In these cells, Hedgehog-dependent removal of PTCH results in increased phosphorylation of SMO and its accumulation at the cell membrane (Denef et al., 2000). These results suggest that although transcriptional expression of Hh genes is increased in senescent cells, the Hh pathway may be inhibited. Cyclopamine-

mediated SMO inhibition decreases the expression of GLI1 and GLI2 proteins, but increases the expression of GLI3 repressor relative to GLI3 activator (Hu et al., 2006). This suggests that the up-regulation of GLI3 observed may be associated with loss of SMO in senescing cells.

In support of the theory that the Hh pathway is inhibited in senescent cells, we observed a down-regulation of *RAB23* in our screen. Depletion of Rab23 (*Ra*s-like in rat *b*rain 23) or expression of dominant-negative Rab23 results in a decrease in SMO protein at the cilium (Boehlke et al., 2010). There are conflicting reports as to whether Rab23 is a positive or negative regulator of the Hh pathway. Analyses of neural tube development in mice indicated a negative role of Rab23 in Hh signalling (Eggenschwiler et al., 2001; Eggenschwiler et al., 2006). This is in contrast to data published in mouse chondrocytes, where down-regulation of Rab23 was shown to decrease the level of Gli1, suggesting a positive role of Rab23 in Gli1 regulation (Yang et al., 2008). Similarly, expression of Rab23 was shown to increase invasion of gastric cancer cells (Hou et al., 2008), and expression of Rab23 suppressed cell proliferation in liver cancer cells (Liu et al., 2007b). Since Hh signalling is activated in gastric and liver cancers (Ma et al., 2005; Huang et al., 2006), these results also imply that Rab23 may be a positive regulator of Hh. The down-regulation of *GLI1* in our senescent cells may be associated with the down-regulation of *RAB23*. GLI1 is a strong transcriptional activator of Hh target genes (Aza-Blanc et al., 2000). Inhibition of GLI1 in brain glioma cell lines, directly by siRNA and indirectly by cyclopamine treatment, results in an up-regulation of p21 (Wang et al., 2010). These results are also consistent with our observations in senescing cells, where we observed a down-regulation in *GLI1* and an up-regulation in *p21*.

GLI2 acts as both a weak transcriptional activator and a repressor of Hh target genes (Sasaki et al., 1999). Gli2 mediates the mitogenic effects of SHh by transcriptional activation of *cyclin D1* (Mill et al., 2003). We observed a significant down-regulation in the expression of *GLI2* but an up-regulation in *CCND1* (Cyclin D1) in our screen. However, up-regulation of *Cyclin D1* has been previously reported in senescent cells (Dulić et al., 1993) and it has been shown that Hh signals induce direct up-regulation of *CCND1* (Kenney and Rowitch, 2000). We also found

that *GLI3* is up-regulated in our screen. GLI3 is a transcriptional repressor of Hh target genes (Aza-Blanc et al., 2000). The up-regulation we see in *GLI3* may be caused by *PTCH1* up-regulation. Stimulation of pancreatic fibroblasts with a SHh ligand results in increased expression of *PTCH1* and increased cytoplasmic expression of *GLI3* (Bailey et al., 2009). We also detected an increase in *BTRC* (β-transducin repeat containing) and *GSK3B* levels in senescent cells. GLI2 is phosphorylated by GSK3 creating docking sites that recruit SCFSlimb/β-TRCP, which promotes its ubiquitination and proteasome degradation (Bhatia et al., 2006). These results suggest that the dysregulation of *GLI* transcription we observed in senescent cells may inhibit transcription of downstream Hh target genes, and thus inhibit the Hh pathway. The dysregulation of *GLI* transcription may also be associated with the up-regulation in *SUFU* we observed in senescent cells. SUFU functions in the negative regulation of Gli proteins (Ding et al., 1999; Stone et al., 1999; Dunaeva et al., 2003). In the absence of Hh ligand, SUFU (suppressor of fused) is localised to the cilium (Haycraft et al., 2005). Through direct binding, SUFU blocks GLI nuclear localization and inhibits *GLI* transcriptional activity within the nucleus (Kogerman et al., 1999; Cheng and Bishop, 2002). These results suggest that the up-regulation of *SUFU* we observed results in the dysregulation of *GLI* transcription in senescing cells.

INTU (Inturned) and *FUZ* (fuzzy) were also up-regulated in our screen. *INTU* and *FUZ* are downstream effectors of the non-canonical Wnt pathway (Park et al., 1996; Collier and Gubb, 1997). Intracellular signalling of the Wnt pathway is classified into two categories: the non-canonical pathway and the canonical/β-catenin pathway (Komiya and Habas, 2008). The canonical Wnt pathway is involved in cell proliferation, differentiation, adhesion and survival (Dravid et al., 2005; Kirstetter et al., 2006; Lee et al., 2006b; Schlange et al., 2007). Non-canonical Wnt signalling diversifies into several pathways including the Wnt-calcium and the planar cell polarity (PCP) pathways (Komiya and Habas, 2008). The Wnt-calcium pathway is involved in embryonic dorsal-ventral patterning, regulating cell migration and development (Westfall et al., 2003; Garriock et al., 2005; Garriock and Krieg, 2007; Cheng et al., 2008). The up-regulation of *INTU* and *FUZ* we observed may be associated with the significant up-regulation of *VANGL2* (van Gogh-like protein 2)

in senescent cells. *VANGL2* is a planar cell polarity gene which localises to the primary cilium. Overexpression of VANGL2 in zebrafish embryos resulted in normal growth and motility of primary cilia but defects in asymmetric basal body positioning (Borovina et al., 2010). Overexpression of VANGL2 in fibrosarcoma cells reduces cell mobility and results in a change in cell morphology, suggesting that excess VANGL2 expression disrupts both cell polarity and motility (Cantrell and Jessen, 2010). *VANGL2* overexpression reduced proliferation and antagonises the canonical WNT pathway in colorectal cancer cells (Piazzi et al., 2013). These results suggest that the up-regulation of VANGL2 may contribute to the reduced proliferation and induction of senescence in our cells.

While our real-time qPCR data strongly suggest that Hh signalling may be altered in senescent cells, we wished to confirm this by investigating the protein expression levels of Hh and Wnt components in proliferating and senescent BJ cells. mRNA expression and protein abundance do not always correlate (Greenbaum et al., 2003; Nie et al., 2006; Brockmann et al., 2007; Guo et al., 2008; Gry et al., 2009; Schwanhäusser et al., 2011). This may be due to post-translational modifications, differing half-lives of mRNA and protein, cleavage or degradation of the protein, inclusion of the protein in transport vesicles or secretion/diffusion of the protein away from the expressing cell/tissue. As previously mentioned, it has been shown although levels of *SMO* RNA are uniform across the epidermis of *D. melanogaster*, levels of SMO protein vary (Denef et al., 2000).

Immunoblot analysis confirmed the general loss of Hh components in senescent cells (Figure 3.11). The increase in expression of β-Gal and p21 proteins confirmed that our cells were senescent, while the increase in expression of VANGL2 protein confirmed our real-time PCR analysis with respect to the Wnt signalling pathway. Together, the qPCR and western blot data show that Hh signalling is down-regulated in senescent cells.

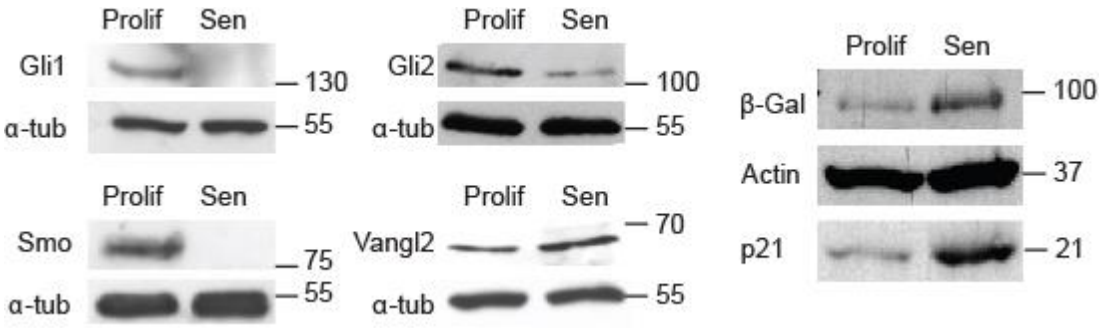

Figure 3.11 The expression of Hedgehog signalling components are altered in senescent human fibroblasts.
A. Immunoblot analysis of the indicated protein expression in BJ cells. Size markers are indicated in kDa.

3.2.5 Loss of Hedgehog signalling leads to increased cilium frequency and length in BJ cells

These data suggested that Hh signalling might be mitogenic in fibroblasts, as it is in human mammary epithelial cells. Hh signalling promotes mitogenesis by suppression of the growth-inhibitory p16. A fragment of GLI2 was found to directly bind and inhibit the *p16* promoter and loss of GLI2 was associated with the induction of stem cell senescence (Bishop et al., 2010). We wanted to test whether inhibition of Hh would have an effect on cilia frequency or length, and cell proliferation. To analyse this we used cyclopamine, a steroidal alkaloid teratogen derived from the plant *Veratrum californicum* (Keeler, 1978). Cyclopamine is a small molecule inhibitor of the Hedgehog family of secreted proteins (Cooper et al., 1998; Incardona et al., 1998). Its inhibition is mediated by direct binding to the heptahelical bundle of Smo, which influences Smo protein conformation. Cyclopamine binding also depends on Ptch function (Chen et al., 2002a). Cyclopamine has anti-tumour activities arising from its ability to specifically block cellular responses to Hh signalling (Taipale et al., 2000; Berman et al., 2002; Kubo et al., 2004; Bar et al., 2007). Cyclopamine treatment has also been found to induce apoptosis in adenoma and carcinoma derived cell lines (Qualtrough et al., 2004).

Proliferating and senescent BJ cells were treated with 20 μM cyclopamine for 48 hours, fixed and stained for Ki67. Ki67 is a marker for cell proliferation (Gerdes et al., 1983). As determined by immunofluorescence microscopy, cyclopamine treatment caused a reduction in the number of $Ki67^{+}$ cells Figure 3.12A. We counted

the frequency of Ki67$^+$ cells in untreated and cyclopamine treated proliferating and senescent cells, Figure 3.12B. We found that after cyclopamine treatment, the number of Ki67$^+$ cells in proliferating populations dropped from 84% to 28%. In the senescent population, this number dropped from 5% to 1%. This result shows that cyclopamine causes a decline in the proliferative index in both young and senescent BJ populations.

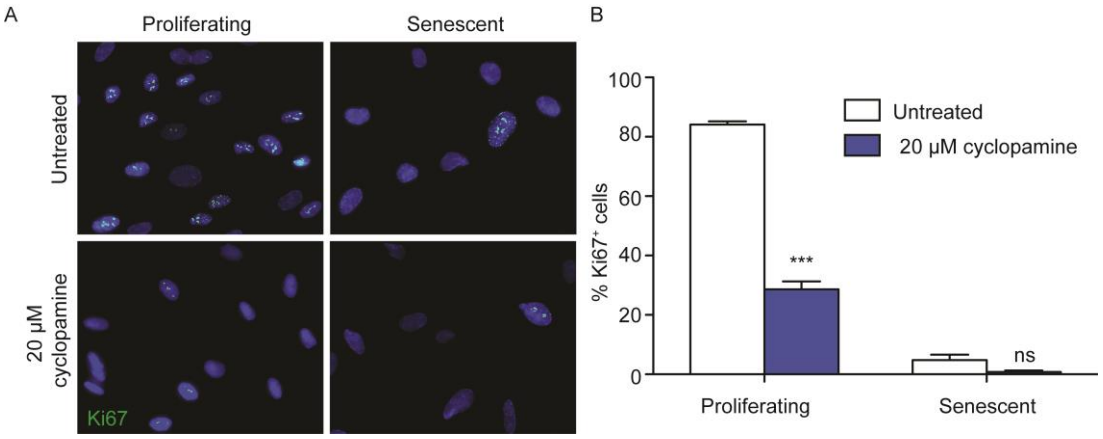

Figure 3.12 Cyclopamine causes a decline in the proliferative index in both proliferating and senescent populations.
A. Immunofluorescence microscopy of BJ cells stained Ki67 antibody (green), used as a marker for proliferation. DNA was visualised with DAPI (blue). Scale bar, 10 μm. **B.** Quantitation of the proliferative index of BJ cells after the indicated treatment, as determined by microscopy analysis of Ki67 signal. Histograms show means ± s.d. of 3 separate experiments in which at least 100 cells were quantitated. ***, P<0.001 by unpaired t-test.

We then went on to examine whether inhibition of Hh had an effect on cilium frequency and length. Proliferating and senescent BJ cells were treated with cyclopamine for 48 hours, fixed and stained for acetylated tubulin and Ki67. We counted the frequency with which cilia arose and found that in proliferating cells, the number of cells with cilia increased from 16% in untreated cells to 45% in cells treated with cyclopamine. In senescent cells this number increased from 49% in untreated cells to 59% in cells treated with cyclopamine (Figure 3.13A). We then measured the length of cilia in untreated and cyclopamine treated cells. As shown in Figure 3.13B, the few Ki67$^+$ cells in the senescent populations had shorter cilia (2 ± 0.5 μm) than those which were no longer in cycle (3 ± 1.4 μm). Notably, the mean cilium length in those young fibroblasts that were no longer proliferating after cyclopamine treatment (2.7 ± 0.5 μm) became as long as it was in senescent cells (3 ± 1.4 μm), while cilia in untreated proliferating cells were the same length in Ki67$^+$

and Ki67$^-$ cells (1.5 ± 0.5 µm and 1.4 ± 0.5 µm, respectively). Together, these data suggest that loss of Hh signalling leads to reduced proliferation and increased ciliation frequency and length.

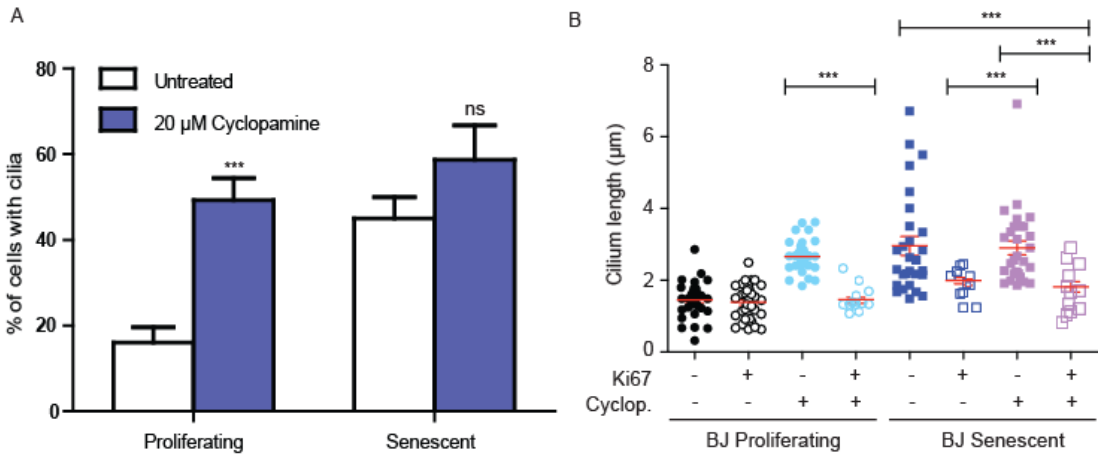

Figure 3.13 Loss of Hh signalling leads to increased cilium frequency and length.
A. Quantitation of the ciliation frequency. Histograms show means ± s.d. of 3 separate experiments in which at least 100 cells were quantitated. **B.** Quantitation of cilium length in the indicated cells. Histograms show means ± s.d. in which the lengths of at least 30 cilia were measured for each condition, even when there were very few such cells. ***, P<0.001 by unpaired t-test.

3.2.6 Cilium length is independent of the growth arrest period and is intrinsic to the cell

We next investigated why senescent BJ cells have longer cilia. Most cells acquire their primary cilium in the stationary or G0 phase of the cell cycle and the cilium is resorbed prior to mitosis (Tucker et al., 1979). This then allows the centrosome to become involved in the formation of the bipolar spindle. The exact timing of ciliary resorption varies between different cells, with some resorbing their cilia in S phase and others not until the G2/M transition (Plotnikova et al., 2009). A more limited time for ciliary elongation is available to proliferating cells than to cells which are no longer cycling. To understand how senescent cells acquire their elongated cilia, we tested if an extended period without cycling was required for such ciliation. To do this experiment, we used the mitotic inhibitor chloral hydrate (CH). Previously-published data have shown that long-term incubation with 4 mM CH removes cilia from *Paramecium caudatum* (Ogura and Takahashi, 1976; Dunlap, 1977) and from the early embryo phase of the sea urchin, *Lytechinus pictus* (Chakrabarti et al., 1998). CH destabilises the cilium at the basal plate, the junction

between the cilium and basal body (Chakrabarti et al., 1998). It has also been shown that CH treatment disturbs the mitotic spindle in mouse oocytes through disassembly of microtubules (Eichenlaub-Ritter and Betzendahl, 1995).

Cells were treated with 4 mM CH for 72 hours to deciliate proliferating and senescent BJ fibroblasts, the drug was washed out and the cells were given a 24 hour recovery period to re-acquire cilia. Cells were fixed and stained for acetylated tubulin. As shown in Figure 3.14A, in proliferating cells the frequency of cilia decreased from 17% in untreated cells to 2% immediately after CH washout. 24 hours post CH washout, 10% of the cilia had grown back. Similarly in senescent cells, the frequency of cilia decreased from 44% in untreated cells to 1% 72 hours post CH treatment. 24 hours post CH washout, 30% of the cilia had grown back. We then measured the length of cilia in proliferating and senescent cells 24 hours post chloral hydrate washout and compared the cilia lengths to those in untreated cells. As shown in Figure 3.14B, the cilia length in senescent cells was re-established at the previously-existing length 24 hours post drug washout. As this is shorter than the cell cycle period in BJ cells (~36 hours), this observation suggests a cell-intrinsic cilium length setting that is independent of the time spent outside cycle.

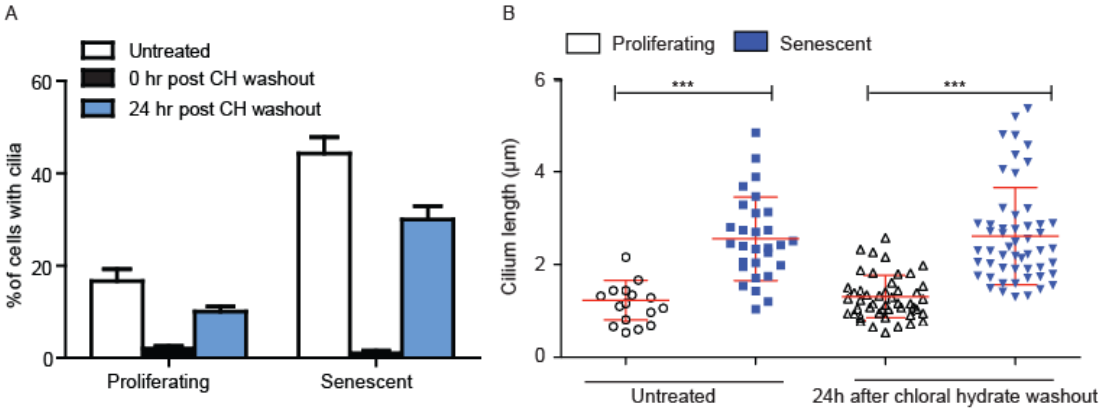

Figure 3.14 The increased ciliary length in senescent cells is not due to their extended time in cell culture.
A. Quantitation of the ciliation frequency. Histograms show means ± s.d. of 3 separate experiments in which at least 100 cells were quantitated. **B.** Quantitation of cilium length in BJ cells, before or 24 h after the washout of 24 h 4 mM chloral hydrate treatment. Histograms show means ± s.d. in which the length of at least 30 cilia were measured. ***, $P<0.001$ by unpaired t-test.

3.2.7 Senescent cells showed reduced levels of the negative cilium length regulator CP110

We next attempted to determine what controls cilium length in senescent BJ cells. CP110 (centriolar coiled-coil protein of 110 kDa) has been described as a negative regulator of ciliogenesis, as its depletion promotes cilium formation in growing cells and in quiescent cells, its overexpression inhibits their ability to form cilia (Spektor et al., 2007; Tsang et al., 2008). CP110 localises to the distal ends of both the mother and daughter centrioles (Chen et al., 2002b). During ciliogenesis CP110 is specifically eliminated from the mother centriole, which provides the basal body, the foundation for formation of primary cilium. The localisation of CEP97 has been shown to be strongly dependent on CP110, suggesting that CP110 recruits CEP97 to the centrosome. Depletion of CEP97 results in the loss of CP110 at the centrosome, promoting cilium formation (Spektor et al., 2007). The kinesin KIF24 was identified as a CP110-CEP97 interaction partner. KIF24 depolymerises centriolar microtubules to prevent premature cilia assembly and the loss of KIF24 promotes ciliogenesis (Kobayashi et al., 2011). In addition, CP110 has also been shown to interact with Cep290 to prevent NPHP6 and Rab8a from initiating ciliogenesis (Tsang et al., 2008). Tau tubulin kinase 2 (TTBK2) is essential for mouse SHh signalling and ciliogenesis. TTBK2 removes CP110 and promotes the recruitment of IFT proteins, which build the ciliary axoneme (Goetz et al., 2012). The distal appendages (DAPs) of centrioles have been proposed to anchor cilia to the plasma membrane. Loss of the DAP protein CEP83 blocks centriole to membrane docking and undocked centrioles fail to recruit TTBK2 or release CP110 (Tanos et al., 2013). The microRNA miR-129-3p has been shown to control cilia assembly by down-regulating CP110 and repressing branched F-actin formation. Blocking miR-129-3p inhibited serum starvation induced ciliogenesis, while its overexpression induced ciliation in proliferating cells and promoted cilia elongation (Cao et al., 2012). These data led us to consider CP110 as a possible candidate for controlling the cilium length setting.

To determine if CP110 is regulating cilia length, we performed quantitative PCR to determine whether the levels of *CP110* expression were altered in senescent BJ cells. The change in expression level was measured by normalising the

expression of *CP110* to a stably-expressed gene (an endogenous control). To identify reliable genes to be used as an endogenous control, for normalisation purposes, we analysed the expression stability of a panel of 11 housekeeping genes (*CYC1*, *SF3A1*, *SDHA*, *ATP5B*, *ACTB*, *GAPDH*, *YWHAZ*, *GADD45A*, *18S rRNA* and *UBCEIF4A2*) using the geNorm algorithim. Figure 3.15 shows the ranking of the 11 housekeeping genes analysed based on their average expression stability (M value). The principle of the geNorm algorithm is the expression ratio of an endogenous control should be the same in all samples regardless of the experimental conditions. An increase in this ratio corresponds to a decrease in stability of the gene. For each of the 12 genes analysed, the expression stability measure (M) was calculated. This is defined as the average variation of a particular gene compared to all the other control genes. The higher the M value, the less stable the gene. The exclusion of genes with the highest M values allowed for the ranking of genes based on their stability and thus identification of the most stable genes for the experiment (Vandesompele et al., 2002).

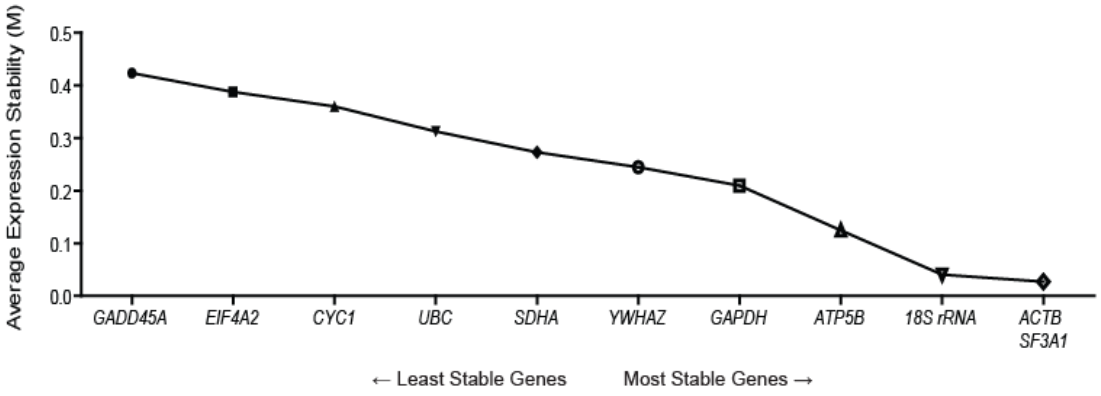

Figure 3.15 Selection of endogenous controls for real-time PCR.
Gene stability of 11 housekeeping genes analysed by geNorm software. M values represent the average expression stability of each gene. The two most stable genes were found to be *SF3A1* and *ACTB*.

We found that in our system *SF3A1* (splicing factor 3a, subunit 1) and *ACTB* (Actin B) were the most stably expressed of the housekeeping genes in proliferating and senescent BJ cells. The *ACTB* gene encodes one of six actin proteins. Actins are highly conserved proteins that are involved in cell motility, structure, and integrity. *ACTB* is a major constituent of the contractile apparatus and one of two non-muscle cytoskeletal actins (Khaitlina, 2001). *SF3A1* encodes subunit 1 of the splicing factor

3a protein complex. The splicing factor 3a heterotrimer is necessary for the *in vitro* conversion of 15S U2 snRNP into the active 17S particle that performs pre-mRNA splicing (Krämer et al., 2005; Tanackovic and Krämer, 2005).

RNA was purified from proliferating and senescent BJ cells, reverse transcribed into cDNA and analysed by real-time PCR. Changes in expression levels for each gene were normalised to the endogenous controls and then compared to the proliferating sample. The fold change in gene expression was calculated using $2^{(-\Delta\Delta CT)}$ (Livak and Schmittgen, 2001). Data from three separate experiments were averaged and plotted on a graph. As shown in Figure 3.16A, *CP110* was down-regulated in senescent cells. Senescent cells expressed 0.26 $\pm$ 0.10 (N = 3) of the levels of *CP110* that were expressed in proliferating controls.

We next investigated the protein expression levels of CP110 in proliferating and senescent BJ cells. Immunoblot analysis of CP110 expression showed a loss of CP110 in senescent cells (Figure 3.16B), with cellular levels of the control protein α-tubulin remaining stable. This in agreement with what we observed at the transcriptional level. These results indicate that CP110 expression is down-regulated in senescent cells.

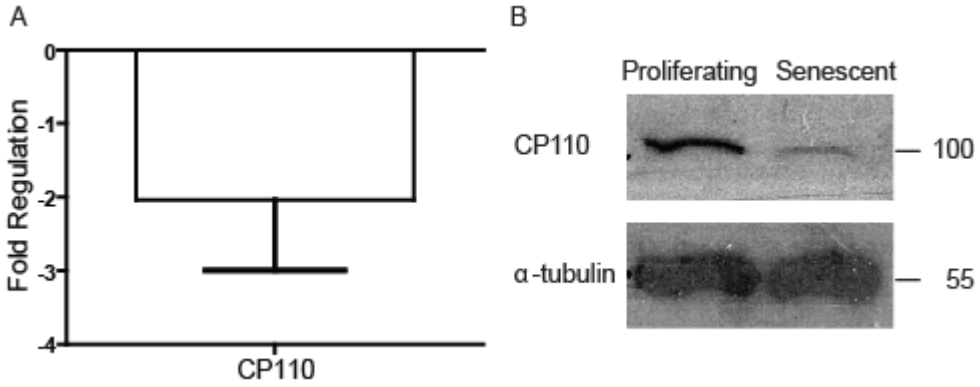

Figure 3.16 CP110 RNA and protein levels are reduced in senescent cells.
A. Quantitation of CP110 gene expression by qPCR. Changes in the expression level for CP110 were normalised to endogenous controls. Quantification is based on the relative expression of a target gene in the senescent sample versus a reference gene in the proliferating sample. **B.** Immunoblot analysis of CP110 expression in BJ cells. Size markers are indicated in kDa.

3.2.8 Overexpression of CP110 caused a decline in the frequency of ciliated cells but did not affect their mean cilium length or the number of proliferating cells

We tested whether restoring CP110 levels would drive the cells out of senescence and back into cycle. To study this we generated a GFP-CP110 fusion protein using genomic DNA isolated from primary human BJ cells. The target cDNA was amplified using primers designed against the *CP110* transcript variant 1 sequence found in the NCBI database (NCBI accession number NM_001199022.1). The plasmid construct was confirmed by sequencing. Proliferating and senescent BJ cells were transiently transfected with the GFP-CP110 construct, fixed 48 hours post transfection and stained with acetylated tubulin. Empty GFP vector was used as a transfection control. Only a low percentage of cells expressed GFP-CP110, due to a low transfection efficiency of primary BJ fibroblasts. In cells expressing GFP-CP110, we were able to see that it localised to the centrosome and co-localised with acetylated tubulin (Figure 3.17A).

As previously mentioned, overexpression of CP110 inhibits cilium formation. We tested whether overexpression of CP110 caused a reduction in the number of primary cilia in proliferating and senescent BJ cells. As shown in Figure 3.17B, in proliferating cells, the frequency of cilia decreased from 13% in untreated cells to 5% in transfected cells, with the empty GFP control having no effect on ciliation frequency. Similarly, in senescent cells, the frequency of cilia decreased from 43% in untreated cells to 14% in transfected cells, with the empty GFP control having no effect on ciliation frequency. These results show that overexpression of CP110 causes a decline in the fraction of ciliated cells.

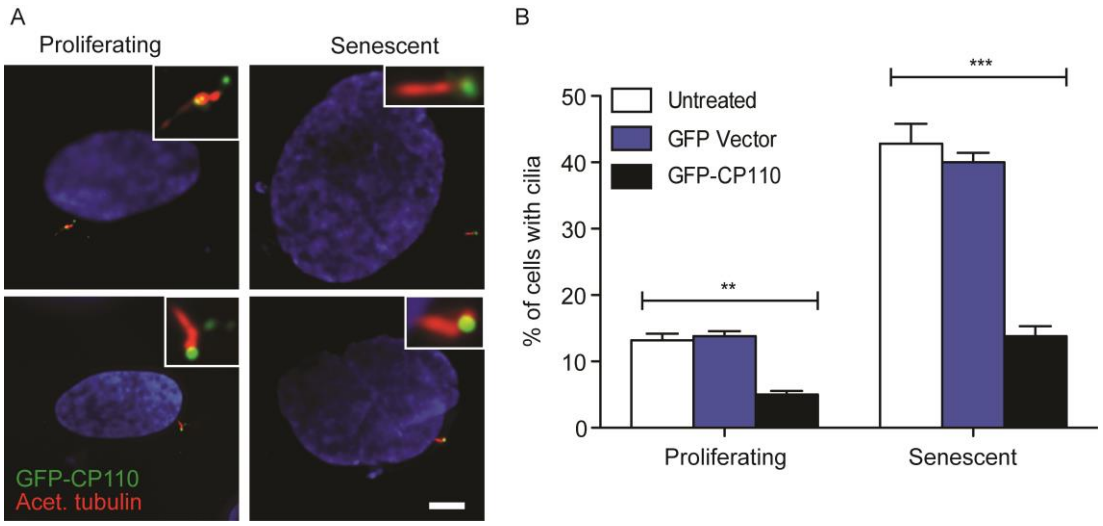

Figure 3.17 Overexpression of CP110 caused a decline in the fraction of ciliated cells.
A. Immunofluorescence microscopy of BJ cells 48 h post transfection with GFP-CP110 (green), co-stained with acetylated tubulin (red). DNA was visualised with DAPI (blue). Scale bar, 10 μm. **B.** Quantitation of the ciliation frequency 48 h post-transfection with GFP-CP110 or control vector. Histograms show means ± s.d. of 3 separate experiments in which at least 100 transfected cells were counted. **, $P<0.01$; ***, $P<0.001$ by unpaired t-test.

We next examined whether overexpression of CP110 had an effect on cilium length. Proliferating and senescent BJ cells were transiently transfected with GFP-CP110 or empty GFP as a control, fixed 48 hours post transfection and stained with acetylated tubulin. We found no difference in the mean cilium length in untreated or transfected, proliferating and senescent populations. These results show that overexpression of GFP-CP110 does not affect the mean cilium length in proliferating or senescent cells (Figure 3.18).

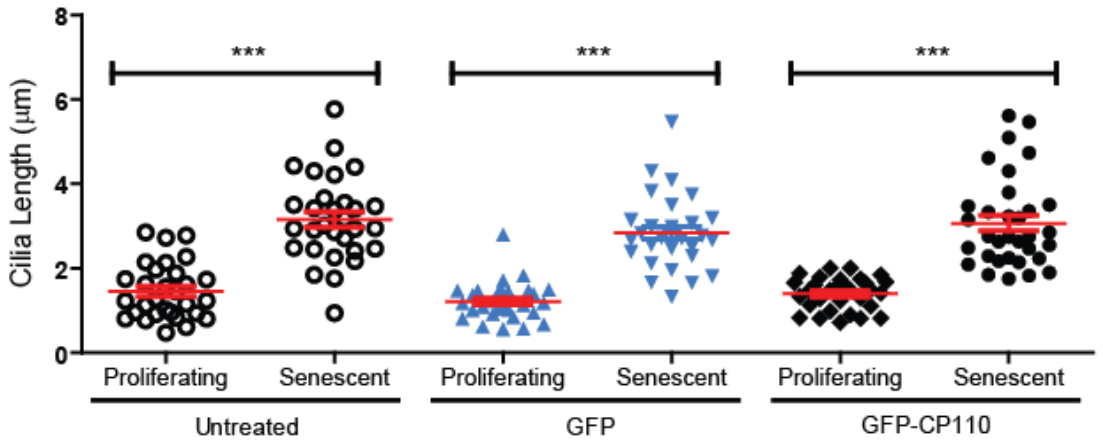

Figure 3.18 Overexpression of CP110 did not affect cilium length.
Quantitation of cilium length in the indicated cells. Histograms show means ± s.d. in which the length of at least 30 cilia were measured for each condition. ***, $P<0.001$ by unpaired t-test.

We then determined if overexpression of CP110 had an effect on the proliferative index in proliferating and senescent cells. Proliferating and senescent BJ cells were transiently transfected with GFP-CP110 or empty GFP as a control, fixed after 48 hours and stained for Ki67. Figure 3.19A shows an immunofluorescence micrograph of cells positive for GFP-CP110 and either $Ki67^-$ (top panels) or $Ki67^+$ (bottom panels). We quantitated the number of $Ki67^+$ cells and found comparable levels of $Ki67^+$ cells in untreated, GFP transfected and GFP-CP110 transfected proliferating and senescent populations (Figure 3.19B). This result shows that overexpression of CP110 does not affect the number of proliferating cells in either senescent or young populations. The results of the overexpression data together suggest that once senescence is established, it cannot simply be reversed through cilium manipulation.

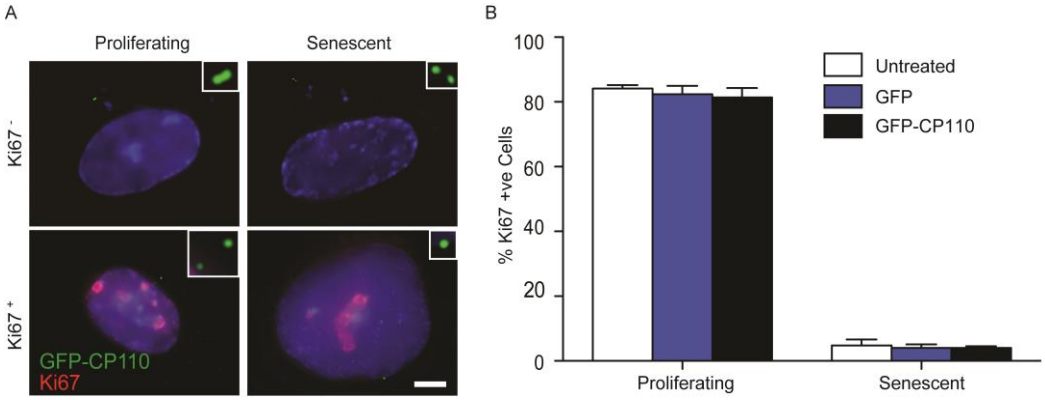

Figure 3.19 CP110 overexpression has no effect on the number of proliferating cells in senescent or young populations.
A. Immunofluorescence microscopy of BJ cells transfected with GFP-CP110 and stained with antibodies to Ki67 (red). DNA was visualised with DAPI (blue). Scale bar, 10 µm. **B.** Quantitation of the proliferative/ senescence index of BJ cells after the indicated treatment, as determined by microscopy analysis of Ki67 signal. Histograms show means ± s.d. of 3 separate experiments in which at least 100 transfected cells were quantitated.

3.2.9 Knockdown of CP110 increased ciliation, reduced proliferation and elevated cellular senescence

As discussed in section 3.2.4, we observed reduced expression of Hedgehog signalling components in senescent cells. The primary cilium in senescent populations may only be responsive to Hedgehog signalling until a threshold length. A lengthening of the cilium past this threshold may contribute to an effective dilution of the signalling capacity, as described by Mahjoub and Stearns (Mahjoub

and Stearns, 2012). They tested the effect of amplified centrosomes on cilium formation and found that cells with extra centrioles often had more than one cilium, had reduced levels of Smoothened at the cilium and had reduced SHh pathway activation (Mahjoub and Stearns, 2012).

To test the impact of increased ciliation on proliferation, we used siRNA to deplete CP110 from BJ fibroblasts. As previously mentioned, depletion of CP110 promotes cilium formation. Proliferating and senescent BJ cells were transiently co-transfected with a carboxyfluorescein (FAM) labelled transfection control and one of two siRNAs inhibitory to CP110 (named CP110 siRNA#1 and CP110 siRNA#2). As a negative siRNA control, we depleted glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). CP110 depletion was tested by qPCR 48 hours post transfection with 50 nM siRNA. Knockdowns were compared to the effect of *GAPDH* siRNA and normalised to *ACTB* and *SF3A1* housekeeping gene expression. As shown in Figure 3.20 A, our qPCR results confirmed the loss of *CP110* in proliferating and senescent cells (Figure 3.20A).

We examined whether CP110 depletion increased cilium frequency in proliferating and senescent BJ cells. Proliferating and senescent BJ cells were treated with 50 nM siRNA for 48 hours, fixed and stained for acetylated tubulin. As shown in Figure 3.20B, we found comparable numbers of cilia in untreated, GAPDH-depleted and CP110-depleted senescent cells. In proliferating cells, we saw comparable numbers of cilia in untreated and GAPDH-depleted cells. However, in proliferating cells, the frequency of cilia increased from 13% in untreated cells to 34% in cells treated with CP110 siRNA#1 and to 48% in cells treated with CP110 siRNA#2. This result shows that knockdown of CP110 caused an increased level of ciliation in proliferating, but not in senescent populations.

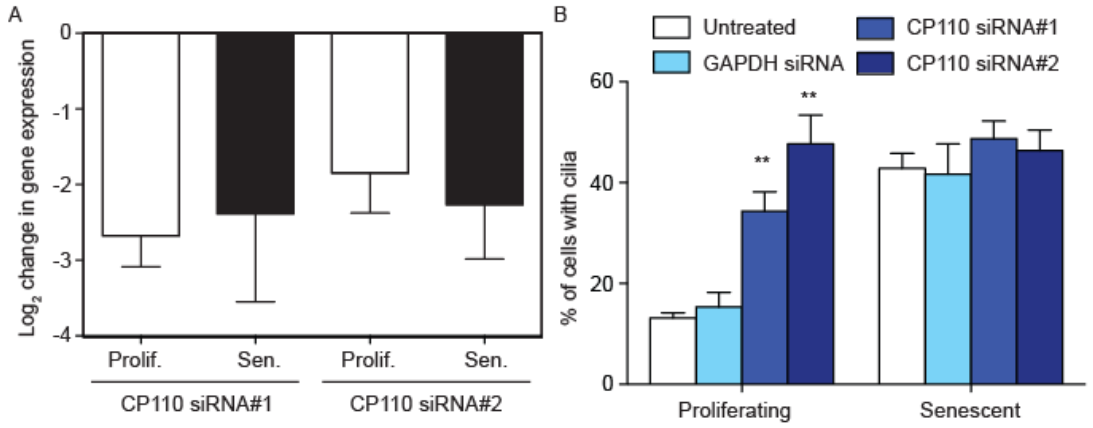

Figure 3.20 CP110 knockdown caused an increase in ciliation frequency in proliferating but not senescent populations.
A. Quantitation of CP110 depletion in BJ cells by siRNA as determined by quantitative RT-PCR. Cells were treated with 50 nM siRNA for 48 hours. Knockdowns are compared to the effect of *GAPDH* siRNA normalised to housekeeping gene expression. Data show the mean – s.d. of 3 separate experiments. **B.** Quantitation of the ciliation frequency. Cells were treated with 50 nM siRNA for 48 hours. Histograms show means ± s.d. of 3 separate experiments in which at least 100 transfected cells were quantitated. **, $P<0.01$ by unpaired t-test.

We then investigated whether CP110 depletion had any effect on cilium length in proliferating and senescent BJ cells. Proliferating and senescent BJ cells were treated with 50 nM siRNA for 48 hours, fixed and stained for acetylated tubulin. As shown in Figure 3.21, we did not observe any significant effect on the mean cilium length in senescent populations after CP110 depletion. However, in the proliferating populations, we observed a small but significant increase in the mean cilium length after CP110 depletion. The length increased from 1.2 ± 0.06 µm in cells transfected with *GAPDH* siRNA to 1.6 ± 0.07 µm in cells transfected with *CP110* siRNA#1 and to 1.4 ± 0.09 µm in cells transfected with *CP110* siRNA#2. This result shows that knockdown of CP110 causes a decrease in the mean cilium length in proliferating but not senescent populations.

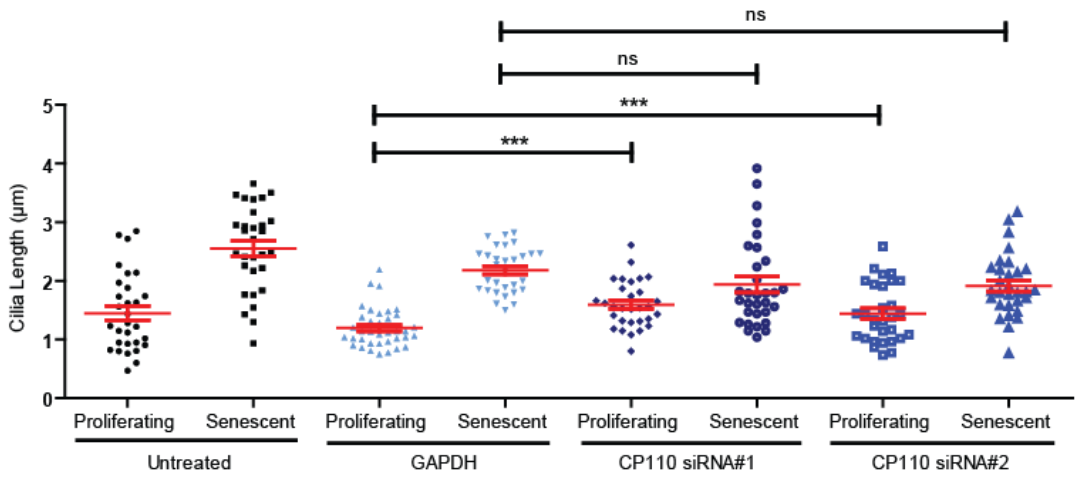

Figure 3.21 CP110 knockdown caused a decrease in cilium length in proliferating but not senescent populations.
Quantitation of cilium length in the indicated cells. Cells were treated with 50 nM siRNA for 48 hours. Histograms show means ± s.d. in which the length of at least 30 cilia were measured for each condition.

We then determined if depletion of CP110 has an effect on the proliferative index in proliferating and senescent cells. Proliferating and senescent BJ cells were treated with 50 nM siRNA for 48 hours, fixed and stained for SA-β-Gal. We then proceeded immediately to immunofluorescence staining for acetylated tubulin. Figure 3.22A shows an immunofluorescence micrograph of a transfected cell, which is stained negative for Ki67 and positive for SA-β-Gal. We counted the frequency of these cells in proliferating and senescent populations. As shown in Figure 3.22B, most of the cells in the senescent populations were $Ki67^-$ and all of the $Ki67^-$ cells stained positive for β-galactosidase (N = 3 experiments of 100 cells each). CP110 knockdown did not have any effect on the number of $Ki67^-$ cells also staining positive for β-galactosidase. However, in the proliferating populations, CP110 knockdown caused a marked increase in the number of $Ki67^-$ cells which also stained positive for β-galactosidase. 70.9 ± 8% of cells transfected with CP110 siRNA#1 and 69.3 ± 7% of cells transfected with CP110 siRNA#2 were $Ki67^-$ and positive for β-galactosidase compared to 15.9 ± 2% in untreated cells. These data suggest that increased ciliation, resulting from the loss of CP110, potentiates cellular senescence in human fibroblasts.

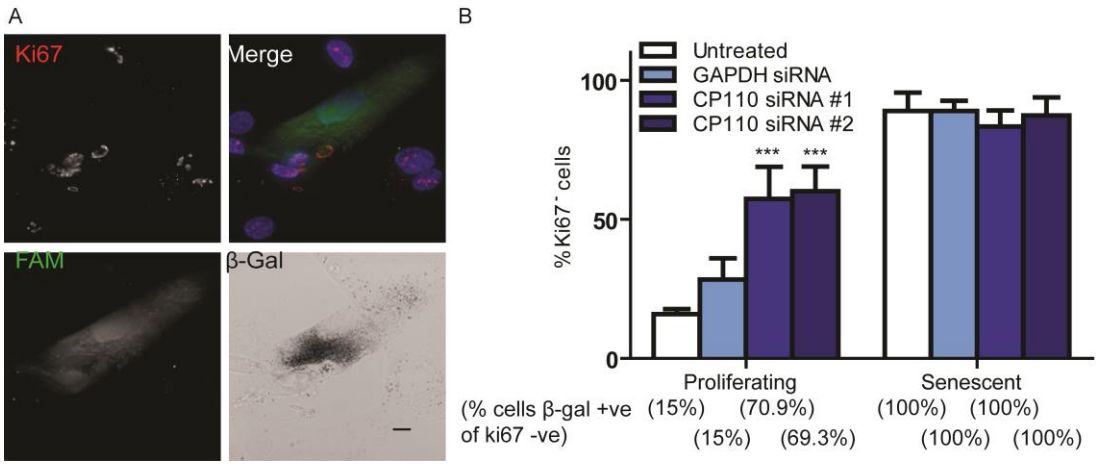

Figure 3.22 CP110 depletion causes increased cellular senescence.
A. Microscopy of the indicated cells stained with antibodies to Ki67 and acetylated tubulin. Fluorescent RNA was co-transfected at a ratio of 1:5 with the siRNA as a transfection control. The brightfield channel shows β-gal staining. Scale bar, 10 μm. **B.** Quantitation of the proliferative/ senescence index of BJ cells after the indicated treatment, as determined by microscopy analysis of Ki67 signal. Histograms show means ± s.d. of 3 separate experiments in which at least 100 transfected cells were quantitated. ***, $P<0.001$ by unpaired t-test.

CHAPTER 4 – DISCUSSION

4.1 Mechanisms of increased ciliary frequency

Cilium formation is closely regulated by and linked to the cell cycle, as cilia must be resorbed to allow the mitotic functioning of centrosomes in bipolar spindle formation. Cellular quiescence, a temporary exit from the cell cycle that can be induced by the removal of growth factors, facilitates ciliogenesis (Seeley and Nachury, 2010; Kobayashi and Dynlacht, 2011). However, the impact of cilia on replicative senescence, an essentially permanent exit from the cell cycle, has not yet been described. To obtain cells that had undergone replicative senescence, BJ, MRC5 and NHDF fibroblast cells were extensively passaged until they stopped proliferating. An increase in senescence-associated β-Galactosidase (SA-β-Gal) activity and senescence-associated heterochromatic (SAHF) staining confirmed that the growth arrest observed was due to the induction of replicative senescence.

We first asked if primary cilia were altered in these senescent cells. When we analysed cilia by immunofluorescence microscopy we observed a significant increase in cilium frequency in senescent populations, compared to proliferating controls. Formation of the primary cilium relies on basal body migration towards the apical surface of the cell and docking to the plasma membrane (reviewed by (Ishikawa and Marshall, 2011; Kim and Dynlacht, 2013)). Actin dynamics appears to have an important role in these processes (Dawe et al., 2009; Kim et al., 2010; Adams et al., 2012; Barbelanne et al., 2013). In fibroblasts, actin filaments may exist as a cortical actin network or actin stress fibres. The cortical actin network provides a scaffold for ciliogenesis, while actin stress fibres inhibit the formation of cilia. Additionally, movement of the actin-myosin network is required for centriole migration (Rosenblatt et al., 2004).

Dysregulation of actin may also be associated with the change in senescent cell morphology (Chen and Ames, 1994). The accumulation of actin has been associated with the induction of senescence (Lim et al., 2000; Gourlay et al., 2004; Kwak et al., 2004). Cell spreading in hTERT-RPE1 cells has been shown to disturb actin architecture, with spread RPE1 cells displaying larger stress fibres (Pitaval et al., 2010). Disruption of the actin network in spread RPE1 cells with cytochalasin D

results in increased cilium frequency and length (Pitaval et al., 2010). These results suggest that the change in senescent cell morphology, brought about by disruption of actin dynamics, may cause an increase in ciliary frequency.

Rho kinase activity is also necessary for the apical positioning of the basal body and the subsequent growth of the primary cilium (Chevrier et al., 2002). Cell spreading has been correlated with an increase in actomyosin contraction (Polte et al., 2004; Engler et al., 2006). Inactivation of Rho kinase relaxes actomyosin contraction of spread RPE1 cells, resulting in an increase in cilium frequency. In these cells, basal bodies are found below the nucleus, in contact with the ventral surface. Rho kinase inhibition does not affect the ventral positioning of basal bodies in spread RPE1 cells, but promotes the growth of ventral cilia (Pitaval et al., 2010). Our results show that *RhoA* is down-regulated in senescent cells (Results section 3.2.4) and interestingly, RhoA dysregulation has been associated with senescence induction in cancer cells from human and mouse (Forti and Armelin, 2007; Park et al., 2007; Costa et al., 2008). Inactivation of RhoA may relax actomyosin contraction in our senescent cells, resulting in an increase in cilium frequency. It would be interesting to examine whether basal body migration or positioning is disrupted in these cells. This experiment could be carried out by staining proliferating and senescent cells for acetylated tubulin and actin, obtaining Z-stack images using immunofluorescence microscopy and comparing their basal body positioning.

In proliferating cells, stabilisation of HEF1, a scaffolding protein which is localised to the basal body, activates Aurora A kinase. Aurora A activates the tubulin deacetylase HDAC6 (histone deacetylase 6), which is localised to the basal body and ciliary axoneme, resulting in destabilisation of axonemal microtubules and initiation of cilium resorption (Pugacheva et al., 2007). Pifo (Pitchfork), a protein which localises to the basal body, also interacts with Aurora A and facilitates its activation (Kinzel et al., 2010). The Never In Mitosis A (NIMA) related kinase NEK2, which is localised to proximal ends of centrioles, is another important mediator essential for cilia resorption at the G2/M transition, but the exact mechanisms involved and its connection to the Aurora A pathway are unclear (Spalluto et al., 2012). It has also been shown that PLK1, which stabilises HEF1, may activate HDAC6 (Lee et al., 2012). Interestingly, down-regulation of Aurora A and, in a separate study PLK1,

have been shown to induce senescence through a p53-dependent pathway (Kim et al., 2011a; Kim et al., 2013). While an increase in cilium frequency is not unexpected in cells which have exited the cell cycle, the mechanism by which this occurs in senescent cells remains unclear. It is possible that the increase in cilium frequency we see is a result of impaired cilium resorption, potentially due to the down-regulation of Aurora A and PLK1. As senescent cells do not enter mitosis, they have no requirement to resorb their cilium. It is also possible that when a cell enters senescence, it does so with its cilium intact, resulting in a higher frequency of cilia in the senescent cell population.

4.2 Mechanisms of ciliary length control

Somewhat surprisingly during our immunofluorescence microscopy analysis, we noted that the primary cilia in senescent cells were consistently longer than those in proliferating cells. The increase in ciliary abnormality we observed suggested that transcriptional control of ciliary gene expression might be altered in senescent cells. A correlation between ciliary gene transcription and ciliary growth has been well documented in *Chlamydomonas* and sea urchins (Lefebvre et al., 1980; Silflow et al., 1982; Harlow and Nemer, 1987; Norrander et al., 1995). For example, Foxj1, a member of the forkhead/winged-helix family of transcription factors, has been shown to be important in regulating genes necessary for cilia function (Jacquet et al., 2009). Interestingly, expression of Foxj1 in mice has been shown to increase ciliary length (Cruz et al., 2010). To identify ciliary genes whose transcription might be affected by senescence, we performed a quantitative real-time PCR screen using a commercial Primary Cilium PCR array. Of the 84 cilium-specific genes analysed, all genes showed a difference in expression in senescent compared to proliferation cells (Results section 3.2.4).

The role of the centrosome in the formation of the bipolar spindle necessitates that the cilium be lost during mitosis, therefore a more limited time for ciliary elongation is available to proliferating cells than to cells which are no longer cycling. To understand how senescent cells acquire their elongated cilia, we tested if an extended period without cycling was required for such ciliation. Our cilia re-

growth assays suggest a cell-intrinsic cilium length that is independent of the time spent outside cycle.

There is growing evidence which suggests that an increase in axonemal length can delay cell cycle re-entry. Several cilium-related proteins have been linked to the cell cycle including mediators of axoneme disassembly. For example, loss of the centrosomal protein Nde1, which is normally expressed at low levels in quiescence and interacts with the axonemal dynein subunit LC8, causes an increase in cilium length and a subsequent delay in cell cycle re-entry (Kim et al., 2011b). Phosphorylation of the cytoplasmic dynein light chain TCTEX1 induces ciliary resorption and promotes S phase entry (Li et al., 2011). Several cell cycle modulators have also been shown to influence ciliogenesis. For example, Aurora A is necessary for cilia disassembly and regulates mitotic entry via spindle organisation (Pugacheva et al., 2007). In addition, the cell cycle modulator and antagonist of CDK1 (cyclin dependent kinase 1), CDC14b (cell division cycle 14 b), has been shown to be essential for proper ciliogenesis and ciliary length regulation (Clément et al., 2012). Similarly, in *Chlamydomonas*, a mutation in the CDK-related kinase Lf2p results in longer cilia (Tam et al., 2007). Also, the spindle checkpoint regulator BubR1 was found to be required for proper primary cilium formation (Miyamoto et al., 2011). Impairment of mitogenic MAP (microtubule associated protein) kinase signalling has also been shown to result in increased ciliary length. For example, in *Chlamydomonas*, null mutants of the MAP kinase Lf4p (Berman et al., 2003b), and in *C. elegans*, mutants of the dyf-5 MAP kinase have increased flagellum/cilium length (Burghoorn et al., 2010). NIMA-related kinases (NRK or NEK) are also involved in the cell cycle and are responsible for cilium length regulation. For instance, in *Chlamydomonas*, knockdown of the NRK Cnk2p by RNAi (Bradley and Quarmby, 2005) and in mice, loss of Nek8 (NPHP9), result in excessively long flagella/cilia (Smith et al., 2006; Sohara et al., 2008). In addition, induction of ciliogenesis upon cell cycle exit has been shown to be accompanied by both activation and proteasomal degradation of Nek8 (Zalli et al., 2012). Taken together, these results suggest that the timing of cilium formation and resorption, along with cilium length regulation, are essential for cell cycle progression. Our data show a down-regulation in *NEK8* and *MAPK1* in senescent cells (Results section 3.2.4).

These results suggest that lengthening of the primary cilium results in down-regulation of cell cycle components and subsequent cell cycle exit, or conversely, that cell cycle exit leads to down-regulation of cell cycle components leading to cilium elongation.

Cilium length can also be regulated through changes in the actin or microtubule network. As mentioned previously, senescent cells exhibit changes in their cell morphology and these changes may be a result of dysregulation of components of the cytoskeleton, such as actin. A functional genomic screen using RNA interference (RNAi) identified positive and negative ciliogenesis modulators, which included molecules involved in actin dynamics and vesicle trafficking (Kim et al., 2010). One of the genes identified, *ACTR3*, is known to encode an actin-related protein required for the nucleation of actin polymers at filament branches (Cooper and Schafer, 2000). *ACTR3* knockdown led to the elongation of primary cilia, suggesting that actin polymerisation has an inhibitory role in primary cilium formation. Similarly, the human ciliopathy Bardet-Biedl syndrome (BBS) proteins play a central role in the regulation of the actin cytoskeleton and control cilia length partly through alteration of RhoA levels (Hernandez-Hernandez et al., 2013). In addition, actin depolymerisation, actin stabilisation and protein kinase A activation result in elongation of the primary cilium (Sharma et al., 2011). Septins are a family of GTPases that interact with the actin-based cytoskeleton and microtubules. SEPT2 forms a complex with SEPT7 and SEPT9, which localises along the entire length of the cilium with MAP4. This complex positively controls cilia length and depletion of any of these septins inhibits ciliogenesis (Ghossoub et al., 2013). Together these results suggest that dysregulation of components of the cytoskeleton, which may also cause changes in the senescent cell morphology, contributes to the lengthening of the primary cilium seen in senescent cells. An interesting experiment would be to test whether actin is involved in the cell shape-associated regulation of increased cilium length in senescent cells. To do this experiment, we would treat senescent cells with cytochalasin D, an inhibitor of actin polymerisation, and measure cilium length by immunofluorescence microscopy.

Since cilia lack the machinery necessary for protein synthesis, the materials required for ciliary axoneme extension must be transported from the cell body

(reviewed by (Pedersen and Rosenbaum, 2008; Satir and Christensen, 2008; Kobayashi and Dynlacht, 2011)). Transport within the cilium is mediated by IFT (Kozminski et al., 1993). During IFT, protein complexes are transported from the base of the cilium to the distal tip by kinesin-2 motors (anterograde transport) (Cole et al., 1998) and from the distal tip back to the cell body by cytoplasmic dynein 2 motors (retrograde transport) (Pazour et al., 1998). Defects in ciliary axoneme elongation caused by loss of intraflagellar transport (IFT) proteins result in cell cycle progression defects. For example, components of the dynein motor have been shown to play a role in regulating ciliary length. The dynein light chain TCTEX1 is a key modulator of cilia length control. Depletion of TCTEX1 results in longer cilia. Suppression of dynein heavy chain-2 (DHC2) is associated with a loss in TCTEX1 and this correlates with an increase in cilia length. Co-depletion of DHC2 and TCTEX1 causes an even greater increase in cilia length (Palmer et al., 2011). Loss of dynein-2 results in lengthened cilia in *Tetrahymena* (Asai et al., 2009). However, in a *Chlamydomonas* mutant with a temperature-sensitive defect in DHC1b (dynein heavy chain 1b) flagella showed a dramatic reduction of retrograde IFT but remained nearly full-length for many hours in culture (Engel et al., 2012). This suggests that retrograde IFT is not required for the maintenance of flagellar length. We observed a trend towards up-regulation of genes involved in intraflagellar transport (IFT) and ciliary morphogenesis in senescent cells (Results section 3.2.4). Moreover, *DNCI1*, a gene encoding intermediate chain 1 of the cytoplasmic dynein, has been shown to be up-regulated in senescent cells (Horikawa et al., 2001). These results make it unlikely that a down-regulation in dynein is contributing to the lengthening of cilia in senescent cells.

Another mechanism of ciliary lengthening may be the binding of doublecortin (DC) domain proteins to microtubules, which facilitates microtubule polymerisation. Microtubules are key components of the cytoskeleton. The arrangement of microtubules around centrosomes is critical for formation of the ciliary axoneme as well as intracellular protein trafficking, mitotic spindle assembly, cell polarity, cell shape and consequently cellular adhesion and mobility (Lüders and Stearns, 2007; Bornens, 2008). Microtubules are assembled by the polymerisation of α- and β-tubulin heterodimers into polarised tubular filaments. Dynein and kinesin

motors bind to microtubules and deliver proteins and regulatory factors to their destinations in the cell (Etienne-Manneville, 2013). Overexpression of the DC-domain containing 2 (DCDC2) protein in fibroblast cells causes an increase in ciliary length (Massinen et al., 2011). Similarly, overexpression of the DC-domain protein RP1 has also been shown to increase cilium length (Omori et al., 2010). Interestingly, disruption of the dynamic instability of microtubules has been shown to induce senescence (Klein et al., 2005). Given the importance of microtubule organisation on cell shape and the effect of cell shape on cilium length, it is possible that binding of DC-domain proteins to microtubules in senescent cells contributes to cilium lengthening.

The mother centriole provides the basal body, a foundation for the formation of the primary cilium (Gerdes et al., 1983; Goetz and Anderson, 2010), while the transition zone is the region where the basal body triplet microtubule structure converts into the axonemal doublet microtubule (Seeley and Nachury, 2010). Basal body and transition zone proteins have been shown to be involved in ciliary lengthening. Proteins associated with the renal ciliopathy, nephronophthisis (NPHP), are localised to the transition zone (reviewed by (Hildebrandt et al., 2009)). There are 10 NPHP genes (*NPHP1-NPHP10*). *NPHP8* depletion in human cells results in the elongation of cilia (Patzke et al., 2010) and in *C. elegans*, NPHP8 is required for proper cilium elongation on ciliated neurons (Liu et al., 2011). Our microscopy analysis showed an increase in centrosome amplification and ciliary abnormality in senescent cells, but otherwise apparently normal centrosome and cilium protein composition (Results section 3.2.3). We found that *NPHP1*, the product of which forms a complex with NPHP4 and NPHP8 (Sang et al., 2011), is up-regulated in senescent cells (Results section 3.2.4). While we have no direct data, these results suggest that NPHP8 is also up-regulated in senescent cells and so is unlikely to be contributing to the lengthening of cilia in senescent cells. Further analysis of NPHP8 by immunofluorescence microscopy and immunoblot would confirm this.

Other data suggest that cell signalling pathways regulate ciliary elongation. Interestingly, dysregulation of cell signalling pathways has also been shown to be involved in cellular senescence. The primary cilium is essential for transduction of the Hedgehog (Hh), Notch and Wnt signalling pathways. Following Notch secretion,

the Notch receptor on the target cell binds to ligands on the surface of the signalling cell (Bray, 2006; Kopan and Ilagan, 2009). Ligand binding promotes two cleavage events in the Notch receptor. This releases the Notch intracellular domain (NICD) from the membrane (Brou et al., 2000; Struhl and Greenwald, 2001). The NICD translocates to the nucleus where it promotes transcription of Notch target genes (Bray, 2006; Kopan and Ilagan, 2009). Hyperactivation of Notch signalling, by overexpressing NICD, results in the elongation of cilia (Lopes et al., 2010). In addition, Notch3 has been shown to be elevated in senescent cells and this up-regulation is required for the induction of p21 expression (Cui et al., 2013). Our results showed an up-regulation in *p21* in senescent cells (Results section 3.2.4). It is possible that elevated Notch signalling components are required for the induction of p21 expression in our senescent cells and also contribute to the increase in cilium length. However, further analysis of Notch signalling components, by quantitative real-time PCR and immunoblot, would have to be carried out to confirm this.

The canonical Wnt pathway is activated when Wnt ligands bind to Fz (Frizzled) or the LDL (Low-density lipoprotein) receptor-related proteins 5/6, which in turn, activate Dvl (Dishevelled) (Cong et al., 2004; González-Sancho et al., 2004; Zeng et al., 2008). Dvl inhibits GSK3β (Glycogen synthase kinase 3 β) and prevents phosphorylation and destruction of β-catenin (van Noort et al., 2002). This allows β-catenin to accumulate in the cytoplasm before translocating to the nucleus where it converts TCF/LEF transcription factors from transcriptional repressors to activators (Behrens et al., 1996; Molenaar et al., 1996) and activates the transcription of Wnt target genes (He et al., 1998; Roose et al., 1999; Tetsu and McCormick, 1999). The Wnt signalling pathway has been shown to be involved in the aging process. Analysis of tissues and organs from young Klotho-deficient mice, a mouse model of accelerated aging, showed a decrease in stem cell number, an increase in progenitor cell senescence and an increase in Wnt signalling. Ectopic expression of Klotho antagonised Wnt activity and accelerated cellular senescence (Liu et al., 2007a). Inhibition of GSK3β with lithium chloride results in elongated flagella in *Chlamydomonas* (Wilson and Lefebvre, 2004). However, in mammalian cells, lithium induces cilium elongation through inhibition of adenylate cyclase III (Ou et al., 2009). Our results showed an up-regulation in *GSK3β* (Results section 3.2.4),

which suggests that Wnt signalling is increased in our cells and may be contributing to the induction of senescence. Our results also showed a down-regulation in *ADCY3* (adenylate cyclase 3), which may be involved in the lengthening of cilia in senescent cells.

Figure 4.1 summaries the mechanisms of ciliary length control in senescent cells. Our results suggest that a down-regulation in dynein, a decrease in Wnt signalling or dysregulation in basal body (BB) proteins are not the cause of an increase in cilium length in senescent cells. However, dysregulation of cytoskeleton components, binding of DC domain proteins to microtubules (MT), an increase in Notch signalling, down-regulation in ADCY3 or down-regulation of cell cycle components may contribute to the lengthening of the cilium in senescent cells. An increase in cilium length results in exit from the cell cycle, or exit from the cell cycle results in an increase in cilium length.

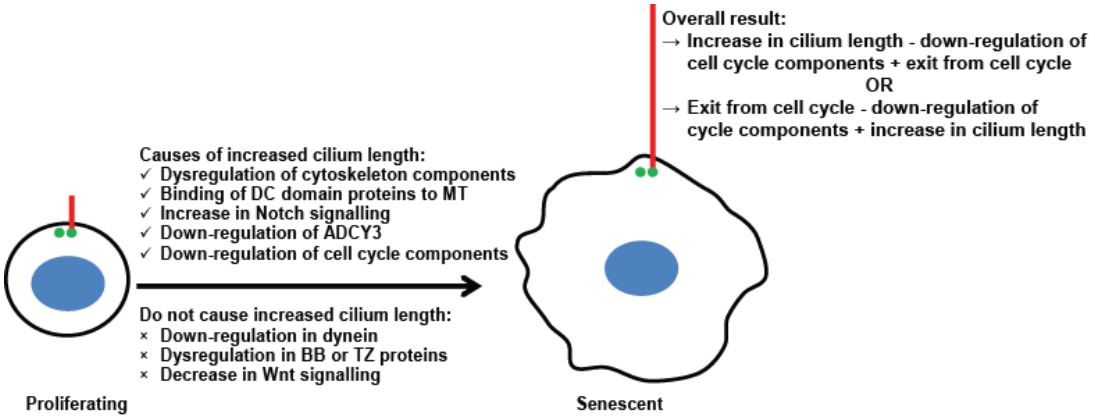

Figure 4.1 Mechanisms of ciliary length control.
A schematic representation of the mechanisms of ciliary length control in senescent cells. Abbreviations: doublecortin (DC); microtubule (MT); adenylate cyclase III (ADCY3); basal body (BB); transition zone (TZ). Diagram not to scale.

4.3 Cause and consequences of centrosome amplification and ciliary abnormality in senescent cells

During our immunofluorescence microscopy analysis we observed that senescent BJ fibroblasts had elevated numbers of centrioles. Centrosome amplification can be induced by *de novo* assembly, overexpression of centrosome duplication proteins, viruses, cytokinesis failure or centrosome fragmentation

(introduction section 1.11). Centrosome duplication proteins or viruses were not ectopically expressed in our senescent cells and so it is improbable that these are the cause of the centrosome amplification observed (Duensing et al., 2000; Bettencourt-Dias et al., 2005). An elevated number of centrioles due to *de novo* assembly is also implausible as this usually only occurs in specialised cells during ciliogenesis or in proliferating cells after removal of centrioles by laser ablation or microsurgery (reviewed by (Dawe et al., 2007; Vladar and Stearns, 2007)). Centrosome amplification may also occur if, for example, cells have unrepaired DNA. Cells with functional p53 activate a checkpoint response if cytokinesis fails and will eventually undergo cell death (reviewed by (Fukasawa, 2008)). In the absence of p53, cells may continue to cycle and undergo repeated cytokinesis failures resulting in tetraploidisation and centrosome amplification (reviewed by (Fukasawa, 2008)). However, senescent cells were not multinucleated, suggesting that it is unlikely that cytokinesis failure was responsible for the increase in the number of centrosomes.

Centrosome amplification can be also be induced by DNA damage (Sato et al., 2000a; Sibon et al., 2000; Sibon, 2003; Dodson et al., 2004; Fletcher and Muschel, 2006). DNA damage induces CHK1-dependent centrosome amplification, which occurs during a prolonged G2 phase delay involving ATM (Dodson et al., 2004; Bourke et al., 2007). Impaired function of the tumour-suppressor p53 has also been shown to cause abnormal centrosome numbers (Meraldi et al., 2002; Tarapore and Fukasawa, 2002). Consistent with this finding, it has also been reported that irradiation induces amplification of centrosomes in cells lacking p53 (Sato et al., 2000b; Kawamura et al., 2006).

Senescence can arise through persistent DNA damage signalling, notably at telomeric sequences that can become exposed after multiple divisions (d'Adda di Fagagna et al., 2003; Herbig et al., 2004; Fumagalli et al., 2012), or through a response to strong mitogenic signalling induced by oncogenes (Serrano et al., 1997; Di Micco et al., 2006). Telomere shortening triggers senescence through the ATM-p53 pathway, up-regulating p21 (also known as CDKN1A, cyclin-dependent kinase inhibitor 1A) and causing growth arrest (Herbig et al., 2004). AKT promotes senescence-like arrest of cell growth via increased transcriptional activity of p53, resulting in up-regulation of *p21* (Miyauchi et al., 2004). Our results show that

CDKN1A, *TP53* (tumour protein 53, also known as p53) and *AKT* are up-regulated in senescent cells (Results section 3.2.4). These data suggest that the centrosome amplification observed in our senescent cells is most likely a consequence of elevated DNA damage signalling induced by telomere shortening, resulting in the up-regulation of *AKT*, *p53* and *p21*. Immunoblot analysis using antibodies against AKT, p53 and p21 would allow us to investigate this further.

Senescence-associated centrosome abnormalities have been reported in other human fibroblast lines (Ohshima and Seyama, 2010). There is a possible association between p53 localisation and the number of amplified centrosomes in replicatively or prematurely senescent cells (Ohshima and Seyama, 2010). An increase in the number of abnormal centrosomes is associated with the entry of late-passage MEFs into senescence. In addition, disruption of the centrosome in early-passage MEFs by depletion of NEDD1 (neural precursor cell expressed developmentally down-regulated gene 1) resulted in centrosome fragmentation and premature entry into senescence (Manning and Kumar, 2010). Our results show apparently normal centrosome protein composition in senescent cells (Results section 3.2.3). It is therefore unlikely that the abnormal number of centrosomes we observed in senescent cells is due to centrosome fragmentation, as seen upon depletion of NEDD1.

Suppression of centrosome amplification after DNA damage depends on the accumulation of p27 (Sugihara et al., 2006). Interestingly, recent results have suggested a role for p27 in senescence induction and maintenance. p27 is induced by growth inhibitory signals that can also induce senescence, suggesting that it participates in mediating senescence signals (Chu et al., 2008). p27 has also been shown to be required for pRb-mediated senescence and ectopic expression of p27 induces senescence in osteosarcoma cells (Alexander and Hinds, 2001). In addition, Cdk2 depletion or inactivation by p27 restores senescence in cells overexpressing MYC (Campaner et al., 2010; Hydbring et al., 2010). Furthermore, AKT-induced premalignant prostatic intraepithelial neoplasia (PIN) shows increased p27 expression and senescence, whereas AKT expression in $p27^{+/-}$ or $p27^{-/-}$ cells led to loss of senescence and cancer progression (Majumder et al., 2008). As mentioned previously, we observed an up-regulation of *AKT* in senescent cells and AKT1

activation has been shown to induce senescence (Nogueira et al., 2008; Astle et al., 2012). Inhibition of Aurora A causes a decrease in AKT1 phosphorylation (Guan et al., 2007). Aurora A is essential for primary cilium resorption (Pugacheva et al., 2007) and down-regulation of Aurora kinases is sufficient to induce senescence (Huck et al., 2010). In our senescent cells, possible down-regulation of Aurora A may lead to a decrease in the phosphorylation of AKT1, resulting in the observed increase in *AKT1* and possible increase in p27, cumulating in the amplification of centrosomes and induction of senescence. Immunoblot analysis using antibodies against Aurora A, p27 and AKT1 would allow us to investigate this further.

The relationship between the centrioles and the primary cilium means that extra centrioles may lead to multiple cilia being formed in a single cell. Mahjoub and Stearns have reported that following PLK4-induced centrosome amplification, multiple primary cilia emerge from a single ciliary pocket in super-ciliated cells. The extra cilia were clustered and shared the same ciliary pocket, suggesting that the ciliary pocket is a rate-limiting structure for trafficking of ciliary proteins (Mahjoub and Stearns, 2012). Interestingly, we observed an increase in the number of abnormal cilia in senescent BJ cells. Ciliary abnormality was scored as more than one ciliary structure per cell, one cilium emanating from overamplified centrosomes, or both. The extended length and higher number of cilia in senescent cells may contribute to an increase in ciliary surface area, as suggested by Mahjoub and Stearns. This may impede Hh signalling by diluting out components of the signalling pathway (Mahjoub and Stearns, 2012). Serial section EM would be useful in determining if the multiple cilia in senescent cells emerge from a single ciliary pocket.

Due to the role of the centrosome as a support for the formation of the primary cilium, dysregulation of the centrosome may lead to defective ciliary assembly and signalling and thus, contribute to cilia-related degenerative diseases. Extra centrosomes have been observed in tissues with mutations in ciliary genes. Meckel-Gruber syndrome is characterised by renal cystic dysplasia, central nervous system malformations and hepatic developmental defects (Blankenberg et al., 1987). Depletion of the centrosomal components Mks1 and Mks3 (Meckel syndrome type 1 and 3), results in centrosome amplification (Tammachote et al., 2009). However, our results show that *MKS* is up-regulated in senescent cells (Results section 3.2.4) and

thus, it is improbable that MKS causes the extra centrosomes and cilia observed in senescent cells.

Having amplified centrosomes and/or more than one primary cilium per cell can have serious cellular consequences. For example, polycystic kidney disease (PKD) is caused by defects in primary cilium function. Loss of Polycystin-1 (PKD1) and (PKD2) cause centrosome amplification (Battini et al., 2008; Burtey et al., 2008) and mutations in *Pkd1* and *Pkd2* cause defects in ciliary signalling (Nauli et al., 2003). Similarly, *Tsc1* and *Tsc2* (Tuberous sclerosis complex 1 and 2) null mice show increased cilia formation and *Tsc2* null MEFs can contain extra centrosomes and cilia, which results in the dilution of ciliary proteins (Astrinidis et al., 2006; Hartman et al., 2009). Furthermore, mutations in the ciliary protein Inversin (Inv) causes nephronophthisis, a form of polycystic kidney disease. Loss of Inv in the developing mouse nephron leads to defective cytokinesis and an increased frequency of binucleate cells that contain extra cilia (Werner et al., 2013). Similarly, loss of Citron kinase in rats causes failed cytokinesis in cortical neural progenitor cells which results in the formation of binucleate neurons that also have amplified centrosomes and multiple primary cilia per neuron (Anastas et al., 2011). Although centrosome amplification has not been directly identified as a cause of these diseases, a change in the centrosome number may have an effect on the structure and function of the primary cilium and thus cause the defects seen in these cells.

Furthermore, control of cilium length is important, as elongated cilia have been associated with age-related diseases. The occurrence of osteoarthritis (OA) increases directly with age and it is the most common cause of chronic disability in the elderly. OA is characterised as joint failure due to progressive changes in several components of the musculoskeletal system (reviewed in (Lee et al., 2013)). OA is associated with increases in cilia length and prevalence, and the increased expression of hedgehog signalling genes (McGlashan et al., 2008; Lin et al., 2009). Chondrocyte senescence has been proposed to contribute to the pathogenesis of OA through increased production of inflammatory mediators and matrix degrading enzymes characteristic of the senescent secretory phenotype (SASP) (Martin and Buckwalter, 2003; Yudoh et al., 2005; Aigner et al., 2007; Loeser, 2009). Interestingly, an increase in primary cilium length was observed in primary

chondrocytes post treatment with inflammatory cytokine interleukin-1 (IL-1), which is up-regulated in OA. This result suggests that the primary cilium influences inflammatory cytokine responses (Wann and Knight, 2012).

4.4 DNA repair pathways in ciliated cells

Previously-published data from our laboratory have shown that ciliated cells had lower levels of Rad51 foci induction than non-ciliated cells, an indication that homologous recombination (HR), and thus DNA repair, may be impaired in ciliated cells (Conroy et al., 2012). However, we found no difference in the levels of Rad51 in ciliated and non-ciliated proliferating and senescent cells, suggesting they respond in the same way to genotoxic stress.

Why ciliated and non-ciliated cells would respond differently to DNA damage remains unclear. However, a small number of proteins have been identified which are involved in both the control of ciliogenesis and the DNA damage response. For example, Nek1 has been implicated in ciliogenesis and DNA damage responses. Overexpression of Nek1 inhibits ciliogenesis and its depletion results in reduced DNA damage repair following IR (Shalom et al., 2008; Pelegrini et al., 2010). Nek1 has also been shown to interact with other proteins which are involved in HR, such as MDC1, MRE11 and ATRX (Surpili et al., 2003). In addition, CEP164 is a centriole appendage protein which is required for primary cilium formation and has also been implicated in the DNA damage response. It interacts with ATM and ATR and is phosphorylated following IR and UV damage. CEP164 is recruited to UV-damage sites on DNA and reduced expression of *CEP164* sensitises cells to UV irradiation (Graser et al., 2007; Sivasubramaniam et al., 2008; Pan and Lee, 2009). Mutations in *CEP164* have been shown to cause Nephronophthisis-related ciliopathies (NPHP-RC), which affect the kidney, retina and brain (Chaki et al., 2012). Furthermore, the ciliary kinase NEK8 has also been linked to the DNA damage response. NEK8 has been identified as an effector of the ATR-mediated replication stress response. NEK8 supresses DNA double strand break (DSB) formation by limiting cyclin A-associated cyclin-dependent kinase (CDK) activity. Cells lacking NEK8 form spontaneous DSBs and NEK8 mutant mice accumulate DNA damage in their kidneys (Choi et al., 2013). Interestingly, another NIMA-

related kinase, NEK4, has been shown to regulate entry into replicative senescence and the response to DNA damage in human fibroblasts. Depletion of NEK4 extends the number of population doublings required to reach replicative senescence and resulted in decreased transcription of the cyclin-dependent kinase inhibitor p21. NEK4-depleted cells also displayed impaired cell cycle arrest in response to DSBs (Nguyen et al., 2012). It has also been shown that down-regulation of NEK4 in ciliated cells results in a decrease in cilium assembly (Coene et al., 2011). Together, these observations implicate Nek4 as a regulator of replicative senescence, the DNA damage response and cilium assembly.

A large number of DNA damage response (DDR) proteins have been shown to localise to the centrosome during the cell cycle (reviewed by (Löffler et al., 2006; Fukasawa, 2007; Shimada and Komatsu, 2009)), suggesting that the centrosome acts as a scaffold at which cellular signals can be amplified, as well as be the eventual target of such signalling. A change in centriole composition may be how ciliated and non-ciliated cells respond differently to the DDR. C-NAP1 depletion causes a change in centriole composition by preventing the recruitment of NEK2 to the proximal ends of centrioles. Loss of NEK2 also impacts on the ciliation capacity of the mother centriole (Spalluto et al., 2012). Furthermore, depletion of ALMS1 (Alström syndrome 1) which is required for the formation and maintenance of the cilium, leads to a reduction of C-NAP1 levels at the proximal end of the centriole (Knorz et al., 2010). Interestingly, our results show that *ALMS1* is down-regulated in senescent cells suggesting that a change in centrosome composition might be how ciliated and non-ciliated proliferating and senescent cells would respond differently to the DDR. It is also possible that because the cilium provides a physical block for cell cycle progression by laying claim to the centrosome, it prevents the DDR proteins localised at the centrosome from becoming active. For example, BRCA1 and BRCA2 are expressed in proliferating, but not quiescent cells (Rajan et al., 1996).

4.5 Loss of Hedgehog signalling leads to increased cilium length in senescent cells

The primary cilium is essential for Hedgehog (Hh) signal transduction (Goetz and Anderson, 2010). Signalling through the Hh pathway requires access of the Hh signal transducer, SMO, to the primary cilium (Corbit et al., 2005). In the absence of Hh signalling, the tumour suppressor Patched (PTCH1) inhibits the activity of SMO and prevents its trafficking to the cilium. In the presence of Hh signalling, Hh binds to PTCH1, relieving the inhibition of SMO and allowing it to move to the cilium and activate the Hh pathway through activation of the Glioma (Gli) family (Chen and Struhl, 1996; Taipale et al., 2002; Bijlsma et al., 2006; Rohatgi et al., 2007). Gli proteins translocate to the nucleus where they activate transcription of Hh target genes (Dai et al., 1999; Kenney and Rowitch, 2000; Kenney et al., 2003; Agren et al., 2004; Bigelow et al., 2004; Pan et al., 2006). Expression of the forkhead transcription factor Foxj1 in mice increases ciliary length and decreases the response to Hh (Cruz et al., 2010). Interestingly, Hh down-regulation has been associated with senescence (Bishop et al., 2010) and age-related diseases such as type 2 diabetes, neurodegeneration, atherosclerosis and osteoporosis (Thomas et al., 2000; Hurtado-Lorenzo et al., 2004; Beckers et al., 2007).

The mother centriole is the foundation for the formation of the primary cilium, and so, extra centrioles may lead to the formation of extra cilia. Mahjoub and Stearns demonstrated that extra centrosomes and cilia negatively impacts on epithelial architecture. Mono-ciliated IMCD-3 (immortalised kidney collecting duct) cells were able to form an organised *in vivo* architecture, while IMCD-3 cells with multiple cilia were not. Furthermore, quantification of GLI-GFP activation showed a dilution of SMO in cilia of super-ciliated cells, demonstrating defective activation of the Hh pathway (Mahjoub and Stearns, 2012).

Our results showed a general loss in Hh components in senescent cells. These data suggested that Hh might be mitogenic in fibroblasts, as it is in human mammary epithelial cells (Bishop et al., 2010). Hh signalling promotes mitogenesis by suppression of the growth-inhibitory p16, a well-established mediator of senescence (Alcorta et al., 1996). A fragment of GLI2 was found to directly bind and inhibit the *p16* promoter and loss of GLI2 was associated with the induction of senescence

(Bishop et al., 2010). We therefore tested whether inhibition of Hh affected proliferation. Hh inhibition reduced proliferation in young cells, with increased cilium length accompanying cell cycle arrest. The increased cilium frequency and length may impede mitogenic signalling by diluting out components of the receptor pathway, as observed by Mahjoub and Stearns (Mahjoub and Stearns, 2012).

4.6 CP110 as a candidate for controlling the cilium length setting in senescent cells

We next wanted to determine what controls cilium length in senescent BJ cells. CP110 (centriolar coiled-coil protein of 110 kDa) is a negative regulator of ciliogenesis. Its depletion promotes cilium formation in growing cells and in quiescent cells; its overexpression inhibits their ability to form cilia (Spektor et al., 2007; Tsang et al., 2008). CP110 localises to the distal ends of both the mother and daughter centrioles (Chen et al., 2002b). During ciliogenesis, CP110 is specifically eliminated from the mother centriole. The localisation of CEP97 has been shown to be strongly dependent on CP110, suggesting that CP110 recruits CEP97 to the centrosome. Indeed, depletion of CEP97 results in the loss of CP110 at the centrosome, promoting cilium formation (Spektor et al., 2007). The kinesin KIF24 was identified as a CP110-CEP97 interaction partner. KIF24 depolymerises centriolar microtubules to prevent premature cilia assembly and the loss of KIF24 promotes ciliogenesis (Kobayashi et al., 2011). In addition, CP110 has also been shown to interact with Cep290 to prevent NPHP6 and Rab8a from initiating ciliogenesis (Tsang et al., 2008). Tau tubulin kinase 2 (TTBK2) is essential for mouse SHh signalling and ciliogenesis. TTBK2 removes CP110 and promotes the recruitment of IFT proteins, which build the ciliary axoneme (Goetz et al., 2012). The distal appendages of centrioles have been proposed to anchor cilia to the plasma membrane. Loss of the distal appendage protein CEP83 blocks centriole to membrane docking and undocked centrioles fail to recruit TTBK2 or release CP110 (Tanos et al., 2013). The microRNA miR-129-3p has been shown to control cilia assembly by down-regulating CP110 and repressing branched F-actin formation. Blocking miR-129-3p inhibited serum starvation-induced ciliogenesis, while its overexpression induced ciliation in proliferating cells and promoted cilia elongation

(Cao et al., 2012). These data led us to consider CP110 as a possible candidate for controlling the cilium length setting.

Our results show that senescent cells express lower levels of *CP110* mRNA and protein. Overexpression of CP110 caused a decline in the fraction of ciliated cells, but did not affect either their mean cilium length or the number of proliferating cells in either senescent or young populations. These results indicate that senescence, once established, cannot be reversed through cilium manipulation. When cells enter senescence, they undergo a series of irreversible morphologic and metabolic changes. They arrest their growth, become resistant to cell death signals, secrete senescence-associated proteins and acquire changes in gene expression (Campisi and d'Adda di Fagagna, 2007). It is possible that restoring CP110 levels does not drive the cells out of senescence because overexpression of CP110 does not also reverse the morphologic and metabolic changes associated with senescence. However, it has been shown that other experimental manipulations can cause some replicatively senescent human cells to proliferate, for example, through the activation of tumour suppressor genes (Gire and Wynford-Thomas, 1998; Beauséjour et al., 2003). In these cases, eliminating p53 function caused senescent cells to resume proliferation, despite having short telomeres, until eventual severe telomere dysfunction drove them into crisis, a state of acute genomic instability. Similarly, inactivation of p21, a p53 target for transactivation and inhibitor of cell cycle progression, allowed the cells to bypass telomere-induced senescence and enter crisis (Brown et al., 1997). Moreover, TERT expression in normal human cells prevents telomere shortening and senescence caused by the end-replication problem (Bodnar et al., 1998). Our results suggest that whilst the senescence growth arrest can be reversed by some experimental manipulations, it cannot be cannot be reversed by experimental manipulation of the primary cilium, at least via CP110.

We then tested the impact of increased ciliation on proliferation. Knockdown of CP110 caused an increased level of ciliation in proliferating, but not in senescent populations. We also observed small but statistically significant increases in the mean cilium length after *CP110* depletion. Strikingly, *CP110* knockdown caused a decline in the number of proliferating cells, with non-proliferating transfected $Ki67^-$ cells also staining positive for β-galactosidase. These data indicate that increased

ciliation, resulting from the loss of *CP110*, is associated with cellular senescence in human fibroblasts. A schematic representation of the regulation of CP110 in senescent cells is presented in Figure 4.2. CP110 expression decreases with age (as does its interaction partners Cep97, Kif24 and Cep290), resulting in failure to disassemble the primary cilium.

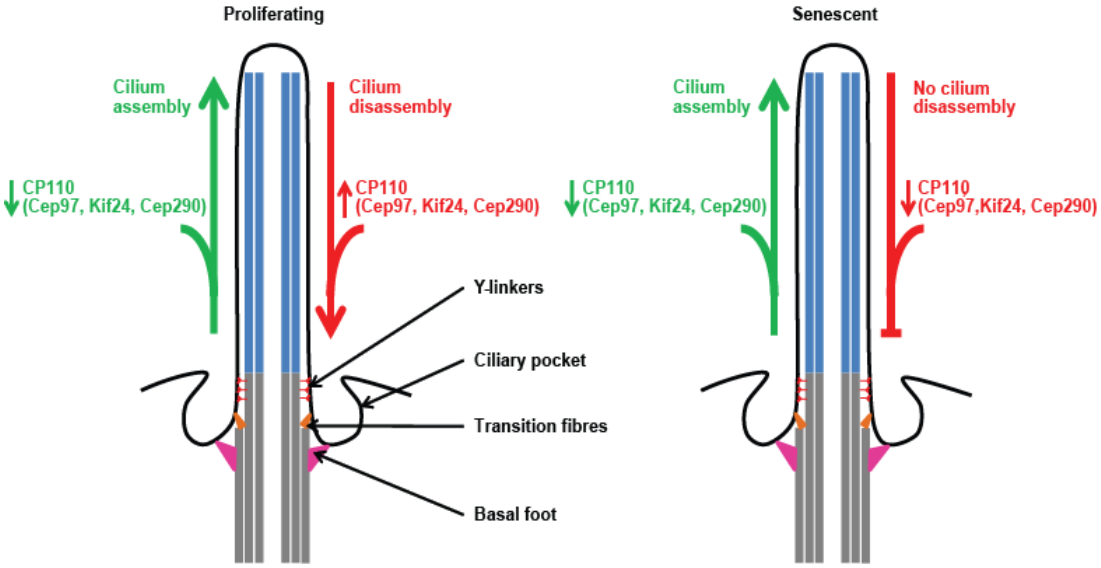

Figure 4.2 Regulation of CP110 in senescent cells.
In proliferating cells, CP110 and its interaction partners Cep97, Kif24 and Cep290 are displaced from the basal body allowing the formation and elongation of the primary cilium. Conversely, CP110 is expressed at the basal body during cilium disassembly. In senescent cells, CP110 expression decreases with age, resulting in failure to disassemble the primary cilium. Diagram not to scale.

We found that expression of Hh signalling pathway components is reduced in senescent populations. Hh signalling promotes mitogenesis by suppression of *p16* through binding of GLI2 to its promoter (Bishop et al., 2010). While our data indicating the involvement of Hh signalling in continued proliferation are consistent with this model, we did not observe the loss of primary cilia in senescent cells that has been described in human mammary epithelial cell (HMEC) cultures (Bishop et al., 2010). Senescing HMECs and fibroblasts have distinct programmes of gene expression, so the impact of ciliary signalling may differ depending on the cell type (Zhang et al., 2004). Although some primary cilia, such as those carried by HMECs and embryonic stem cells, drive pro-mitogenic Hh signalling (Kiprilov et al., 2008), current evidence suggests that ciliation may be associated more generally with reduced proliferation (Goto et al., 2013). Tumour cells frequently lack cilia (Seeley

et al., 2009). The mitotic kinase Aurora A causes disassembly of the primary cilium (Pugacheva et al., 2007) and cilium-dependent cell cycle exit through Aurora A inhibition has been described as a means of inducing quiescence in human cells (Inoko et al., 2012).

While the acquisition of a primary cilium may be a transient event in a proliferating cell population (Seeley and Nachury, 2010), extending cilium length may be a novel mechanism to establish senescence. If a threshold length of cilium is still responsive to a mitogen such as Hh, a lengthening of the structure may contribute to an effective dilution of this signalling capacity (Mahjoub and Stearns, 2012) and a blunting of the signal. Such a model implicates the regulation of CP110 in the control of cellular senescence. As shown in Figure 4.3, we propose that CP110 expression decreases as cells age resulting in longer and/or multi-ciliated cells. This in turn results in a dilution of Hh signalling which ultimately contributes to the induction of senescence.

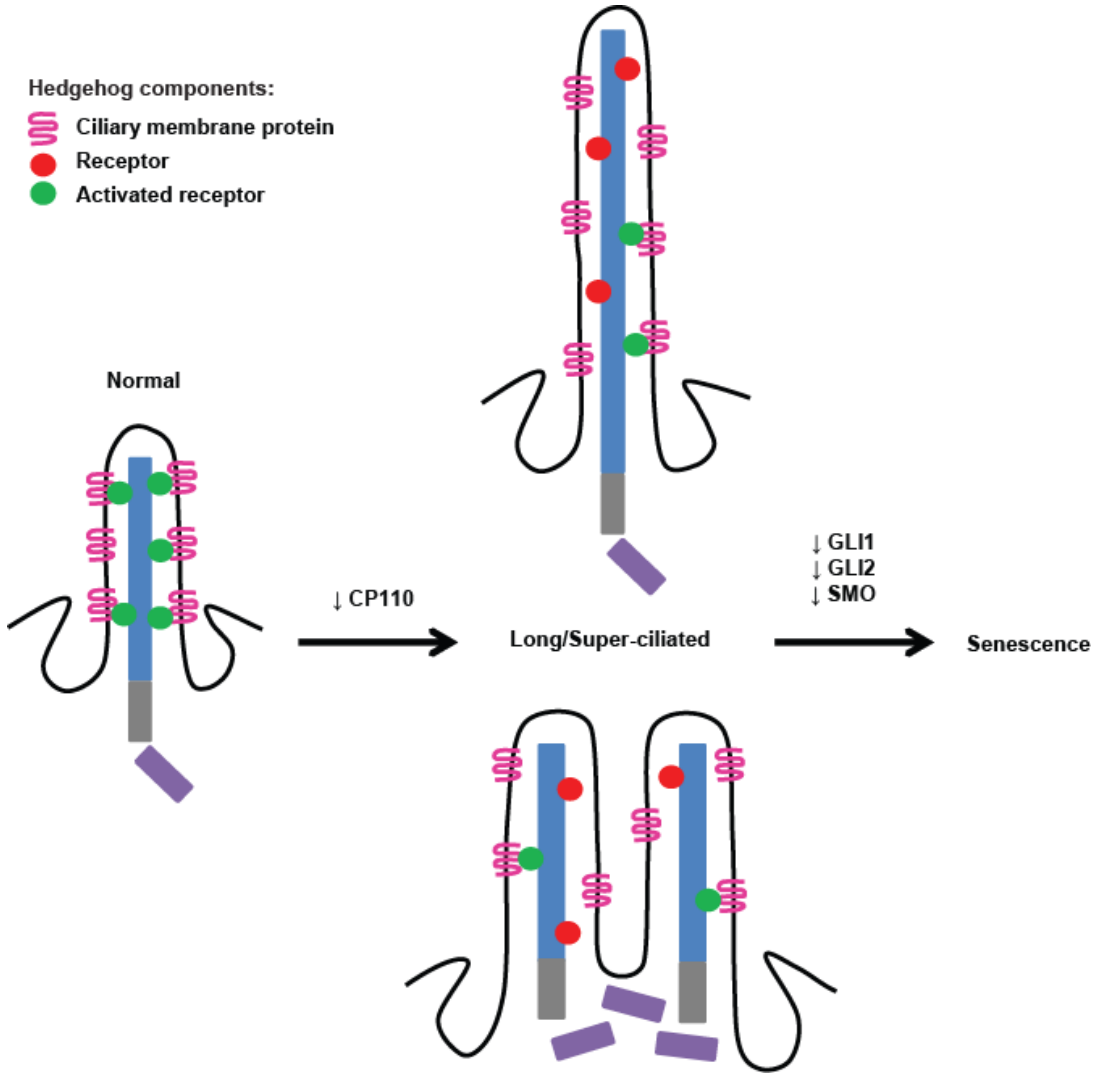

Figure 4.3 Increased ciliation, resulting from the loss of CP110, is associated with the loss of Hh and induction of senescence.
Our model proposes that ciliary dysfunction is caused by a decrease in CP110 expression with age. This causes a dilution of activated Hh receptors in long/super-ciliated cells, culminating in the induction of cellular senescence. Diagram not to scale.

4.7 The primary cilium, Hedgehog signalling, aging and cancer

Hh signalling has been identified as one of the fundamental signal transduction pathways in embryonic development and morphogenesis. However, in adult life, Hh down-regulation is associated with senescence (Bishop et al., 2010) and age-related diseases such as type 2 diabetes, neurodegeneration, atherosclerosis and osteoporosis (Thomas et al., 2000; Hurtado-Lorenzo et al., 2004; Beckers et al., 2007). Although aging is a multifactorial process, a reduced propensity to regenerate tissues contributes to the process and this appears to be a consequence of reduced

integrity of stem cell compartments in the body (Rando and Chang, 2012). With aging comes tissue, organ, bone, brain and skin deterioration. Maintenance of these tissues requires the continuous replenishment of stem cells (Weissman, 2000). Hh signalling has been shown to be essential for stem cell maintenance and senescence has been associated with reduced stem cell function (Liu et al., 2006; Han et al., 2008; Gao et al., 2009; Michel et al., 2012).

Hh signalling is required for the maintenance of stem cells in a range of tissues, including cartilage and bone. During adult life a decrease in bone volume is accompanied by an increase in adipose tissue due to an imbalance in differentiation of osteogenic and adipogenic mesenchymal stem cells (MSCs) (Moerman et al., 2004). Hh signalling is down-regulated during adipocyte differentiation. Hh interferes with the transcription of CCAAT/enhancer binding protein α (C/EBPα) and the peroxisome proliferator-activated receptor gamma (PPARγ), which enhances adipogenesis at the expense of osteogenesis (Fontaine et al., 2008). Hh signalling is also required for the maintenance of neuronal stem cells (Han et al., 2008) and persistent Hh signalling is necessary for neuronal stem cells to acquire their identity during development (Briscoe et al., 1999). LRP2 is a SHh receptor (McCarthy et al., 2002) that has been associated with Alzheimer's disease through its protective role in the choroid plexus (Bolós et al., 2010). Hh has been proposed as a counteractor of the Alzheimer's disease process through increased stem cell activity (Paganelli et al., 2001) and increased stem cell activity has been shown to improve memory loss (Wang et al., 2006; Yamasaki et al., 2007; Blurton-Jones et al., 2009). Hh signalling also plays an important role in protecting neurons from external insults. Oxidative stress is one pathological mechanism in neurodegenerative diseases (Barnham et al., 2004). Hh protects cortical neurons against oxidative stress (Dai et al., 2011). Hh signalling is also required for the maintenance of skin stem cells. A decreased capacity for skin repair is associated with reduced activation of Hh signalling in bulge cells, a population of stem cells in the skin (Rittié et al., 2009). Lentiviral-mediated overexpression of GLI1 increases human hair follicle stem cell markers in human keratinocytes (Rittié et al., 2009). These data show that Hh is involved in maintenance of various tissues and also in preventing their senescence. Hh may act

as an anti-aging signal and its reduced expression with advanced aging may be a trigger for aging.

The influence of Hh signalling on stem cells suggests why activation of the pathway has been found in many human tumours. It is thought that tumours contain a population of cells that expresses normal stem cells markers and so, have the capacity to self-renew (Tan et al., 2006). Abnormal activation of Hh is associated with Basal cell carcinoma (BCC) and medulloblastomas in the stem cell and precursor cell population, respectively (Hutchin et al., 2005; Schüller et al., 2008). BCC cells are often ciliated, while cerebellar granule precursors (GMPs) require the primary cilium and Hh signalling for proliferation and can give rise to medulloblastomas. Ciliary ablation in mice inhibits both the formation of BCC-like tumours and medulloblastomas induced by activated SMO. However, ciliary ablation accelerates tumour growth in BCC-like tumours and medulloblastomas induced by activated GLI2. These results suggest the primary cilium can either activate or repress tumourigenesis, depending on the nature of the oncogenic-initiating event, playing a dual role in activating and repressing Hh signalling (Han et al., 2009; Wong et al., 2009).

In contrast to these ligand-dependent tumours, other tumours associated with Hh signal activation depend on ligand production, either by the tumour or the surrounding stromal cells (Teglund and Toftgård, 2010). Increased Hh ligand expression leads to increased Hh signalling in the activated target cell. This can occur through two mechanisms: autocrine and paracrine (Scales and de Sauvage, 2009). In the autocrine mechanism, malignant cells both secrete and respond to the Hh ligand. Recent studies have demonstrated that Hh signalling supports and enhances cancer cell growth (Berman et al., 2003a). Hh signalling has also been shown to promote Warburg-like glycolytic metabolism, found in many tumours, suggesting a possible role for Hh within the tumour cells (Teperino et al., 2012). In the paracrine mechanism, the secreting cell and recipient target cell are different. Hh promotes the tumour microenvironment by signalling to the stroma, which then signals back to the tumour (Yauch et al., 2008). This mechanism is favoured over the autocrine mechanism, based on the evidence that tumour cells do not respond to Hh ligand themselves (Yauch et al., 2008; Tian et al., 2009). One proposed explanation

for this is that these tumour cells frequently lack the primary cilium needed for Hh signalling (Seeley et al., 2009). The exact function of Hh in these tumours and whether it signifies dysregulation of normal stem cell maintenance remains to be determined.

CHAPTER 5 – CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Overall conclusions

In this project we show that increased cilium frequency and length accompanies senescence in primary human fibroblasts and that ciliation induced by depletion of the centriolar protein CP110 causes senescence. Our data demonstrate that primary cilium length regulation through CP110 is a potential novel determinant of cellular proliferative capacity.

5.2 Future work

We propose that CP110 expression decreases as cells age resulting in a lengthening of the primary cilium. This in turn results in a dilution of Hh signalling which ultimately contributes to the induction of senescence. To increase the specificity of the link between the primary cilium and senescence it would be interesting to manipulate the primary cilium in another way, for example by overexpressing and depleting Cep97, which interacts with CP110. If our model is correct, we would expect that Cep97 manipulation would have a similar effect as CP110 manipulation. Another interesting experiment would be to analyse cilia length and CP110 expression over time. This would provide evidence for any correlation between CP110 expression levels and cilium length over time in culture. If our model is correct, the two should be negatively correlated.

To test our model in vivo, we could generate knockout mice for genes involved in cilium assembly and function (such as *Ccp110* or *Ift88*), harvest mouse embryonic fibroblasts (MEFs) and compare cilium length and senescence kinetics to wild-type mice over time in culture. If our model is correct, Ccp110-depleted MEFs would senesce more rapidly than wild-type MEFs, whilst MEFs lacking Ift88, and hence functional cilia, would continue to proliferate for longer.

Our results show that loss of Hh leads to reduced proliferation and increased cilium frequency and length. It would be interesting to activate Hh signalling in senescent cells and examine whether this has the opposite effect on cilium length and senescence kinetics. This experiment could be achieved by purifying Hh protein and adding it to senescent cells (Martinez-Chinchilla and Riobo, 2008). The Hh pathway

could also be activated by the overexpression of a Hh pathway component. For example, over-expression of LRP2 has been shown to increase Hh signalling capacity (Christ et al., 2012). Activation of the Hh pathway could then be measured by immunoblot analysis, quantitative real-time PCR and/or a GLI reporter assay.

5.3 Future perspectives

Our data present an interesting view on the potential role of the primary cilium and Hedgehog signalling in cellular senescence. This leads us to some exciting perspectives for future work:

1. Does our model apply in vivo and does this contribute to the pathogenesis of age-related disease? The presence and SASP of senescent chondrocytes has been shown to contribute to the development of osteoarthritis (OA) (Roberts et al., 2006; Shane Anderson and Loeser, 2010). Interestingly, treatment of primary chondrocytes with IL-1, a component of the SASP, results in an increase in primary cilium length (Wann and Knight, 2012). These findings provide a correlative link between the primary cilium, the SASP and OA. It is therefore conceivable that the primary cilium contributes to the pathogenesis of age-related diseases.
2. Does Hh signalling have a general role in rejuvenating cells and is this what triggers the induction of senescence in our model? A consequence of reduced stem cell function is a reduced ability to regenerate tissues (Rando and Chang, 2012). Hh signalling has been shown to be essential for stem cell maintenance and senescence has been associated with reduced stem cell function (Liu et al., 2006; Han et al., 2008; Gao et al., 2009; Michel et al., 2012). It is possible that lengthening of the primary cilium with age and subsequent loss of Hh signalling contributes to senescence through reduced stem cell function and subsequent inability to regenerate tissues.
3. Is it possible to stabilise the primary cilium and activate the Hh pathway without activating its undesirable effects? Abnormal activation of Hh is associated with the formation of tumours (Hutchin et al., 2005; Schüller et al., 2008). The primary cilium can either activate or repress tumourigenesis, depending on the nature of the oncogenic-initiating event, playing a dual role in activating and

repressing Hh signalling (Han et al., 2009; Wong et al., 2009). By stabilising the primary cilium, it may also be possible to stabilise Hh signalling and restore the ability to regenerate tissues.

4. Can we exploit the Hh pathway or the primary cilium to prevent/treat age-related diseases (Lauth and Toftgård, 2007)? This could be achieved by using compounds which selectively treat ciliated cells, for example, to prevent lengthening of the primary cilium and thus loss of Hh signalling capacity.

The hope is that these experiments may allow more insight into the link between the primary cilium, Hedgehog signalling, senescence and degenerative diseases and thus help in the identification of new therapeutic targets.

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APPENDIX I

Table A1 presents relative quantification (RQ) values measuring the change in expression of the indicated genes in senescent BJ fibroblasts relative to the same genes in proliferating BJ fibroblasts, and normalised to the housekeeping genes. RQ analysis was performed using quantitative real-time PCR. The data presented are mean plus standard error of the mean (SEM) of three biological replicates.

Genes highlighted in orange: These gene's average threshold cycle is relatively high (> 30), meaning that its relative expression level is low in both proliferating and senescent samples.

Genes highlighted in red: This gene's average threshold cycle is either not determined or greater than the defined cut-off value (35), in both proliferating and senescent samples, meaning that its expression was undetected, making this fold-change result un-interpretable.

Table A1 RQ of ciliary genes analysed in senescent BJ cells

Symbol	Description	RQ	SEM	p-value
ADCY3	Adenylate cyclase 3	-1.48	-2.98	ns
ADCY7	Adenylate cyclase 7	-1.48	-1.5	ns
AHI1	Abelson helper integration site 1	1.35	1.33	ns
AKT1	V-akt murine thymoma viral oncogene homolog 1	1.36	1.35	ns
ALMS1	Alstrom syndrome 1	-1.5	1.34	ns
ARL13B	ADP-ribosylation factor-like 13B	1.35	1.34	ns
ARL6	ADP-ribosylation factor-like 6	2.71	1.34	ns
AVPR2	Arginine vasopressin receptor 2	-1.53	-1.55	ns
AXIN2	Axin 2	1.35	1.33	ns
BBS1	Bardet-Biedl syndrome 1	1.36	1.35	ns
BBS2	Bardet-Biedl syndrome 2	1.36	1.35	**
BBS4	Bardet-Biedl syndrome 4	1.35	1.33	ns
BBS7	Bardet-Biedl syndrome 7	1.33	1.34	ns
BTRC	Beta-transducin repeat containing	2.66	1.33	ns
CC2D2A	Coiled-coil and C2 domain containing 2A	1.35	-1.53	ns
CCND1	Cyclin D1	2.67	1.33	*
CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)	1.36	-1.49	ns
CDK5RAP2	CDK5 regulatory subunit associated protein 2	-1.48	1.33	ns
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.35	1.34	*
CEP290	Centrosomal protein 290kDa	1.34	1.35	**

DVL1	Dishevelled, dsh homolog 1 (Drosophila)	1.35	-1.47	ns
DYNC2LI1	Dynein, cytoplasmic 2, light intermediate chain 1	1.34	1.33	**
FAT4	FAT tumour suppressor homolog 4 (Drosophila)	-1.48	1.34	ns
FJX1	Four jointed box 1 (Drosophila)	1.36	-1.51	ns
FOS	FBJ murine osteosarcoma viral oncogene homolog	1.35	1.33	ns
FUZ	Fuzzy homolog (Drosophila)	1.35	1.34	ns
FZD1	Frizzled family receptor 1	1.37	-1.5	ns
GLI1	GLI family zinc finger 1	-1.47	-2.94	ns
GLI2	GLI family zinc finger 2	-2.93	-5.94	**
GLI3	GLI family zinc finger 3	-1.48	1.33	ns
GLIS2	GLIS family zinc finger 2	1.35	-1.49	ns
GSK3B	Glycogen synthase kinase 3 beta	2.69	1.34	ns
HNF1B	HNF1 homeobox B	1.21	1.28	ns
HTR6	5-hydroxytryptamine (serotonin) receptor 6	1.24	1.31	ns
IFT172	Intraflagellar transport 172 homolog (Chlamydomonas)	1.35	1.35	*
IFT20	Intraflagellar transport 20 homolog (Chlamydomonas)	1.36	1.34	ns
IFT74	Intraflagellar transport 74 homolog (Chlamydomonas)	1.35	1.33	**
IFT80	Intraflagellar transport 80 homolog (Chlamydomonas)	1.35	1.34	ns
IFT88	Intraflagellar transport 88 homolog (Chlamydomonas)	2.68	1.34	**
IGF1	Insulin-like growth factor 1 (somatomedin C)	-1.48	-3.02	ns
IHH	Indian hedgehog	1.38	1.24	ns
INS	Insulin	1.5	-	ns
INTU	Inturned planar cell polarity effector homolog (Drosophila)	2.68	1.35	ns
INVS	Inversin	1.35	1.34	ns
IQCB1	IQ motif containing B1	1.34	-1.5	ns
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1.35	1.34	ns
KIF3A	Kinesin family member 3A	1.35	1.34	**
KIF3B	Kinesin family member 3B	1.35	1.33	**
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.37	-1.49	ns
LRP2	Low density lipoprotein receptor-related protein 2	10.61	10.73	*
MAP2K1	Mitogen-activated protein kinase kinase 1	1.36	1.33	*
MAPK1	Mitogen-activated protein kinase 1	-1.47	-1.49	ns
MKKS	McKusick-Kaufman syndrome	1.34	2.65	*
MKS1	Meckel syndrome, type 1	1.29	1.47	ns
MOS	V-mos Moloney murine sarcoma viral oncogene homolog	1.34	1.32	ns
MTOR	Mechanistic target of rapamycin (serine/threonine kinase)	-1.5	1.34	ns
NEK8	NIMA (never in mitosis gene a)- related kinase 8	1.35	-1.48	ns
NPHP1	Nephronophthisis 1 (juvenile)	2.72	1.35	**
NPHP3	Nephronophthisis 3 (adolescent)	1.35	-1.48	ns

OFD1	Oral-facial-digital syndrome 1	1.35	1.34	ns
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	1.34	2.68	ns
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	2.73	1.35	ns
PKD1	Polycystic kidney disease 1 (autosomal dominant)	1.34	-1.49	ns
PKD2	Polycystic kidney disease 2 (autosomal dominant)	1.35	1.33	ns
PKHD1	Polycystic kidney and hepatic disease 1 (autosomal recessive)	-	-	ns
PRKCA	Protein kinase C, alpha	1.35	1.34	ns
PTCH1	Patched 1	1.35	1.35	ns
PTPN5	Protein tyrosine phosphatase, non-receptor type 5 (striatum-enriched)	1.35	2.71	ns
RAB23	RAB23, member RAS oncogene family	1.36	-1.48	ns
RHOA	Ras homolog gene family, member A	1.36	-1.5	ns
ROCK2	Rho-associated, coiled-coil containing protein kinase 2	-1.5	-1.51	ns
RPGRIP1L	RPGRIP1-like	1.34	1.33	ns
SHH	Sonic hedgehog	1.45	1.38	ns
SMO	Smoothened, frizzled family receptor	2.7	1.34	**
SSTR3	Somatostatin receptor 3	1.26	1.67	ns
SUFU	Suppressor of fused homolog (Drosophila)	1.36	1.34	ns
TMEM67	Transmembrane protein 67	1.36	1.34	ns
TP53	Tumour protein p53	2.67	1.34	ns
TSC1	Tuberous sclerosis 1	1.36	-1.48	ns
TSC2	Tuberous sclerosis 2	1.35	-1.48	ns
TTC8	Tetratricopeptide repeat domain 8	1.34	1.34	**
VANGL2	Vang-like 2 (van gogh, Drosophila)	5.41	2.69	**
WNT9B	Wingless-type MMTV integration site family, member 9B	1.43	1.32	ns
WWTR1	WW domain containing transcription regulator 1	1.35	1.35	ns

Table A2 Ct values of the housekeeping genes

Symbol	**Description**	**C_T values**					
B2M	Beta-2-microglobulin	22.94	20.92	22.92	19.92	19.92	21.92
HPRT1	Hypoxanthine phosphoribosyltransferase 1	25.91	25.91	26.91	26.9	26.9	26.91
RPL13A	Ribosomal protein L13a	21.91	21.9	22.91	22.93	21.9	23.91
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	19.9	19.9	19.9	19.89	19.89	20.9
ACTB	Actin, beta	18.92	18.9	19.89	18.9	18.93	20.92

Figure A.1 The expression of cilium-related genes in senescent human fibroblasts.