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IDENTIFICATION OF NOVEL CHROMATIN AND NUCLEAR FACTORS REGULATING ONCOGENE-INDUCED SENESCENCE

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Publications

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Statement of originality

All experiments included in this thesis were performed by myself unless otherwise stated.

Abstract

Cellular senescence is a general stress response that encompasses a stable proliferation arrest achieved partly due to the activation of tumour suppressor pathways. In addition to becoming stably arrested, senescent cells undergo other important changes that include profound chromatin reorganization and the production of a complex mix of proinflammatory factors. The relevance of senescence is highlighted by the multitude of processes it regulates from embryogenesis to cancer. For this reason, the discovery of novel senescence regulators could not only improve our knowledge about senescence but also provide insights useful to understand or treat many pathologies.

To identify novel genes regulating oncogene-induced senescence (OIS), spatial proteomics was performed with IMR90 ER:RAS fibroblasts previously fractionated into nuclear soluble, insoluble (chromatin) and cytoplasm. The chromatin-associated proteome presented the biggest differences between growing and senescent cells. To address the functional relevance of the observed changes we performed siRNA screens to target chromatin remodelers and identified several genes potentially regulating OIS. Preliminary validation steps confirmed a role for 6 of those factors TRIM28, ENY2, OGT, TAF10, TAF12 and GTF3C4 in regulating senescence.

Thus, using unbiased large-scale approaches this work identified novel senescence regulators and provided an initial characterization for their role in OIS. Further experiments will be essential to investigate how these genes regulate senescence and whether or not they could play a role in senescence-associated diseases such as cancer or fibrosis.

Resumo

A senescência celular é uma resposta geral ao stress que consiste num bloqueio da proliferação celular em parte, devido à activação de vias de genes supressores tumorais. Para além do bloqueio da proliferação, as células senescentes sofrem alterações importantes que incluem uma reorganização profunda da cromatina e a produção de uma mistura complexa de factores pró-inflamatórios.

A senescência torna-se relevante quando se avalia a variedade de processos nos quais está envolvida, desde a embriogénese ao cancro. Por esta razão, a descoberta de novos genes reguladores da senescência poderá expandir o nosso conhecimento sobre o processo em si assim como o dos variados contextos biológicos e patologias aos quais está associada.

Com o objectivo de identificar novos genes que regulam a senescência induzida por oncogenes (OIS), fibroblastos IMR90 ER:RAS foram fraccionados e as amostras contendo as proteínas nucleares solúveis, insolúveis (cromatina) e citoplasmáticas foram submetidas a uma análise proteómica. A análise proteómica revelou que as células senescentes expressam diferentes proteínas relativamente às células em crescimento, principalmente ao nível das proteínas associadas à cromatina. De forma a avaliar a relevância funcional dos resultados obtidos, usaram-se *screens* de siRNAs para silenciar a expressão de vários reguladores da cromatina. Vários genes com potencial na regulação do bloqueio da proliferação e da expressão do p16 foram identificados e após vários passos de validação, confirmou-se o papel dos genes TRIM28, ENY2, OGT, TAF10, TAF12 e GTF3C4 na senescência.

Assim, este trabalho identificou, usando abordagens de larga escala, novos genes envolvidos na senescência celular e apresentou uma caracterização inicial do seu papel neste processo. Experiências futuras são essenciais para desvendar o modo pelo qual estes genes regulam a senescência celular e se podem contribuir para as doenças que lhes estão associadas como o cancro ou a fibrose.

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Abbreviations

4-OHT	4-hydroxy tamoxifen
A	Adenine
Ac	Acetylated
ACTG	Actin, Gamma 1
ACTL	Actin-Like
ACTR3	ARP3 Actin-Related Protein 3 Homolog (Yeast)
AKT	v-akt murine thymoma viral oncogene homologue
ALG	Asparagine-Linked Glycosylation
AOF2	KDM1A
ARF	Alternative reading frame
ARGL1	Arginine And Glutamate-Rich Protein 1
ARID	AT-rich interactive domain
ARP	Actin related protein
ARRB1	Arrestin, Beta 1
ASF	Anti-silencing function
ASH2	Absent, Small, Or Homeotic
ASH2L	Absent, Small, Or Homeotic-Like
ATCC	American Type Culture Collection
ATM	Ataxia telangiectasia mutated
ATP	Adenosine tri-phosphate
ATR	Ataxia telangiectasia and Rad3 related
ATXN	Ataxin
AUF1	AU-Rich Element RNA Binding Protein 1
bp	**Base pair**
BPTF	Bromodomain PHD finger transcription factor
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA	Breast cancer gene
BRD	Bromodomain containing
BrdU	5-bromo-2-deoxyuridine
BRG1	Brahma related gene 1
BSA	Bovine serum albumin

C	**Cytosine**
CAPG	Capping Protein (Actin Filament), Gelsolin-Like
Ca^{2+}	Calcium ion
C/EBP	CCAAT/enhancer binding protein
C. elegans	*Caenorhabditis elegans*
c-FOS	Cellular-FBJ osteosarcoma oncogene
CBX7	Chromobox protein homolog 7
CCL	Chemokine (C-C motif) ligand
CDC	Cell division cycle
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CDKN1	Cyclin dependent kinase inhibitors, family 1
CDKN2	Cyclin dependent kinase inhibitors, family 2
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CHK	Checkpoint kinase
CLDN11	Claudin 11
c-MYC	v-myc avian myelocytomatosis viral oncogene homolog
CpG	—C—phosphate—G—
CSC	Clinical Sciences Centre
CT	Cytoplasm
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
C3D	Cross-Conditions Cluster Detection
DAPI	**4,6-diamidino-2-phenylindole**
DDR	DNA damage response
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide DNA Deoxyribonucleic acid
DNAase	Deoxyribonuclease
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
ds	Double strand
DTT	Dithiothreitol

DUB	Deubiquitinase
E.coli	***Escherichia coli***
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EHMT	Euchromatin histone methyltransferase
ENY2	Enhancer Of Yellow 2 Homolog (Drosophila)
EP300	E1A binding protein p300
EP400	E1A binding protein p400
Esc	Embryonic stem cells
ER	Oestrogen receptor
ERK	Extracellular signal-regulated kinases
ETS	E26 oncogene homolog
EZH2	Enhancer of zeste homolog 2
FACT	**Facilitates chromatin transcription (complex)**
FALZ	BPTF
FBS	Foetal bovine serum
FDR	False discovery rate
FOX	Forkhead box proteins
FUBP	Far Upstream element-Binding Proteins
g	**Gram**
G	Guanine
Gadd45a	Growth Arrest and DNA-damage-inducible protein
GCN5	Histone acetyltransferase GCN5
GO	Gene ontology GR Glucocorticoid receptor
GTF3C4	General Transcription Factor IIIC, Polypeptide 4, 90kDa
GTP	guanosine triphosphate
GTPase	GTP hydrolase
Gy	Gray
H	**Histone**
HAT	Histone acetyl-transferase
HCA	High content analysis
HCF1	Host cell factor C1
HDAC	Histone deacetylase

HDM2	Human double minute 2 homolog
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGPS	Hutchinson–Gilford progeria syndrome
HIRA	Histone repressor A
HO-GSVD	Higher-Order Generalized Singular Value Decomposition
HMG	High mobility group
HMT	Histone methyltransferase
HP	Heterochromatic protein
HP1BP	Heterochromatin Protein 1, Binding Protein
hr	Hour
HRAS	Harvey rat sarcoma gene
HSC	Hepatic stellate cells
hTERT	Human telomerase reverse transcriptase
HTM	High throughput microscopy
HUCA	HIRA/UBN1/CABIN1/ASF1a complex
HuR	ELAV-like protein 1
ID	**Inhibitor of DNA binding**
IF	Immunofluorescence
IL	Interleukin
INK4	Inhibitor of CDK4
INT	Integrator
iPS	Induced pluripotent stem cell
JARID	**Jumonji, AT Rich Interactive Domain**
JMJD	Jumonji domain containing
K	**Lysine**
KAKA	KRAB- and KAP1-associated
KAP1	KRAB-associated protein 1 (same as TRIM28)
Kb	Kilo base
kDa	Kilo dalton
KDM	Lysine demethylase
KLF4	Krüppel-like factor 4
KMT	Lysine methyltransferase
KRAB-ZFPs	Krüppel associated box- Zinc finger proteins

L	**Litre**
lncRNA	Long non-coding RNA
LMN	Lamin
LRIQ1	Leucine-Rich Repeats And IQ Motif Containing
LTR	Long terminal repeat
Lys	Lysine
m	**Milli**
M	Molarity
MAD	Maximum absolute deviation
MAPK	Mitogen-activated protein kinases
MAP1A	Microtubule-Associated Protein 1A
MA2A1	Mannosidase, Alpha, Class 2A, Member 1
Mb	Mega base pair
MDM2	Mouse double minute 2 homolog
me	Methylation
MEF	Mouse embryonic fibroblast
MEK1	Mitogen-Activated Protein Kinase Kinase 1
MGAT5	Mannosyl (Alpha-1,6-)-Glycoprotein Beta-1,6-N-Acetyl-Glucosaminyltransferase
min	Minute
miR	MicroRNA
MLL	*Myeloid-lymphoid leukemia protein*
MMP	Matrix metallopeptidase
MPU1	Mannose-P-Dolichol Utilization Defect 1
mRNA	Messenger RNA
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
MW	Molecular weight
NBEA	**Neurobeachin, Lysosomal-Trafficking Regulator 2**
NCOR1	Nuclear Receptor Corepressor 1
NF-κβ	Nuclear factor kappa B
NI	Nuclear insoluble fraction
NS	Nuclear soluble fraction

NuRD	Nucleosome remodeling and deacetylation
OCT4	**Octamer-binding transcription factor 4**
OGA	O-GlcNAcase
OGT	O-Linked N-Acetylglucosamine (GlcNAc) Transferase
O-GlcNAc	O-Linked N-Acetylglucosamine
OIS	Oncogene induce-senescence
OSKM	Oct4, Sox2, Klf4 and c-Myc
OSTC	Oligosaccharyltransferase Complex Subunit (Non-Catalytic)
PABP2	**Poly(A) Binding Protein, Nuclear 1**
PAI	Plasminogen activator inhibitor
PAXIP1	PAX Interacting (With Transcription-Activation Domain) Protein 1
PAF1C	RNA polymerase-associated factor 1 complex
PBRM	Polybromo
PBS	Phosphate buffered saline
PB1	Polybromo
PCAF	P300/CBP-associated factor
PcG	Polycomb group
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PEX1	Peroxisomal Biogenesis Factor 1
PFA	Paraformaldehyde
PML	Promyelocytic Leukemia
Pol I, II or III	RNA polymerase I, II or III
PRC	Polycomb repressive complex
PRDM	PR Domain Containing
PSMD	Proteasome (prosome, macropain) 26S subunit, non-ATPase
PTEN	Phosphatase and tensin homolog
R	**Pearson correlation**
RAD23B	RAD23 Homolog B
RB	Retinoblastoma
RBP	RNA binding protein
RLS	Replicative life span
RNA	Ribonucleic acid

RNAi	RNA interference
RNase	Ribonuclease
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RPL	Ribosomal protein
rpm	Rotations per minute
RPN	Ribophorin
RPN	Ribonucleoprotein particle
RPS14	Ribosomal protein S14
rRNA	Ribosomal RNA
RRBP1	Ribosome Binding Protein 1
RRP	Ribosomal RNA processing protein
RS	Ribosomal protein
RTF1	Paf1/RNA polymerase II complex component
RT-qPCR	Quantitative reverse transcription PCR
s	**Second**
SA-β-gal	Senescence-associated β-galactosidase
SAGA	Spt-Ada-Gcn5 Acetyltransferase complex (yeast)
SAHF	Senescence-associated heterochromatic foci
SALRNA1	Senescence Associated Long Non-Coding RNA 1
SASP	Senescence associated secretory phenotype
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Ser	Serine
SET1	SET Domain Containing 1A
shRNA	Short hairpin RNA
SILAC	Stable isotope labelling with amino acids in cell culture
siRNA	Small interfering RNA
SMAD	Mothers against decapentaplegic homolog
SMARC	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin
SMCA	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin
SNF	Sucrose non-fermenting

snRNA	Small nuclear RNA
SOX2	SRY (sex determining region Y)-box 2
SQSTM1	Sequestosome 1
SSRP1	Structure Specific Recognition Protein 1
STAGA	SPT3-TAF(II)31-GCN5L acetylase (human)
STT3	Subunit Of The Oligosaccharyltransferase Complex
SUPT(x)H	Suppressor Of Ty (x) Homolog
SUZ12	Suppressor Of Zeste 12
SWI	Switch
T	**Thymine**
TASCC	TOR autophagy spatial coupling compartment
TAF	TBP-associated factor
TBA3E	Tubulin, Alpha 3e
TBB6	Tubulin, Beta 6 Class V
TBP	TATA binding protein
TET	Ten-eleven translocation methylcytosine dioxygenase
TF	Transcription factor
TFTC	TBP-free TAF complex
TGF	Tumour growth factor
TREX-2	Transcription and Export complex 2
TRIM28	Tripartite Motif Containing 28
tRNA	Transport RNA
Trx	Trithorax
TSS	transcription start site
U	**Unit (of enzyme activity)**
UBP22	Ubiquitin carboxyl-terminal hydrolase 22
UDP	Uridine diphosphate
UV	Ultraviolet
µ	Micro
VHL	**Von Hippel-Lindau tumour suppressor**
VSV-g	Vesicular stomatitis virus glycoprotein
WB	**Western Blot**
WCL	Whole cell lysates
w/v	Weight/volume

WNT	Wingless (Wg) and integration site (INT) gene
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XPO	Exportin
γH2AX	**Phosphorylated histone 2, variant X**
Zmpste24	**Zinc Metallopeptidase STE24**

Chapter 1. Introduction

1.1 Senescence

Over half a century ago Hayflick and Moorhead described the finite capacity of 25 primary human fibroblast strains to replicate in culture (Hayflick and Moorhead, 1961). They showed that, as opposed to malignant cells, these fibroblasts would undergo a maximum of 50±10 population doublings before irreversibly arresting, what they attributed to “unknown” intrinsic factors (Hayflick and Moorhead, 1961). The observation that cells from adult tissues had less doubling potential than those of fetal donors, and the independence of such phenotype from culture conditions (as opposed to quiescence), prompted the establishment of the concept of cellular senescence or aging as a consequence of accumulation of damage to a certain cellular component (Hayflick, 1965). It is now well established that the cellular component Hayflick unknowingly mentioned were telomeres, that due to incomplete replication upon several cell cycle rounds, become irremediably short, posing a blockade to proliferation (Harley et al., 1990; Levy et al., 1992). Despite being arrested senescent cells are metabolically active, suggesting a dynamic role in biological processes. In following years it was discovered that senescence can be triggered prematurely by additional stresses, such as activated oncogenes. Oncogene-induced senescence (OIS) constitutes an important barrier against malignant transformation (Collado et al., 2005; Serrano et al., 1997). Thus, senescence is overall a response to stress, be it telomeric, oncogenic or genotoxic, that protects against the spreading of damaged genetic information. Despite its “good intentions” senescence has also a dark side and is behind several ageing associated diseases. Hence, senescence is a complex phenotype with multiple implications and its study has proven fruitful in providing insights into many biological scenarios ranging from embryonic development to aging.

1.1.1 The senescent phenotype

The main hallmark of senescent cells is their intrinsic inability to resume proliferation, being typically arrested at the G1 phase of the cell cycle (**Figure 1**). This differentiates them from quiescent cells, that undergo cell cycle exit (G0) due to lack of growth stimuli, but can restart proliferation once physiological stimuli are optimal (Campisi and d'Adda di Fagagna, 2007). Behind the cell cycle arrest there is a specific genetic program that encompasses activation of major tumour suppressive networks, mainly involving pRb and p53 as central mediators. Despite the involvement of p53 both in senescence and apoptosis, senescent cells are frequently resistant to apoptotic stimuli, what might account for their long-time stability (Seluanov et al., 2001). Several factors ranging from stress level, post-translational modifications of p53 or other cell type specifications seem to contribute to determine cell fate (Childs et al., 2014).

Morphologically, senescent cells can be distinguished in culture by their flat, vacuolated and enlarged morphology, which underlies cytoskeleton changes (Nishio et al., 2001). An additional feature routinely used to identify senescent cells *in vivo* is the increased senescence-associated-β-galactosidase (SA-β-gal) activity measured at a sub-optimal pH 6.0, what reflects increased lysosomal activity (**Figure 1**) (Dimri et al., 1995). Despite its importance for detecting senescent cells, SA-β-gal is not relevant for the establishment of senescence and can be induced by other stresses such as prolonged confluence in culture. Therefore, its use as a tool to identify senescence cells must be complemented by the monitoring of other markers (Lee et al., 2006).

Molecularly, senescence is characterized by changes in expression of cell cycle regulators **(Figure 1)** (Mason et al., 2004; Shelton et al., 1999). Specifically, as part of the senescent program for the establishment of the cell cycle arrest, senescent cells upregulate several cyclin-dependent kinases inhibitors (CDKi) such as p16, p15 and p21 and repress genes involved in proliferation most of which are targets of E2F transcription factors, such as cyclins A and B and the proliferating cell nuclear antigen (PCNA) (Pang and Chen, 1994; Seshadri and Campisi, 1990; Stein et al., 1991).

Additionally, senescent cells actively secrete a plethora of factors, collectively known as the senescence-associated secretory phenotype (SASP) (**Figure 1**). The SASP has been shown to influence not only the surrounding neighboring cells but also the extracellular matrix. The cocktail of secreted factors include, cytokines (*i.e.* IL6), chemokines (*i.e* IL8) and growth factors, which can have both anti- or pro-tumorigenic effects depending of the cellular contexts (Coppe et al., 2010; Kuilman and Peeper, 2009).

Adding up to the altered genetic program, senescent cells undergo deep epigenetic changes (**Figure 1**). Specifically, senescent nuclei display foci of heterochromatin (senescence-associated heterochromatin foci- SAHFs) that are enriched for specific proteins and chromatin marks such as HP1γ, HMGA1 and 2, H3K9me3 and H3K27me3 and depleted of H3K4me or H3K9ac (Narita et al., 2006; Narita et al., 2003).

Finally, the increased SA-β-gal activity reflects increased lysosomal activity, which has been, on its turn, associated with autophagy (Gerland et al., 2003). Autophagy (generally referring to macroautophagy) is a cellular catabolic process whereby autophagosomes engulf cytoplasmatic components and deliver them to lysosomes for bulk degradation (Mizushima and Komatsu, 2011). Previous reports showed increased autophagic activity in senescence (Capparelli et al., 2012; Dorr et al., 2013; Narita et al., 2011; Sasaki et al., 2010; Young et al., 2009). Although rather paradoxical, it appears that activation of autophagy, a catabolic process, is closely related with the establishment of the SASP, characterized by active protein biosynthesis. Specifically, previous work has shown that autolysosomal vesicles localize adjacently to the trans side of the Golgi apparatus, forming the TOR-autophagy spatial coupling compartment (TASCC), where mTOR also accumulates, to coordinate protein synthesis (Narita et al., 2011). This spatial organization involving catabolic and anabolic centers arises possibly to allow the constant supply of basic molecular components for protein biosynthesis. Additionally, a recent study postulated that *en mass* production of secreted factors leads to accumulation of misfolded proteins (proteotoxic stress) that can affect cellular viability (Dorr et al., 2013). Thus, increased autophagy, and thereafter lysosomal activity, has a dual role: establishing the secretory phenotype and protecting the cell from proteotoxic stress. It is however relevant to mention that

other studies have shown that autophagy counteracts senescence, thus a deeper investigation is needed to understand how both processes are interconnected (Gewirtz, 2013). It has also been suggested that lipid catabolism (fatty acid oxidation) increases in senescence possibly to lower the energy spent in biosynthesis of macromolecules that are not needed once cells are not proliferating (Jiang et al., 2013; Perez-Mancera et al., 2014; Quijano et al., 2012).

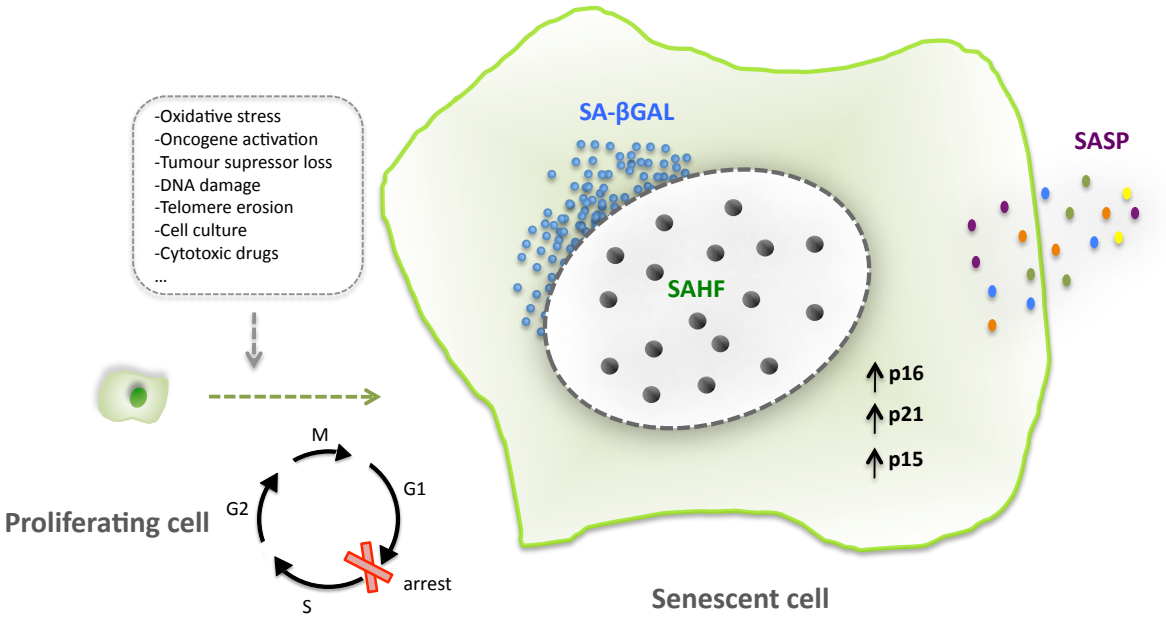


Figure 1. The Senescent Phenotype. Multiple stresses can induce senescence. Senescent cells have several characteristics that allow their discrimination from additional forms of cell cycle arrest (*i.e.* quiescence). As cells undergo senescence they arrest in G1, increase lysosomal activity, express SA-β-Gal activity and rearrange their chromatin with concomitant formation of senescence-associated heterochromatin foci (SAHF). Typical genes upregulated during senescence include cyclin-dependent kinases inhibitors (*i.e.* p16) and secreted factors (SASP).

1.1.2 Replicative senescence

When Hayflick and Moorhead first described that cultured fibroblasts could only undergo a finite number of population doublings the idea of a “mitotic clock” was established (Hayflick and Moorhead, 1961). A series of discoveries from the description of the DNA double helix and the “end-replication problem” by James Watson to the revelation of the existence of an organized and repetitive structure at the chromosome ends (telomeres), culminated with the finding that telomere

shortening, at every cell cycle round, was the phenomenon behind the Hayflick limit and cellular ageing (Hayflick, 1979; Moyzis et al., 1988; Shay and Wright, 2000; Watson, 1972).

Telomeres consist of double stranded repetitions of a single DNA motive, TTAGGG, extending from the 5' toward the 3' chromosomal end, which ends in a single stranded stretch (Nandakumar and Cech, 2013). In humans, telomeres encompass 10 to 15 Kb in average, while in mice they are longer, extending along 20 to 50 Kb (Nandakumar and Cech, 2013). Owing to the inability of the DNA polymerase to fully replicate the 3' end of chromosomes, there is a shortening of telomeres at every cell cycle round. This has been referred as the "end replication problem" and is observed in most adult somatic cells (**Figure 2**) (Levy et al., 1992; Watson, 1972). The observation that some cells, like stem cells or cancer cells, overcome this limitation suggested that a compensatory mechanism exists in these cells. Indeed, in immortalized cells telomeres are constantly maintained through 3' end binding of telomerase that contains both, a reverse transcriptase enzymatic activity (TERT) and the RNA template needed for telomere extension (Feng et al., 1995; Kim et al., 1994; Morin, 1989). More than 90% of all cancers show TERT upregulation (Heidenreich et al., 2014). Additionally introduction of telomerase in normal cells resulted in lifespan extension, suggesting that the lack of telomerase in non-immortal cells is behind their limited proliferation (Bodnar et al., 1998).

The single stranded 3' end of telomeres is not only needed for telomerase association but also for binding of specific proteins involved in telomere capping, composing the shelterin complex (Nandakumar and Cech, 2013). The shelterin complex encompasses 6 proteins, amongst which TRF1 and TRF2 that directly bind telomeric DNA protecting both single and doubled stranded DNA from DNA damage and end-to-end telomere fusions by non-homologous end joining (NHEJ) (**Figure 2**). Thus, consecutive telomere erosion ultimately leads to a stage where shelterin can no longer find its binding motive, therefore leaving telomeres uncapped and vulnerable to DNA damage which leads to replicative senescence through activation of ATM, p53 and p21 (Diotti and Loayza, 2011; Herbig et al., 2004). This can be replicated when subunits of the shelterin complex are inactivated, for instance by expression of a double mutant TRF2 leading to

telomere dysfunction-induced foci (TIF) of persistent DNA damage (Hiroyuki Takai and Titia de, 2003; Zhang et al., 2006).

Confirmation that senescence as a consequence of telomere attrition does happen *in vivo* arrived from several studies showing that not only senescent cells do accumulate in older individuals, as these cells have shorter telomeres and co-localized with DNA damage foci (Cristofalo et al., 2004; Dimri et al., 1995; Herbig et al., 2006).

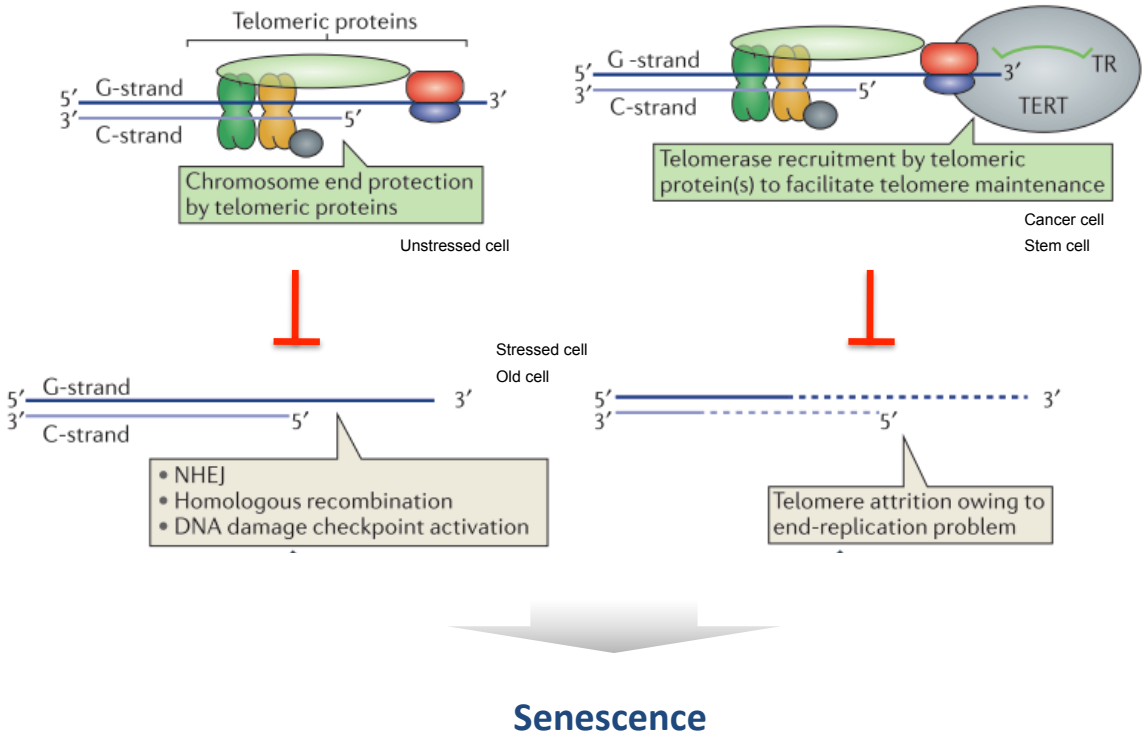


Figure 2. Replicative senescence. The existence of telomerase in cancer cells and telomeric proteins protects telomeres from erosion and DNA damage. Somatic cells do not have active telomerase and therefore cannot efficiently replicate chromosomal ends. Once cells reach the Hayflick limit and/or telomeric proteins uncap the telomeres, telomeric DNA accumulates DNA damage that triggers senescence and therefore irreversible cell cycle arrest (Nandakumar and Cech, 2013).

1.1.3 Premature senescence

Despite its earlier association with telomeres and aging, senescence has been subsequently described to occur also in response to other triggers. Thus, due to the dissociation of such types of senescence from the cell replicative status, they are collectively referred to as “premature senescence”, as it happens before the Hayflick limit is reached (Kuilman et al., 2010). Premature senescence represents therefore a robust cell defense mechanism to several stresses in order to impede perpetuation of damaged genetic content.

1.1.3.1 Stress-induced senescence

The induction of senescence in response to triggers other than shortened telomeres has suggested that senescence is above and foremost a cellular response to stress. Although the recent observation of senescence during embryonic development challenged the idea that senescence is exclusively an endpoint of a stress response, in most cases senescence is a reaction to stress (Perez-Mancera et al., 2014).

Murine cellular models were key for defining premature senescence, as mouse cells have long telomeres and activated telomerase, but still undergo senescence via telomere independent mechanisms (Kipling and Cooke, 1990; Perez-Mancera et al., 2014; Prowse and Greider, 1995). Indeed stress- and DNA damage-inducing triggers such as chemotherapeutical drugs (Therapy-induced senescence), activated oncogenes or γ-irradiation, induce senescence (Dorr et al., 2013; Rodier et al., 2009; Schmitt et al., 2002; Serrano et al., 1997). Interestingly, these stresses frequently lead to persistent DNA damage in the telomeric regions (TIF, or telomere dysfunction-induced foci), irrespective of the replicative status, which is irreparable due to the presence of the capping complex proteins, such as TRF2, that do not allow for assembly of the repair machinery (Fumagalli et al., 2012; Herbig et al., 2006).

An additional cause for premature senescence is oxidative stress (Kuilman et al., 2010). In fact, the association between oxygen levels and senescence has long been appreciated as a cause of the “culture shock” observed upon cell culture together with disruption of cell–cell contacts, lack of interactions between different

cell types, medium-to-cell ratio, lack of appropriate growth factors as well as plating on plastic (Sherr and DePinho, 2000). Reactive oxygen species (ROS) are central to oxidative stress and have been reported as both cause and consequence of senescence. Treatment with anti-oxidants can reverse the senescent phenotype (Chen et al., 1995; Lee et al., 1999; Macip et al., 2002; Passos et al., 2010). ROS arise from mitochondrial dysfunction and have been shown to engage mainly on a p38 MAPK/p21-mediated positive feedback loop with DDR to maintain senescence, although p16/pRb pathway might play a role as well (Passos et al., 2010; Sun et al., 2007).

Several studies have shown that the mitochondrial function is significantly altered in senescence (Salama et al., 2014). Whereas some studies suggest that senescent cells shift their metabolism from glycolysis to mitochondrial respiration in senescence with consequent mass energy production (mainly OIS and TIS), others report that mitochondrial function and energy production, is impaired in senescent cells that show increased mitochondrial biogenesis (Dorr et al., 2013; Kaplon et al., 2013; Moiseeva et al., 2009; Passos et al., 2007a; Passos et al., 2007b; Quijano et al., 2012). Nevertheless in both cases, increased ROS production seems to underlie altered mitochondrial function in senescence (Moiseeva et al., 2009; Passos et al., 2007a; Passos et al., 2007b). Increases in reactive oxygen species in senescence can also be due to a decrease in NADPH (Jiang et al., 2013).

1.1.3.2 Oncogene-induced senescence

A type of premature senescence requiring special mention is that induced by activated oncogenes (**Figure 3**). It had long been appreciated that introduction of oncogenes into cells *in vitro* could result in a cell cycle arrest (Franza et al., 1986; Land et al., 1983). The explanation to this phenomenon came in 1997, when Serrano and co-workers observed that human fibroblasts transduced with the oncogene H-Ras^{V12} presented features overlapping with those of cells that had undergone replicative senescence (Serrano et al., 1997). Ras expressing cells were cell cycle arrested, expressed p53 and p16 and were positive for SA-β-gal. These observations were subsequently extended to other oncogenes such as

MEK or RAF (Lin et al., 1998; Zhu et al., 1998). Subsequent studies confirmed that this type of senescence was independent from the replicative status of the cells, as introduction of telomerase could not revert the phenotype, thus postulating that oncogenes could induce senescence prematurely, at least *in vitro* (Wei et al., 1999). The relevance of oncogene-induced senescence *in vivo* came only in 2005 when several groups independently showed that oncogenes could be found in murine and human benign lesions (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Lazzerini Denchi et al., 2005). Specifically, animal models expressing K-Ras^{v12} and Eμ-N-Ras presented benign lung lesions and non-lymphoid neoplasias, respectively, with characteristics of senescence. Interestingly, the malignant counterparts did not present senescence-like features suggesting that OIS could function as a barrier against tumorigenesis (Braig et al., 2005; Collado et al., 2005). Moreover in the case of the latter, depletion of either p53 or Suv39h1 (H3K9 methyltransferase) resulted in bypass of senescence (Braig et al., 2005). The overexpression of BRAF^{E600} and E2F3 in human and murine lesions overlapped with appearance of senescence, in the skin and pituitary gland, respectively (Lazzerini Denchi et al., 2005; Michaloglou et al., 2005). The case of BRAF^{E600} benign skin lesions (nevi) is a remarkable example of how oncogene-induced senescence contributes to the stable repression of malignancy for several years (de Keizer et al., 2010; Lazzerini Denchi et al., 2005). OIS has since been observed in other pre-malignant lesions, as for instance in K-RAS^{G12D} serrated colon benign lesions (Bennecke et al., 2010). In addition, more than 50 oncogenes are able to induce senescence (Gorgoulis and Halazonetis, 2010). For instance c-Myc in the presence of stroma derived TGF-β triggers senescence and prevents B-cell lymphoma (Reimann et al., 2010). The extent to which oncogenes trigger senescence to the detriment of proliferation depends on whether oncogene expression exceeds a certain threshold (Junttila et al., 2010; Sarkisian et al., 2007).

Senescence upon oncogene activation arises in part due to DNA hyper-replication, single- and double-strand DNA breaks (SSB and DSB, respectively) and consequent DNA damage, although this might not be a universal feature (Bartkova et al., 2006; Bianchi-Smiraglia and Nikiforov, 2012; Di Micco et al., 2006). A senescent benign lesion can also be triggered upon tumour suppressor loss. A

prototypical case of tumour suppressor loss is that of PTEN- loss-induced cellular senescence (PICS) in prostate lesions (Alimonti et al., 2010; Chen et al., 2005; Lin et al., 2010). This type of senescence differs from OIS as although it relies on p53 expression it occurs in the absence of proliferation and visible DDR (Alimonti et al., 2010; Lin et al., 2010). Loss of NF1,VHL (von Hippel–Lindau tumour suppressor gene) and Rb can also induce senescence in neurofibromas, and kidney and thyroid tumours, respectively (Courtois-Cox et al., 2006; Shamma et al., 2009; Young et al., 2008).

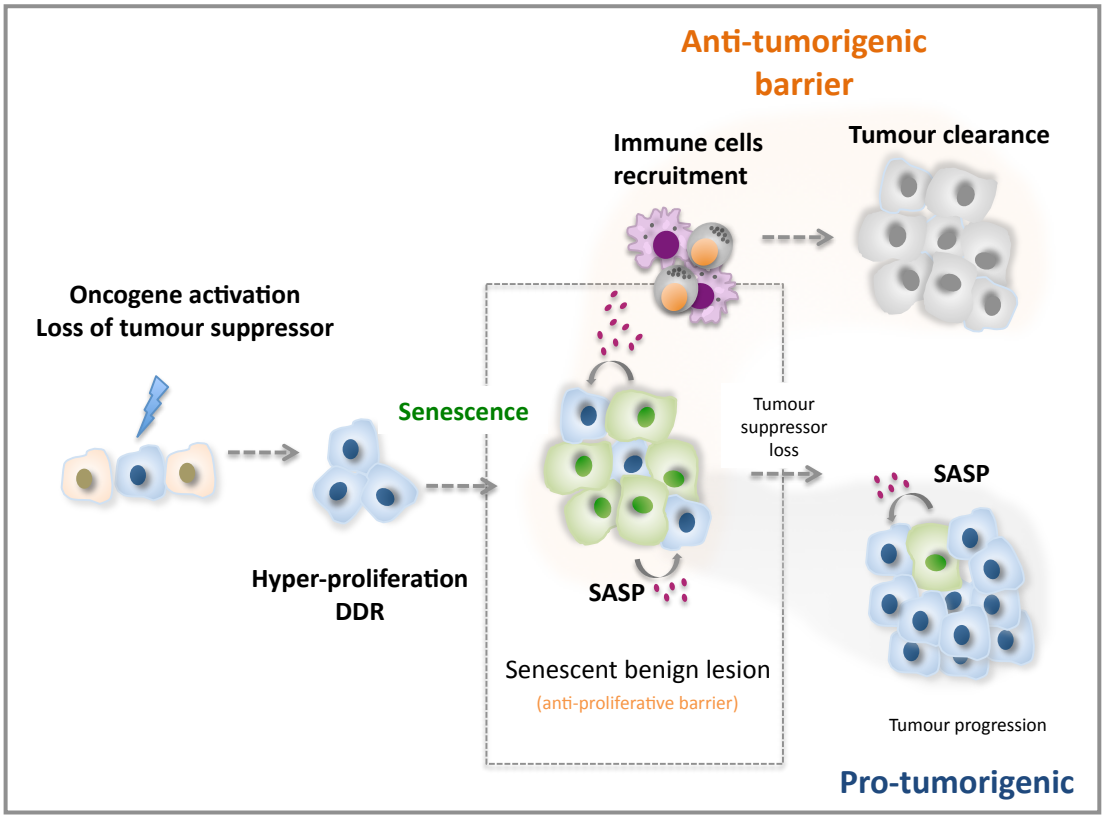


Figure 3. Oncogene-induced senescence. Activated oncogenes lead to DNA hyperreplication, proliferation and consequent DNA damage accumulation, triggering senescence. Benign tumours are frequently populated by senescent cells, which are thought to provide an anti-proliferative barrier for tumour progression. Additionally, senescent cells secrete proinflammatory cytokines that attract the immune system leading to tumour clearance, for what senescence is considered an anti-tumorigenic barrier. However, if cells manage to bypass the senescence barrier, the SASP might confer pro-tumorigenic properties to senescent cells and reinforce tumour progression.

1.1.3.3 Other types of senescence

Despite its long-term association with aging, senescence has been shown to occur in early phases of the embryonic development. Specifically, two recent manuscripts demonstrated that senescence is a developmentally programmed

event during embryogenesis (**Figure 4**) (Munoz-Espin et al., 2013; Storer et al., 2013).

SA-β-gal had already been reported in the mesonephros (embryonic kidney) of quail embryos, however, although hypothesized, a direct connection with senescence was not established (Nacher et al., 2006). In 2013, two independent studies corroborated these results by showing that SA-β-gal positive cells do indeed accumulate in the mesonephros and endolymphatic sac of the inner ear, and the apical ectodermal ridge (AER, important for limb outgrowth and patterning) and the neural roof plate, respectively, of mice and chick (Munoz-Espin et al., 2013; Storer et al., 2013). They showed that these cells have characteristics of senescence: they are arrested at G1, show increased expression of specific senescence-associated markers such as p21, p15 and p27 and have senescence-associated chromatin and nuclear features (H3K3me, HP1γ, PML) (Munoz-Espin et al., 2013; Storer et al., 2013). Moreover, gene expression of those cells largely overlapped with that of oncogene-induced senescence namely in the induction of factors involved in the SASP, suggesting that already in the embryo senescence might be triggered to influence surrounding tissues (Storer et al., 2013). Despite the overlap of gene expression, developmental senescence appears to be more rudimental, as it relies strictly on p21 expression with no DDR, p53 or p16 associated expression (Munoz-Espin et al., 2013; Storer et al., 2013). Nevertheless, failure in establishing senescence in the mesonephros of mice embryos, led to developmental defects in the adult animals, namely in the incomplete removal of vaginal septa, which can lead to infertility (Munoz-Espin et al., 2013). Moreover, the observation that cells with the aforementioned characteristics were also present in human embryos suggests that developmental senescence is an evolutionary conserved process (Munoz-Espin et al., 2013).

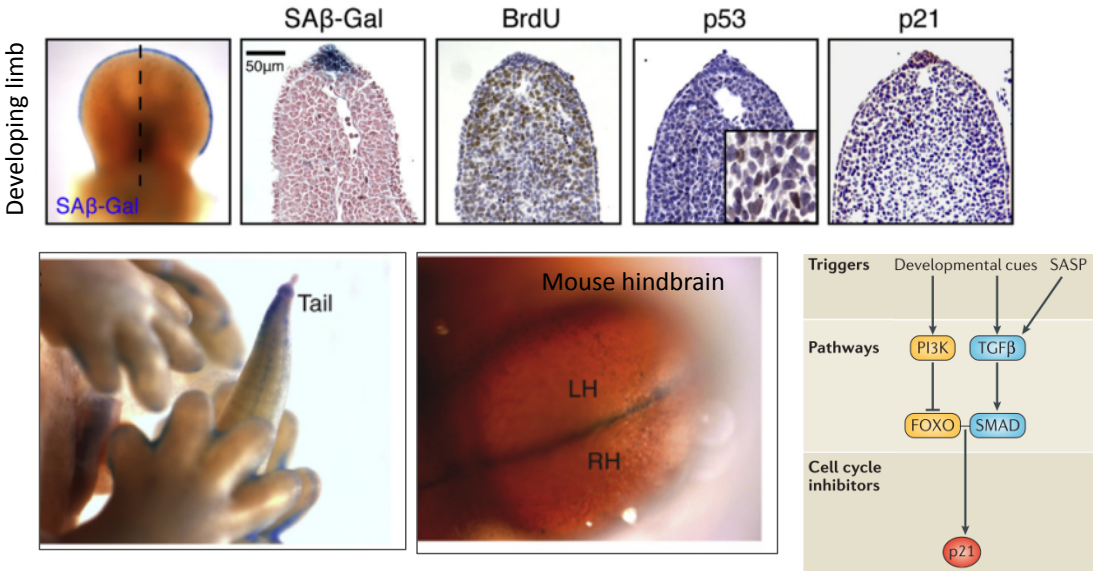


Figure 4. Senescence is developmentally programmed. Several embryonic structures stain positive for SA-β-Gal during phases E11.5 to E14.5 of embryonic development. Cells show charateristics of senescence such as lack of proliferation (BrdU), expression of the CDK inhibitor p21, independently of p53, and expression of SASP-associated factors. Developmental senescence is independent of DNA damage and is rather activated through the TGFβ/SMAD-mediated pathway. (Adapted from Munoz-Espin et al., 2013 and Storer et al., 2013)

Somatic cells can be reprogrammed into induced pluripotent stem cells (iPS) though the expression of four transcription factors, OCT4, SOX2, KLF4 and c-MYC. However the rate of reprogramming to iPS is low (Takahashi and Yamanaka, 2006). Several studies in 2009, showed that expression of the 4 factors can trigger a DDR response as well as the expression of known senescence mediators, p16, p53 and p21 suggesting that limited reprogramming could arise due to the establishment of a senescence-associated barrier (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009). This becomes relevant in the context of cancer stem cells, suggesting that senescence could function as a barrier for the unlimited self-renewal capacity of these cells (Reya et al., 2001). A recent study however, showed that addition of 2 other factors, specifically NANOG and LIN28, allowed for full reprogramming of senescent fibroblasts, suggesting that optimization of reprogramming methods might permit senescence bypass (Lapasset et al., 2011).
Finally, one of the main characteristics of senescence is its ability to produce secreted factors with both pro- and anti-inflammatory properties, able to affect the surrounding tissues (Coppe et al., 2010; Kuilman and Peeper, 2009). Recent reports showed that secreted factors, specifically IL-1α and IL-1β and members of

the TGFβ family of ligands, can act on neighboring non-stressed cells and induce senescence with properties very similar to that of origin cells (Acosta et al., 2013a; Hubackova et al., 2012). “Paracrine senescence” is therefore a new form of senescence that will certainly elicit further studies, as it reinforces the importance of the SASP and consequently the ability of senescent cells to ”spread” the phenotype and halt the expansion of damaged cells.

Of note, any stress able to induce the *INK4/ARF* locus, which encodes major regulators of senescence is likely to induce cell cycle arrest and will be discussed later (Peters, 2008).

1.1.4 Molecular pathways controlling senescence

1.1.4.1 Tumour suppressor pathways

The principal hallmark of senescence is the lack of proliferation, and despite the multitude of senescence triggers it all seems to come down to the activation of two main tumour suppressor pathways involving p53 and pRb, respectively (**Figure 6**) (Courtois-Cox et al., 2008).

Retinoblastoma protein (pRb) is a tumour suppressor gene, highly mutated in cancer which belongs to the “pocket” protein family that bind members of the E2F transcription factors family, involved in cell cycle progression (Burkhart and Sage, 2008). Despite the similarity of pRb with other pocket proteins (*i.e.* p107 and p130) pRb has specific functions in senescence regulating a particular subset of E2F target genes (Chicas et al., 2010).

Regulation of pRb activity is mediated by phosphorylation. Specifically, as cyclin D and E increase during G1, they start forming complexes with cyclin dependent kinases (CDK) CDK4 and 6 and CDK2, respectively. Once activated, CDKs are able to phosphorylate pRb, releasing E2F transcription factors, which then bind the promoters and upregulate the expression of cell cycle genes, such as cyclins (*i.e.* cyclinA2, cyclin E etc) resulting in progression into S-phase (Chicas et al., 2010);(Spitkovsky et al., 1997; Weinberg, 1995).

pRb hyperphosphorylation is counteracted by CDK inhibitors (CDKi), such as p16, p21, p15 and p27 which are essential for the establishment of hypophosphorylated

pRb (Munoz-Espin and Serrano, 2014). Concomitant with an important role in cell cycle regulation, CDK inhibitors are often downregulated or mutated in cancer (Chu et al., 2008; Krimpenfort et al., 2007; Okamoto et al., 1994). Conversely, these CDKi accumulate during senescence (Kuilman et al., 2010). Particularly, the p16-pRb pathway is of extreme relevance for both replicative and oncogene-induced senescence in humans. The CDK4/6 inhibitor p16 which is not normally expressed in adult tissues, is increasingly expressed during aging (see below, regulation of the *INK4/ARF* locus) and upon oncogene activation and its depletion impairs the establishment of senescence (Alcorta et al., 1996; Brookes et al., 2004; Brookes et al., 2002; Burd et al., 2013; Lowe and Sherr, 2003; Serrano et al., 1997; Zindy et al., 1997). Additionally, p16 is often deleted in cancer and mutations are mutually exclusive with those for pRb, reinforcing that these molecules act in a linear pathway to inhibit cell cycle and induce senescence (Burkhart and Sage, 2008; Okamoto et al., 1994). Inactivation of p16 leads to extended, although finite, lifespan as cells can only proliferate for another few rounds of replication until they undergo telomere crisis and cell cycle arrest, mediated by a second pathway involving p53 and p21 (Brookes et al., 2004; Chicas et al., 2010; Shay and Wright, 2000). Of note both p16 and telomerase inhibition are needed for immortalization of cultured fibroblasts, what per se already suggests the existence of at least 2 pathways controlling senescence (Kiyono et al., 1998). A recent report showed that p16 inhibition could result in increased telomere instability and further DDR signaling, p53 and p21 activation and cell cycle arrest (Wang et al., 2013). Altogether, this suggests that p16 not only has a protective role in ageing as it communicates with the p53/p21 pathway for reinforcing replicative senescence (Kuilman et al., 2010; Martin et al., 2014; Takeuchi et al., 2010).

Other key tumour suppressor is p53 which has a central role in cell fate decision controlling several processes such as senescence and apoptosis, for what it is considered the guardian of the genome. It is therefore not surprising that more than 50% of all human cancers have mutations on p53 (Bieging et al., 2014; Rinn and Huarte, 2011). p53 acts as a transcription factor and directly (via binding to p53 consensus response element- p53RE- on target promoters) or indirectly regulates the expression of several downstream targets (Rinn and Huarte, 2011). One of such genes is the CDK2/CDK1 inhibitor p21, which accumulates in

response to p53 activation (el-Deiry et al., 1993). By doing so p53 controls pRb phosphorylation levels and induces cell cycle arrest at the G1 and G2 phases by negatively interfering with E2F target genes expression such as cyclin A2 and directly inhibiting cyclinB/CDK1 complexes, respectively (Abbas and Dutta, 2009; Brugarolas et al., 1995; Deng et al., 1995; Dutta and Anindya, 2009; Spitkovsky et al., 1997). In mice, p53 can mediate senescence independently of p21 as murine cells lacking p21 can still undergo cell cycle arrest (Pantoja and Serrano, 1999). However this does not apply to human cells as p21 depletion leads to a senescence bypass, suggesting that it is an essential modulator of p53-mediated senescence in humans (Brown et al., 1997). Indeed, p53 regulates the expression of a plethora of genes relevant for senescence such as MDM2, its negative regulator, PAI-1, 14-3-3σ and Gadd45a (Bieging et al., 2014; Lanigan et al., 2011). Also p21 has been shown to be regulated in a p53-independent and Chk2-dependent fashion and induce senescence in human cells (Aliouat-Denis, 2005).

p53 is mainly regulated at the post-translational level. Specifically, one of its target MDM2 (HDM2 in humans) is a E3 ubiquitin ligase that regulates p53 by both directly inhibiting its transcriptional activity and sending it for degradation (Xu, 2003). In response to DNA damage p53 is rapidly phosphorylated by ATM (Ataxia telangiectasia mutated serine/threonine kinase) and Chk2 (checkpoint kinase 2) on serines 15 and 20, respectively, and less so by ATR/Chk1 on Ser 15 what competes with MDM2 binding therefore leading to protein stabilization and increased half-life (Qian and Chen, 2013). Direct MDM2 phosphorylation by ATM synergizes to increase p53 stability that is also enhanced by acetylation. Different acetylating events might direct p53 to decide between cell fates (*i.e.* apoptosis vs senescence) that additionally seem to depend on different transactivation domains (Brady et al., 2011; Qian and Chen, 2013).

DNA damage response (DDR) is key for p53 activation and it is often involved in cellular senescence (**Figure 5**) (d'Adda di Fagagna, 2008). Telomere erosion, genotoxic stress (*i.e.* γirradiation), or DNA hyper-replication upon oncogene signaling, can lead to the exposure of single stranded DNA or double strand breaks (DSB) triggering a DDR signaling and consequent cell cycle arrest (Bartkova et al., 2006; d'Adda di Fagagna et al., 2003; Di Micco et al., 2006; Fumagalli et al., 2012; Herbig et al., 2004). These abnormalities lead to the

recruitment of Ataxia Telangiectasia and Rad3-related (ATR) and ATM, phosphorylation of the histone variant H2Ax (γH2Ax), deposition of mediator of DNA-damage checkpoint 1 (MDC1) and p53-binding protein 1 (53BP1) that reinforce a positive feedback loop which culminates with ATM/CHK2 and ATR/CHK1 mediated p53 phosphorylation and senescence (d'Adda di Fagagna, 2008). This is exacerbated by concomitant phosphorylation and inactivation of CDC25 (cell-division cycle 25), which is itself a target of p53 mediated repression (Clair and Manfredi, 2006; Mailand et al., 2000).

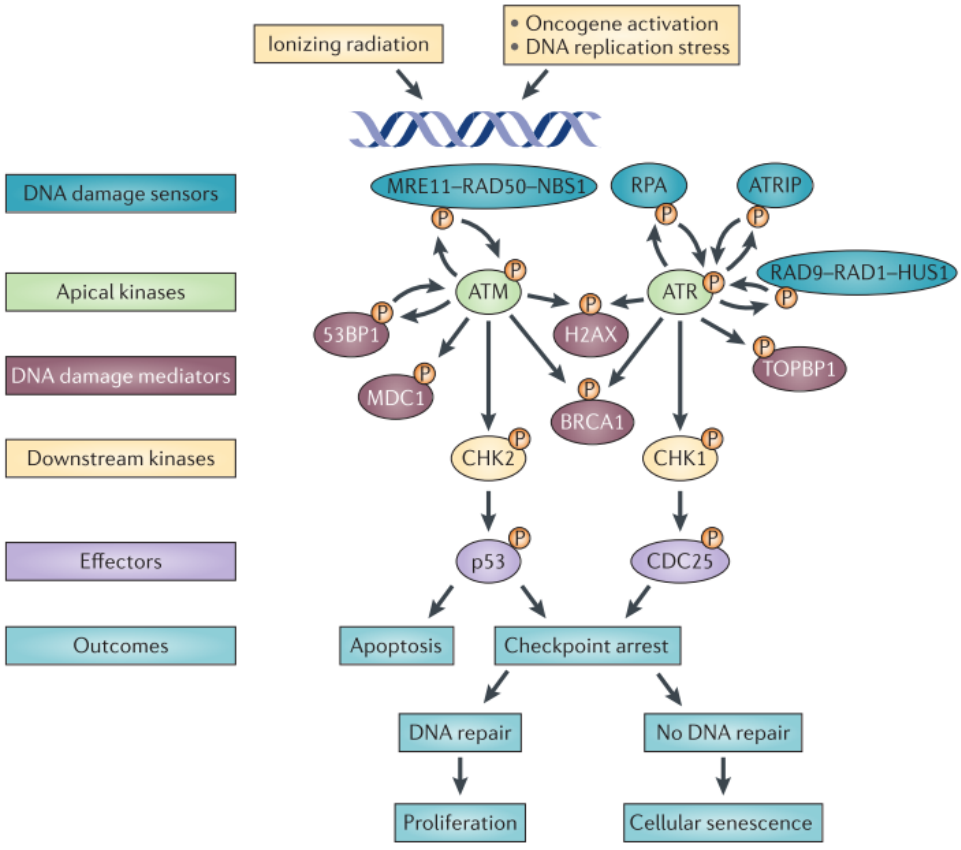


Figure 5. The DNA damage response. MRE11, RAD50 and NBS1 are sensors of double strand breaks and lead to activation of the kinase ataxia-telangiectasia mutated (ATM) and consequent amplification of the response by recruitment of other DNA damage signaling proteins. ATM acts as a kinase of several proteins including MDM2, MDM4, p53 and checkpoint-2 (CHK2), which phosphorylates p53 and other proteins. Single strand breaks are sensed by replication protein A (RPA) and the RAD9–RAD1–HUS1 (9-1-1) complex that attract the ataxia-telangiectasia and Rad3-related (ATR) - interacting protein (ATRIP) which phosphorylates the 9-1-1 complex (constituted by RAD9, RAD1 and HUS1) further activating ATR. Active ATR phosphorylates p53, MDM2, checkpoint-1 (CHK1) and other substrates. Both ATM and ATR phosphorylate several proteins important for sustaining the DNA damage response (*i.e.* γH2Ax, 53BP1). DNA damage signaling ultimately spreads away from the damaged site, culminating with activation of effector proteins such as p53, CDC25 or structural maintenance of chromosomes (SMC1). Depending of cellular context and levels of DNA damage final outcome might encompass DNA repair and proliferation resuming, cell death or apoptosis (Sulli et al., 2012)

p53 can also be activated through direct phosphorylation by p38-regulated/activated protein kinase during Ras-induced senescence, in a DDR independent way (PRAK) (Sun et al., 2007). Finally an important regulator of p53 in mice is $p19^{ARF}$ ($p14^{ARF}$ in humans). ARF is a negative regulator of MDM2, by impeding its ubiquitinase activity against p53 as well as promoting its degradation (Honda and Yasuda, 1999; Pomerantz et al., 1998; Tao and Levine, 1999; Zhang et al., 1998). $p19^{ARF}$ is upregulated during Ras-induced senescence and its depletion led to a bypass of OIS in rodent cells (Kamijo et al., 1997; Palmero et al., 1998; Wei et al., 2001). Whereas it seems to have a prominent role during OIS in murine models, its role in human cells is not so prominent (Michaloglou et al., 2005; Wei et al., 2001).

Additionally, in fibroblasts induced to senesce by $BRAF^{E600}$ expression, p16 is upregulated but other factors such as FOXO4/p21 seem to play a key role in inducing cell cycle arrest (de Keizer et al., 2010; Michaloglou et al., 2005). Thus, both the DDR/p53/p21 and p16/pRb pathways are likely to contribute to the establishment of different types of senescence, with cell specificities possibly dictating the relative contribution of each route for the observed phenotype.

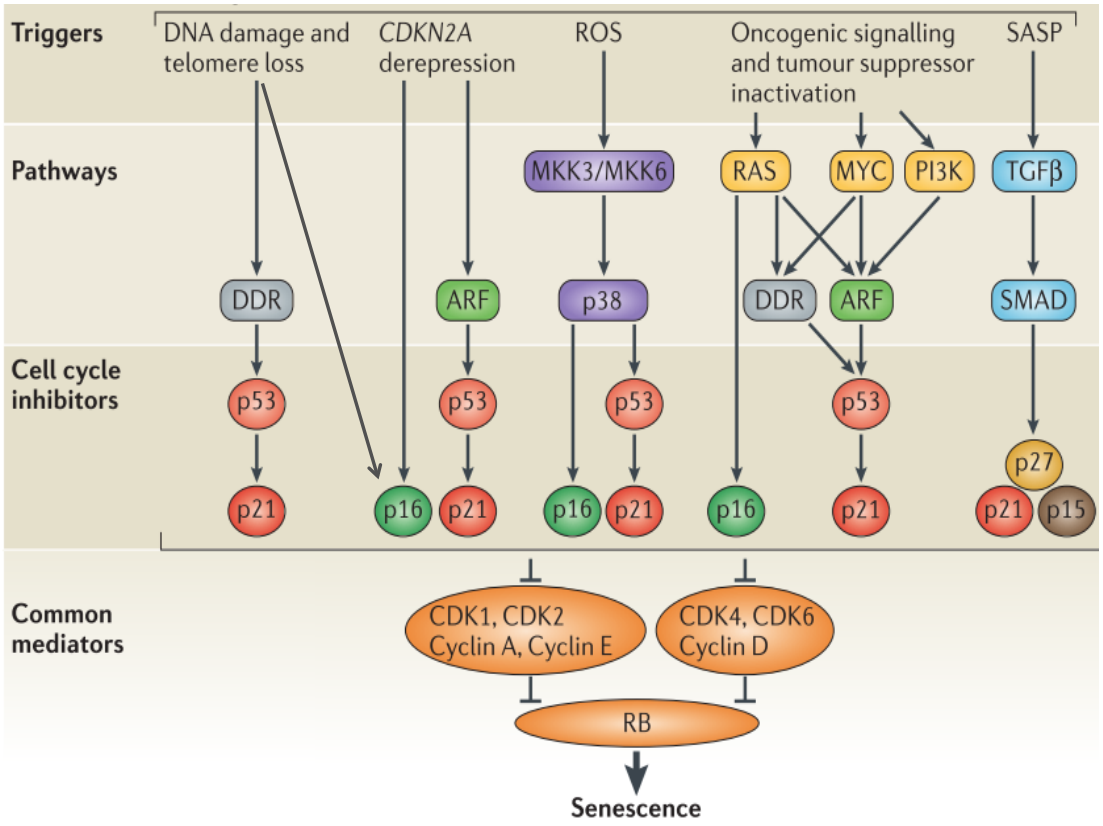


Figure 6. Molecular routes regulating senescence. Several stresses, from damaged telomeres, oncogene signaling or oxidative stress induce senescence invariably through activation of the p53/p21 or p16/pRb pathways, culminating with CDKi mediated hypophosphorylated pRb and consequent downregulation of E2F transcription factors target genes. Upstream signaling pathways can encompass DNA damage signaling as in the case of damaged telomeres and oncogenic signaling, ARF-mediated p53 activation (mainly in murine models) upon oncogene signaling or CDKN2A derepression or MAPK signaling. Paracrine senescence is activated by the SASP and is thought to signal through the TGFβ/SMAD signaling pathway (Adapted from Munoz-Espin and Serrano, 2014).

1.1.4.2 Regulation of the senescence-associated secretory phenotype (SASP)

In addition to the pathways regulating the cell cycle arrest, a parallel, however intertwined network of genes regulates the SASP, which consists of a mixture of secreted factors, including cytokines, chemokines, proteases, extracellular matrix components and growth factors. As these factors are involved in extracellular signaling, then senescent cells can influence surrounding cells (Coppe et al., 2010; Kuilman and Peeper, 2009). Prototypical SASP factors include the cytokine IL6 and several of the CXCR2 ligands such as IL8, and GROα. Their induction has been shown to be dependent on NF-κβ and C/EBPβ (Acosta et al., 2008; Chien et al., 2011; Kuilman et al., 2008). Although SASP induction upon persistent DNA

damage signaling has been reported, the DDR response does not appear absolutely necessarily as DDR-independent induction of the SASP is also observed (Freund et al., 2011; Orjalo et al., 2009; Rodier et al., 2009). Additional regulators of the SASP include, mTOR, p38MAPK and PARP-1 (Freund et al., 2011; Narita et al., 2011; Ohanna et al., 2011). Also, while p16 seems unnecessary, p53 can affect the production of IL6 and IL8 (Coppe et al., 2011). Specifically, Coppe et al. showed that p53 acted to restrain the SASP (Coppe et al., 2008). The idea that p53-mediated signaling played a role in SASP regulation, was reinforced by a recent work from Lowe and colleagues showing that p53 depletion in hepatic stellate cells leads to quantitative and qualitative alterations of the secretome and consequently a shift in the attracted macrophage population from a anti- to protumorigenic, with functions in angiogenesis and tissue remodeling (Lujambio et al., 2013). Recent work identified that the inflammasome also regulates the SASP (Acosta et al., 2013).

As the network of SASP regulators continues to build, it is now clear that secreted factors can act both in an autocrine or paracrine way (**Figure 7**). Specifically, studies by both Gil and Pepper and colleagues demonstrated that IL6, and CXCR2 ligands (*i.e.* IL8, GROα) not only are upregulated in OIS, as signal through their receptors to reinforce senescence in a p15 and p53-mediated way, respectively (Acosta et al., 2008; Kuilman et al., 2008). Autocrine reinforcement of senescence includes an additional positive feedback loop mediated by IL1 signaling and the inflammasome leading to further accumulation of IL6 and IL8 (Acosta et al., 2013; Orjalo et al., 2009).

As previously mentioned, the SASP can act on the surrounding microenvironment to induce senescence in non-stressed cells, what has been denominated “paracrine senescence” (Acosta et al., 2013). Senescence can be induced on bystander cells by soluble factors, among which IL-1α and IL-1β and TGFβ and related family members, via SMAD signaling, play a role both *in vitro* and *in vivo* (**Figure 6, Figure 7**) (Acosta et al., 2013; Hubackova et al., 2012). Thus, paracrine senescence can propagate the tumour suppressive environment to the neighboring cells.

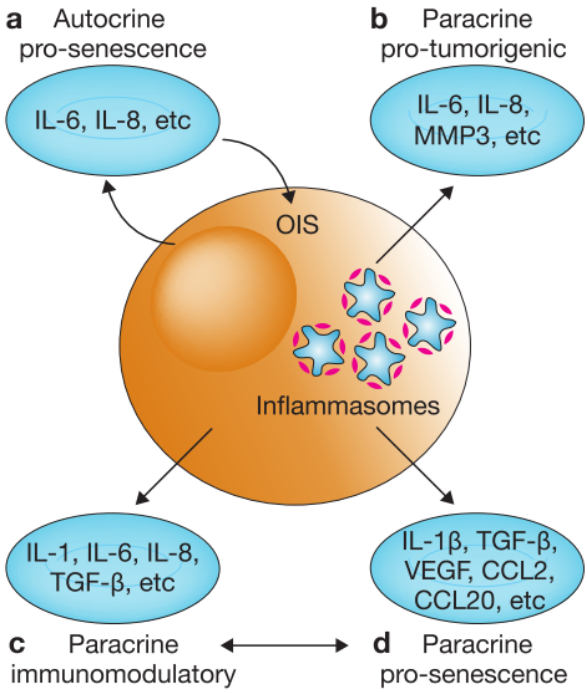


Figure 7. The senescence-associated secretory phenotype. Senescent cells secrete a plethora of proinflammatory cytokines, chemokines and growth factors which is regulated by transcription factors such as NF-κβ and C/EBPβ and other cellular components like the inflammasome. These factors can reinforce senescence autonomously (*i.e.* IL-6 and Il-8) or have paracrine effects that range from pro-senescence functions, to immunomodulatory (regulating immune system mediated clearance of senescent cells) or pro-tumorigenic depending on the cellular context (Hoare and Narita, 2013).

1.1.5 Epigenetic mechanisms controlling senescence

Epigenetic control of gene expression involves mechanisms that are independent of the DNA sequence, but instead depend on alterations occurring at the chromatin level. Changes in the status of chromatin condensation are achieved by a combinatorial action of several effectors from histones, to “writers” (such as methyltransferases) and “readers” (*i.e.* chromodomain containing proteins) (Barth and Imhof, 2010). Histones and DNA are the basic components of the chromatin. Core histones (H2A, H2B, H3 and H4) associate with around 147 bp of DNA to form the nucleosomes. DNA between nucleosomes is called linker DNA and is bound by H1 histone. Histone N and C tails can be modified by “writers” (Barth and Imhof, 2010). Histone acetylation is usually associated with the induction gene expression, and histone methylation with both transcriptional activation and

repression. For instance, H3K9me di- and trimethylation and H3K27me3 are typical marks of repressed promoters and chromatin. However, H3K4me for instance is normally associated with active chromatin (Rai and Adams, 2012). Finally once a chromatin mark is created it can be “read” by proteins with specific domains, for example bromodomain containing proteins (usually detect acetylated histones- *i.e.* SWI/SNF) or proteins with chromodomains (usually recognize methylated residues- *i.e.* HP1) that translate the chromatin language to other enzymatic complexes. Nevertheless additional histone marks and readers exist (Barth and Imhof, 2010).

Regulation of several senescence effectors, such as p21, p14, p16 or p15 is achieved mostly at the transcriptional level. Furthermore, the three latter present an interesting scenario as they are expressed from the same locus, known as the *INK4b-ARF-INK4a* locus, which is under stringent epigenetic regulation. Additionally, high-order chromatin-remodeling events are known to take place in senescence, and thought to contribute to the stability of the phenotype.

1.1.5.1 *INK4b-ARF-INK4a* locus

The *INK4b-ARF-INK4a* (also known as *INK4/ARF* locus) locus extends along 25 Kb of the human chromosome 9p21 (mouse chromosome 4), and encodes two related cyclin dependent kinases inhibitions, p15 (INK4b) and p16 (INK4a), and the unrelated gene ARF, all with relevant functions in senescence (**Figure 8**) (Gil and Peters, 2006). While p16 and p15 induce senescence by negatively regulating CDK4 and CDK6 and consequently allowing pRb mediated negative regulation of E2F target genes, ARF is an upstream regulator of p53, that by negatively regulating MDM2 (HMD2) increases p53 stability and half-life (Honda and Yasuda, 1999; Pomerantz et al., 1998; Quelle et al., 1995; Tao and Levine, 1999; Zhang et al., 1998) The name ARF derives from the fact this gene arises from an alternative reading frame (ARF) from that of p16, with which it shares two exons. However, p15 is encoded by an independent and adjacent gene (Quelle et al., 1995).

The genomic region containing the *INK4/ARF* locus is one of the most altered in cancer (Esteller, 2008; Gil and Peters, 2006). Indeed, data from murine models suggest all three genes are bona fide tumour suppressors and senescence

regulators (Lanigan et al., 2011; Sharpless, 2005). However some differences exist regarding the relevance of each gene in mouse models and humans. While ARF is upregulated in murine models and its deletion results in senescence bypass and tumorigenesis, the same is not true in human cells (Kamijo et al., 1997; Michaloglou et al., 2005; Palmero et al., 1998; Voorhoeve and Agami, 2003; Wei et al., 2001). Indeed, although existent, ARF-specific mutations in human tumours are rarer than those for p16 (Sharpless, 2005). Conversely, the expression of p16 is upregulated as tissues age and upon several stresses, such as oncogenes, genotoxic stress and telomere erosion, and is one of the most mutated genes in human cancer (Alcorta et al., 1996; Burd et al., 2013; Jacobs and de Lange, 2004; Okamoto et al., 1994; Serrano et al., 1997; Sun et al., 2007; Yamakoshi et al., 2009). The role of p15 has been less studied than those of its neighboring genes. However p15 is also a tumour suppressor mainly in hematological malignancies and is upregulated and regulates senescence (Boultwood and Wainscoat, 2007; Collado et al., 2005; Erickson et al., 1998; He et al., 2008; Kim and Sharpless, 2006; Krimpenfort et al., 2007; Malumbres et al., 2000).

Owing to the importance of this locus in aging and cancer its regulation must be tightly tuned to keep its expression low in young tissues but immediately inducible under stressful events. Several transcription factors have been reported to directly bind and regulate the expression the *INK4b-ARF-INK4a* locus, as for instance MYC, AP1 and E2F family members (reviewed in Gil and Peters, 2006). The best described transcription factor-mediated mechanism regulating INK4a expression is that involving Ets1 and Ets2, and ID1 positively and negatively regulating p16, respectively (Alani et al., 2001; Lyden et al., 1999; Ohtani et al., 2001).

However, the tight regulation and plasticity of expression of the *INK4/ARF* is achieved mainly via epigenetic mechanisms (**Figure 8**) (Gil and Peters, 2006; Lanigan et al., 2011; Popov and Gil, 2010).

The prototypical epigenetic regulators of the *INK4/ARF* locus are Polycomb proteins which organize into two complexes, PRC1 and PRC2. PRC1 is composed by a Pc (Polycomb) protein (*i.e.* CBX7,CBX8), a PSC (Posterior sex combs) protein (*i.e.* BMI1/ MEL18), a RING (Really Interesting New Gene) protein, a PH (Polyhomeotic) protein and an SCML (Sex combs on mid-leg) protein, whereas PRC2 is made up by EZH2 (Enhancer of Zeste2), Embryonic Ectoderm

Development (EED) and SUZ12 (Suppressor of Zeste-12) (Bracken and Helin, 2009). These complexes are involved in chromatin repression and achieve so via a multistep process that involves first the tri-methylation of H3K27 by EZH2 (PRC2), its reading by PRC1 and consequent H2A monoubiquitination of lysine 119 mediated by RING proteins (Bracken and Helin, 2009).

In unstressed young cells Polycomb proteins exert their repressive function on the *INK4/ARF* locus, through deposition of H3K27me3. However as cells reach senescence, EZH2, which is downstream of the pRb/E2F pathway, is downregulated, Polycomb complexes are displaced and the *INK4/ARF* locus expressed (Agherbi et al., 2009; Bracken et al., 2007; Bracken et al., 2003). Moreover, senescence as a consequence of oncogenic Ras displayed identical dynamics, indicating that loss of Polycomb-mediated repression of the *INK4/ARF* locus is a common feature in senescence (Barradas et al., 2009). Supporting their role in senescence, previous work showed that overexpression of Polycomb genes CBX7 and CBX8 resulted in extended lifespan (Gil et al., 2004; Dietrich et al., 2007), while knockdown of BMI1, CBX7, CBX8, and MEL18 induced senescence, due to *INK4/ARF* upregulation (Dietrich et al., 2007; Gil et al., 2004; Itahana et al., 2003; Jacobs et al., 1999; Maertens et al., 2009). Considering their role in repressing potent tumour suppressors, such as p16, it is therefore not surprising that Polycomb genes are frequently overexpressed in cancer and confer oncogenic properties (Bracken et al., 2003; Chang et al., 2011; Yang et al., 2010).

Localization of Polycomb complexes to the *INK4/ARF* locus has been shown to be potentiated by several mechanisms (**Figure 8**), as for instance binding to transcription factors like the Zinc Finger Protein Zfp277 and the DNA replication factor CDC6, that interact with BMI1 (Agherbi et al., 2009; Negishi et al., 2010). Recently also Homeobox genes (*i.e.* HLX1) have been shown to mediate p16 repression through Polycomb recruitment (Martin et al., 2013a; Martin et al., 2013b). Additionally, binding to long non-coding RNAs (ncRNAs), like ANRIL, plays a role in correct targeting of Polycomb proteins to the regulatory region of the INK4a locus. Specifically ANRIL is a *cis* transcript of the *INK4/ARF* locus and has been implicated in recruiting CBX7 containing complexes and contributing for Polycomb mediated senescence inhibition (**Figure 8**) (Yap et al., 2010).

Polycomb eviction from the *INK4/ARF* locus during senescence is achieved due to concerted action of several mechanisms. In one hand, EZH2 is a target for E2F

mediated transcription. As during senescence E2F transcription factors are tethered by hypophosphorylated pRb, their transcriptional targets are consequently downregulated (Bracken et al., 2004; Bracken et al., 2003). Additionally, other chromatin remodeling complexes also play a role. That is the case of the SWI/SNF chromatin remodeling complex (mediating nucleosome repositioning) that has been associated with senescence partly due to its role in promoting displacement of Polycomb complexes from the *INK4b-ARF-INK4a* locus (**Figure 8**) (Alessio et al., 2010; Burrows et al., 2010; Kia et al., 2008; Oruetxebarria et al., 2004; Wilson et al., 2010). Specifically, SWI/SNF targeting to the *INK4/ARF* promoter appears to depend on SNF5, leading to chromatin remodeling by subsequent action of the SWI/SNF catalytic subunit BRG1, and upregulation of p16 and p15 (Kia et al., 2008). Moreover SWI/SWF positioning within the *INK4/ARF* promoters mediates recruitment of Trithorax group proteins, like MLL, leading to formation of active chromatin marks such as H3K4me3, and transcriptional activation of both p16 and p15 genes (Kia et al., 2008; Kotake et al., 2009). Finally, an additional consequence of SWI/SNF recruitment is the eviction of DNA methyltransferase DNMT3B and consequent loss of repressive CpG island-associated methylation, on the promoter of the p16 gene (**Figure 8**) (Kia et al., 2008). Since the *INK4/ARF* locus is hypermethylated in cancer and several DNA methylases have been described to localize to the regulatory region of Polycomb target genes, it is likely that other DNMTs might also contribute for repression of *INK4/ARF* enconded genes (Esteller et al., 2001; Mohammad et al., 2009; Vire et al., 2006). Also histone demethylases can negatively regulate senescence by specifically demethylating the H3K36me2 active chromatin mark within the INK4b (p15) locus upon Ras activation (He et al., 2008).

Finally, in 2009 two groups independently characterized the role of a H3K27me3 histone demethylase, JMJD3, that counteracts Polycomb mediated repression during senescence triggered upon RAS or BRAF overexpression or irradiation (**Figure 8**) (Agger et al., 2009; Barradas et al., 2009). Once again suggesting the different importance of ARF in mouse and humans, JMJD3 does modulate ARF expression in MEFs but not in HDF, where it specifically upregulates p16 and possibly p15 (Barradas et al., 2009).

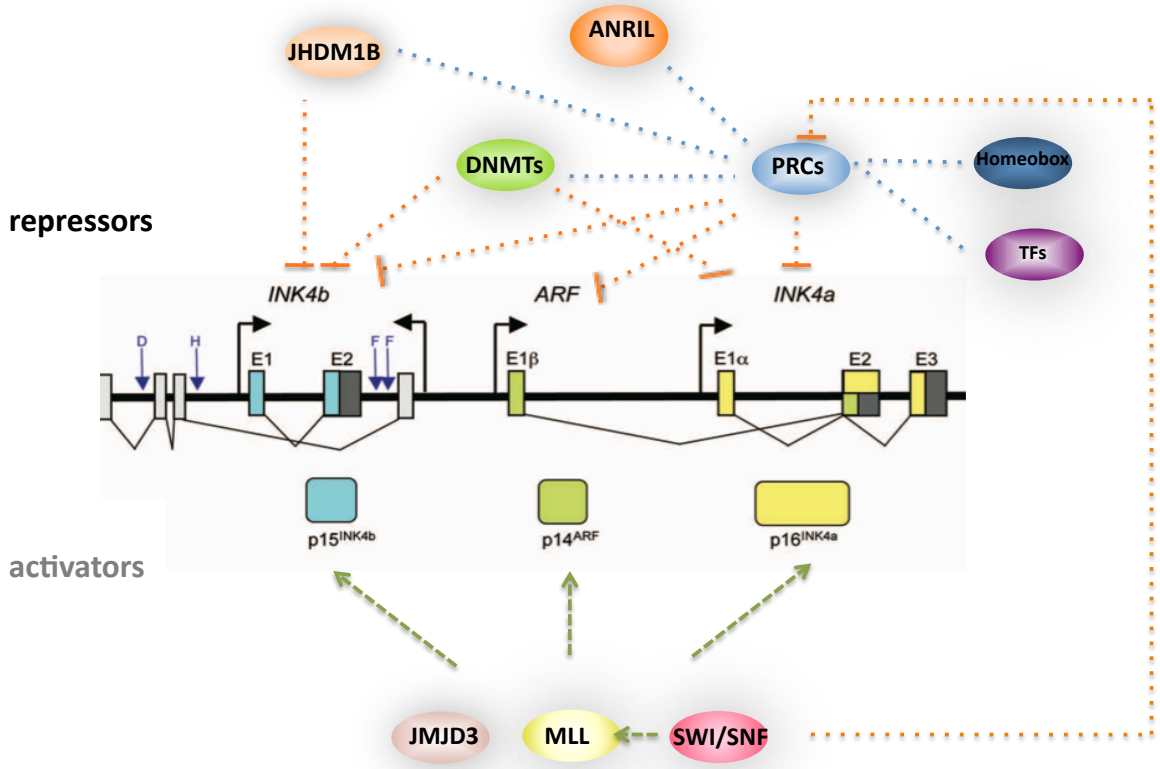


Figure 8. Epigenetic regulation of the *INK4/ARF* locus. The *INK4/ARF* locus encodes for the CDK inhibitors, p15 and p16 as well as ARF and, in young cells, is normally under epigenetic repression mediated by a plethora of regulators. The best described mechanism of epigenetic repression of the *INK4/ARF* locus is that mediated by Polycomb proteins that regulate the formation of the chromatin repressive mark H3K27me3 (by PRC2) and H2A119ub (PRC1), leading to transcriptional repression. Polycomb proteins are recruited to the *INK4/ARF* locus through several mechanisms: binding to homeobox proteins, anchoring to transcription factors (TF) (*i.e.* CDC6) and tethering by long non-coding RNAs (*i.e.* ANRIL). Additional negative regulators of the *INK4/ARF* locus include DNA methyltransferases –DNMTs- (*i.e.* DNMT3B) and histone demethylases (*i.e.* JHDM1B). In old or stressed cells PRC2 member EZH2 is downregulated and Polycomb repression is progressively released. This is reinforced by action of several transcriptional activators, such as the H3K27me3 demethylase JMJD3, the H3K4me methylating complex MLL and the chromatin remodeling complex SWI/SNF that competes with Polycomb proteins for promoter binding (Image adapted from Popov and Gil, 2010).

1.1.5.2 Chromatin and structural changes in the nuclei

As part of the senescence program, cells undergo global chromatin reorganization (**Figure 9**). Specifically when compared with proliferating cells, senescent cells display DAPI-dense foci that correspond to areas of heterochromatin and are known as SAHF (senescence-associated heterochromatin foci) (Narita et al., 2003). In 2003 Narita and co-workers saw that cells undergoing senescence

presented chromatin foci with characteristics of heterochromatin: enrichment for H3K9me3 and HP1 (heterochromatin protein) and depletion of H3K4me and H3K9ac (Narita et al., 2003). Additionally, these foci were seen to form upon pRb/p16 signaling and include repressed E2F target genes such as *cyclin A*, for what they have been considered essential in the establishment of the cell cycle arrest during senescence (Narita et al., 2003). Following work has helped clarifying the formation and composition of the SAHF. Senescence-associated heterochromatin foci additionally include the high mobility group A proteins, HMGA1 and HMGA2, which are key for SAHF formation as well as the histone variant macroH2A, that is necessary for their maintenance (**Figure 9**) (Narita et al., 2006; Zhang et al., 2005). SAHF formation is a multi-step process that involves recruitment of histone chaperones ASF1a (antisilencing function 1a) and HIRA (histone repressor A), members of the HUCA H3.3 chaperone complex, which undergoes a previous and vital incorporation step into PML bodies, before SAHF formation (Ye et al., 2007; Zhang et al., 2005). PML (promyelocytic leukemia) is a tumour suppressor that is upregulated in senescence and nucleates into nuclear bodies to regulate p53 post-translational activation and function and recruitment of pRb/E2F complexes leading to abolishment of E2F target genes expression (Bischof et al., 2002; Ferbeyre, 2002; Pearson and Pelicci, 2001; Vernier et al., 2011).

Each SAHF is thought to represent a single chromosome and recent work has described its multilayer nature, with rearrangement of repressive histone marks H3K9me3 and H3K27me3 into non-overlapping structural layers (**Figure 9**) (Chandra et al., 2012; Funayama et al., 2006; Zhang et al., 2007). While heterochromatin foci become more visible during senescence, the overall levels of heterochromatin actually decrease as cells enter senescence (Chandra et al., 2015; De Cecco et al., 2013; Swanson et al., 2013). Indeed, two recent works showed that bona fide areas of constitutive heterochromatin such as centromeric and peri-centromeric chromatin become open during senescence leading to satellite expression and transcription of transposable elements (De Cecco et al., 2013; Swanson et al., 2013). Thus, rather than from *de novo* heterochromatinization events, SAHFs result from spatial repositioning of pre-existing repressive chromatin areas which become loose due to loss of lamina-

associated heterochromatin (lamina-associated domains, LADs) (Chandra et al., 2012; Chandra et al., 2015).

Despite the initial reports that SAHFs participated in the pRb/p16^{INK4A} tumour suppressive pathway leading to repression of pro-proliferative genes (Narita et al., 2003, Narita et al., 2006), recent studies suggest that SAHF are a consequence of Ras activation and can persist even after resuming proliferation with p53 depletion (Di Micco et al., 2011). The authors suggested that instead, SAHF had a role in restraining DDR signaling and contribute for tumorigenesis by not allowing local amplification of signals.

In addition to changes in compaction, senescence encompasses changes in chromatin composition. Specifically, some histone variants are depleted from the senescent chromatin. That is the case of the linker histone H1 which is lost during senescence, what is thought to allow HMGA2 recruitment into the SAHFs (Funayama et al., 2006). Additional work has shown that histone H3 and H4 biosynthesis is globally decreased during senescence (O'Sullivan et al., 2010). Moreover, lysosomal processing of budded-off-nuclei chromatin was observed during senescence suggesting a complete make-over of the chromatin landscape during senescence (Ivanov et al., 2013). Although the relevance of such dramatic event is still to be elucidated, it seemed to be accompanied by loss of lamin B and nuclear disintegration (Ivanov et al., 2013).

Lamin B1 is a component of the nuclear lamina that together with other B-type lamins (lamin B2) and A-type lamins (lamin A and C) have functions in nuclear envelope structure and mechanics, chromatin organization and transcription, among other roles (Vlcek and Foisner, 2007). Nuclear envelope perturbations are characteristic of progeria syndromes, such as the Hutchison–Gilford progeria syndrome (HGPS) which is caused by accumulation of a mutated form of lamin A (progerin) (Eriksson et al., 2003). Progerin accumulation leads to accelerated telomere shortening and premature senescence in human fibroblasts and progressive heterochromatin loss (Huang et al., 2008; Shumaker et al., 2006). In the last years several reports showed that also lamin B1 is downregulated in senescence (Dreesen et al., 2013; Freund et al., 2012; Sadaie et al., 2013; Shah et al., 2013; Shimi et al., 2011). Lamin B1 is depleted particularly from H3K9me3 enriched regions leading to loss of peri-nuclear chromatin, what is thought to contribute to SAHF formation upon clustering of constitutive heterochromatic

regions (Chandra et al., 2012; Chandra and Narita, 2013; Sadaie et al., 2013). Despite the overall loss of lamin B1, new lamin B1-associated domains (LADs) are formed with enlarged H3K4me3 and H3K27me3-enriched regions. Loss of large H3K27me3 areas also takes place upon lamin B1 depletion, what correlates with upregulation of senescence-associated genes (*i.e.* SASP, cell cycle genes) (Shah et al., 2013). Whereas most published works suggest that lamin B1 depletion induces senescence, additional publications on the matter report that lamin B1 overexpression triggers a senescent phenotype in primary fibroblasts, for what the specific contribution of different lamin B1 levels for senescence is still on the way to be understood (Barascu et al., 2012; Dreesen et al., 2013).

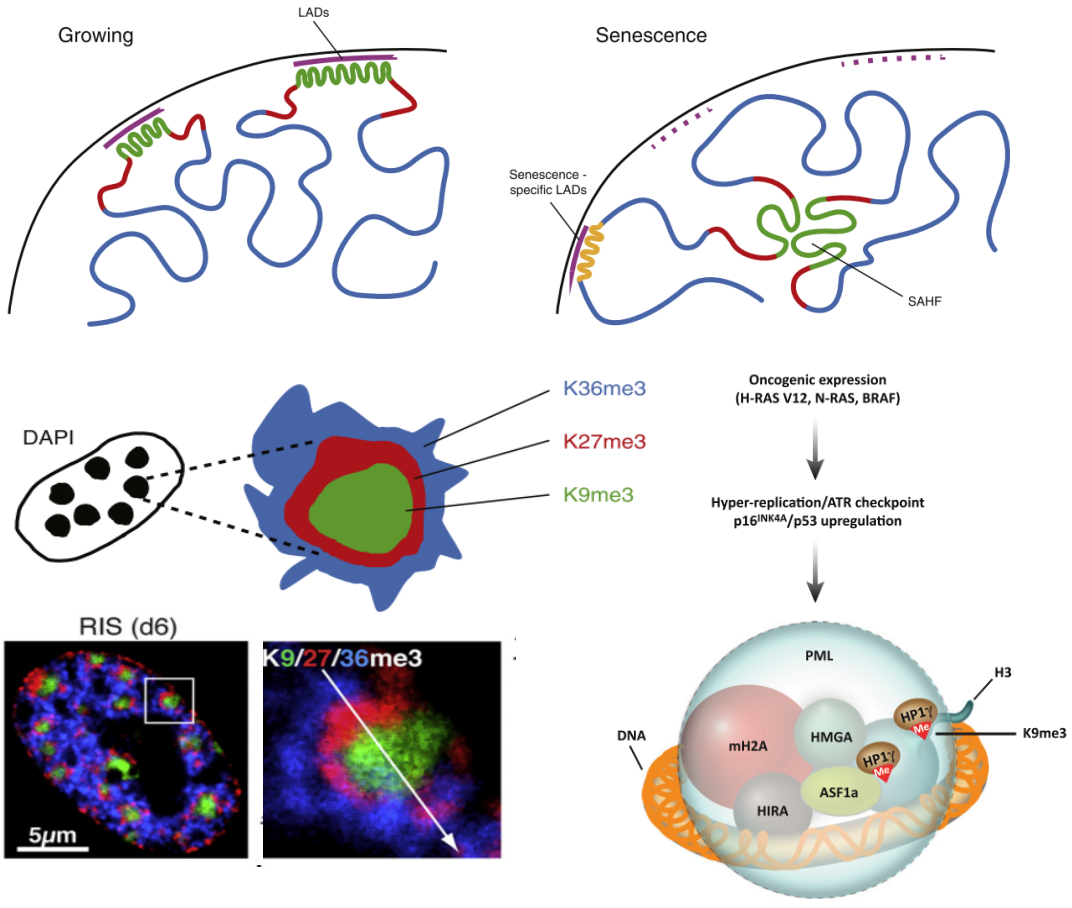


Figure 9. Chromatin structural rearrangement during senescence (SAHF). During senescence the contacts between constitutive heterochromatin (mainly H3K9me3 enriched areas) and the nuclear lamina are progressively lost leading to structural rearrangement of the chromatin and clustering of heterochromatic regions potentiating SAHFs (senescence-associated heterochromatin foci) formation. SAHFs are organized in layers of different types of chromatin, with constitutive heterochromatin (cHC, enriched for H3K9me3) in the center, followed by a layer of facultative chromatine (fHC) enriched for H3K27me3 and finally surrounded by a sheet of euchromatin (K3K36me3). SAHFs are enriched for several proteins such as HP1, macroH2A and HMGA1 and 2 and its formation is dependent on the action of histone chaperones HIRA and ASF1a. (O'Sullivan and Karlseder, 2012).

1.1.6 Senescence *in vivo*: health and disease

Despite being arrested, senescent cells are metabolically active and produce a variety of secreted factors. The senescent secretome not only reinforces senescence autonomously as it also acts in a paracrine manner affecting surrounding cells (Kuilman and Peeper, 2009). Senescence can occur at several stages of an individual's life cycle contributing to wider biological processes.

The recent discovery that senescence is developmentally programmed suggested that senescence is not just associated with pathology and stress, but is also an important developmental process (Munoz-Espin et al., 2013; Storer et al., 2013). Indeed, ablation of senescence in murine embryos led to visible developmental defects, with impact on fertility (Munoz-Espin et al., 2013). In terms of gene expression, senescence in the embryo, largely overlaps with oncogene-induced senescence, but lacks activation of major OIS pathways, such as DDR/p53 and p16/pRb, relying on TGFβ/ SMAD and PI3K/FOXO mediated upregulation of p21 (Munoz-Espin et al., 2013; Storer et al., 2013). Thus, it is possible that due to the controlled nature of development, a "controlled" senescence is triggered. During pathological scenarios such as telomere attrition or oncogene-activation, adult cells "remember" the embryonic senescence but engage more powerful venues to stall proliferation of damaged cells. An interesting observation of the studies by Keyes' and Serrano's labs was the temporary nature of embryonic senescence, occurring between E11.5 and E14.5. Senescent cells secrete factors that resemble those of "adult" senescent cells, what attracts macrophages that deplete the senescent population (Munoz-Espin et al., 2013; Storer et al., 2013). These studies therefore concluded that embryonic senescence could serve two roles: eliminating structures through macrophage-dependent clearance, for proper development as well as and achieving an accurate balance between cell populations (Munoz-Espin et al., 2013).

In the adult, physiologically controlled senescence was reported to occur in two cell types megakaryocytes and placental syncytiotrophoblasts (Munoz-Espin and Serrano, 2014). In addition senescence is associated with multiple pathologies such as aging and associated diseases and cancer. The observation that benign lesions were highly populated by senescent cells catapulted senescence to fame

as a potent tumour suppressor mechanism (Collado et al, 2005.) Thus, by blocking the cell cycle, senescent cells are able to restrict the spreading of damaged cells. But the beneficial aspects of senescence go beyond their ability to stop proliferation. Indeed, probably the most relevant feature of senescence, in the context of a multi-cellular environment, is the SASP. Not only the SASP helps stalling damaged cells by reinforcing senescence autonomously, as it spreads senescence to the neighboring and potentially damaged cells (Acosta et al., 2008; Acosta et al., 2013; Hoare and Narita, 2013; Kuilman et al., 2008). An additional beneficial effect of the SASP is its ability to modulate the immune system to clear off senescent cells what is known as “senescence surveillance” (Acosta et al., 2013a; Chien et al., 2011; Iannello et al., 2013; Kang et al., 2011; Lujambio et al., 2013; Rakhra et al., 2010; Xue et al., 2007). This has been elegantly demonstrated in murine models not only in the context of pre- and malignant liver tumours and T-cell lymphoma and B-cell leukemia, as well as in the context of liver fibrosis derived from damaged hepatic stellate cells (Iannello et al., 2013; Lujambio et al., 2013; Xue et al., 2007). Both the innate and adaptative immune system play a role in senescence surveillance, with NK cells, neutrophils, CD4+ T-cell lymphocytes and macrophages being attracted to senescent cells due to the secretion of specific factors, such as CSF1, CCL2, IL1α and IL6 and IFNγ, respectively, amongst others. Interestingly, recruitment of an appropriate immune response capable of clearing senescent cells appeared dependent on p53 integrity (Iannello et al., 2013; Lujambio et al., 2013; Xue et al., 2007). An immune system-mediated clearance of senescent cells is desirable and one of the aims of therapy induced senescence (TIS) for cancer treatment (Nardella et al., 2011). However, we are still in the beginning of the journey towards a full understanding of how and what drives the immune system to target senescent cells. Also, the heterogeneity of senescence and the SASP, and the fact that the process in murine models might slightly differ from that in humans, may provide some difficulties for the clinical application of senescence surveillance (van Deursen, 2014).

Senescence also plays a beneficial role in restricting the atherosclerotic plates, hypertension and wound healing of skin as well as in limiting, renal cardiac and liver fibrosis (Munoz-Espin and Serrano, 2014). Specifically, senescence is activated in hepatic stellate cells (HSC) as a consequence of accumulated

damage due to uncontrolled proliferation. These cells expressing SA-β-gal and other senescence-associated markers ultimately accumulate in the periphery of the scar (Krizhanovsky et al., 2008). The effects of senescence go beyond limiting the spread of the fibrotic tissue, as senescent cells induce a state of immunesurveillance, activating macrophages and subsequent elimination of the damaged cells (Lujambio et al., 2013).

Despite being traditionally regarded as an anti-tumorigenic process, senescence has also detrimental effects on surrounding cells. That should not come as a surprise as some of the secreted factors have roles in tumorigenesis, such as IL6 or the metalloproteinase-3 (MMP3) (Ancrile et al., 2007; Parrinello et al., 2005). Indeed it has long been shown that senescent cells can, by means of the secreted factors they produce, promote or reinforce tumorigenesis of adjacent pre- and malignant cells (Krtolica et al., 2001). This proinflammatory network of secreted factors can shift into a more pro-tumorigenic force once key senescence mediators such as p53 are inactivated (Coppe et al., 2008; Lujambio et al., 2013; Pribluda et al., 2013). Thus, senescence has a dark side in tumorigenesis as well, intrinsically associated with its secretory phenotype (Coppe et al., 2010).

Additional age-related conditions in which senescent cells have been shown to accumulate and aggravate the phenotype are sarcopenia (degenerative loss of muscular mass), type 2 diabetes, obesity and pulmonary fibrosis (Munoz-Espin and Serrano, 2014). An ingenious study recently showed how drug-induced clearance of p16 positive cells from a murine model of accelerated ageing ($BubR1^{h/h}$) could alleviate several age associated diseases (Baker et al., 2011).

The “good-bad” dichotomy of senescence might lie on the distinction between acute and chronic senescence. Thus in acute situations such as developmental senescence or senescence of pre-malignant tumours in young tissues, where the immune system is not compromised, senescence seems to play an important role in tissue homeostasis, while senescence due to chronic damage, normally associated with age with concomitant decrease in immune system function might contribute for tumorigenesis and debilitating diseases (**Figure 10**) (Munoz-Espin and Serrano, 2014; van Deursen, 2014). Therefore the accumulation of senescence in specific tissues along time should be monitored for better understanding its role in the most various processes. Some studies have already

tried to monitor senescence using for example a luciferase knock-in mouse model to follow p16 expression along time and shown its accumulation in pre-neoplastic lesions (Burd et al., 2013). Additional approaches should now emerge to monitor accumulation of senescent cells by assessing additional features such as the SASP and its correlation with overall immune system health.

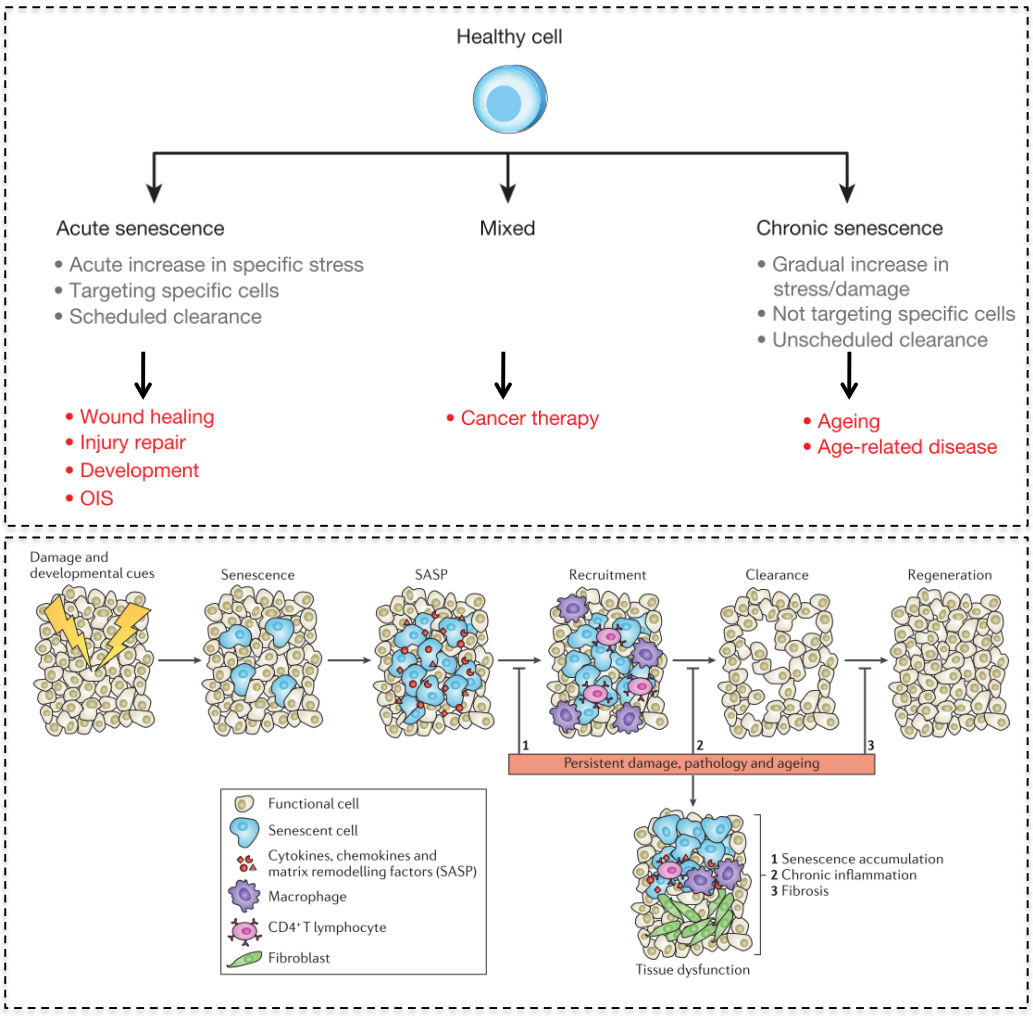


Figure 10. Senescence and its biological implications. Senescent cells accumulate *in vivo* during several stages of life due to programmed processes or random stressful events. In young, unstressed tissues senescent cells are thought to accumulate due to acute stresses and serve an important role in several biological processes from embryonic development, wound healing and fibrosis. Due to their ability to attract the immune system, senescent cells are thought to be ultimately cleared by the immune system leading to tissue regeneration and homeostasis. Additionally owing to their properties (*i.e.* SASP, high metabolism) senescent cells offer great opportunities for anti-cancer therapeutical approaches. However senescent cells also accumulate along time due to chronic damage, for what there are not perceived by the immune system, whose fitness tends to decline with age. Failure with senescent cells clearance or tissue regeneration leads to persistent accumulation of senescent cells what can contribute for chronic inflammation and fibrosis (Adapted from Munoz-Espin and Serrano, 2014 and van Deursen, 2014).

1.2 Large-scale approaches to discover regulators of cancer and senescence

As technology develops, the knowledge of the gene networks expressed during specific biological scenarios is growing, leading to data overload but also to a better and detailed understanding of biological processes and diseases (*i.e.* cancer or senescence). The integrative view of gene networks and their functionality have been made possible by outstanding technological milestones such as the complete characterization of the human genome or the development of RNA-sequencing and microarray technology, allowing an insight into the complete transcriptomes (Lizardi et al., 2011; Mohr and Perrimon, 2012; Zanella et al., 2010). Indeed, these technologies have proved valuable in the characterization of alternative splicing, lncRNAs and gene profiling in both cancer and senescence (Abdelmohsen et al., 2013; Beer et al., 2002; Eswaran et al., 2013; Nelson et al., 2014; Shelton et al., 1999; Yoon et al., 2004; Zhang et al., 2003). The development of proteomics came to reinforce the spectrum of large-scale approaches to unravel genes with actual functional relevance, and has also been successfully applied for cancer and senescence research (Aan et al., 2013; Acosta et al., 2013a; Acosta et al., 2013b; Benvenuti et al., 2002). Although the unveiling of transcriptomes, proteomes and metabolomes, set the basis for a functional approach of cellular processes it did not per se shed light on the specific function of the observed expression changes. This came to change with the development of both RNAi technology and high-throughput microscopy and screenings, allowing for a more detailed functional analysis of genome-wide gene sets in more complex biological scenarios, (*i.e. in vivo*) (**Figure 11**) (Zanella et al., 2010). RNAi and compound-based screenings have contributed to identify novel regulators of senescence and associated pathways such as p53, p16/Rb and the SASP (Acosta et al., 2013a; Berns et al., 2004; Bishop et al., 2010; Lahtela et al., 2013; Rovillain et al., 2011). An exciting area is that of *in vivo screens* where the RNAi technology is used directly in model animals to target genes and observe their function in a physiological background. These studies were first carried out by ex vivo infection of cells with shRNAs previous to their implantation in mice, however studies from the Fuchs and Zender labs, in 2013, pioneered the direct *in vivo* usage of RNAi

technology to identify regulators of epidermal and liver oncogenic growth and regeneration, respectively (Beronja et al., 2013; Wuestefeld et al., 2013; Zender et al., 2008).

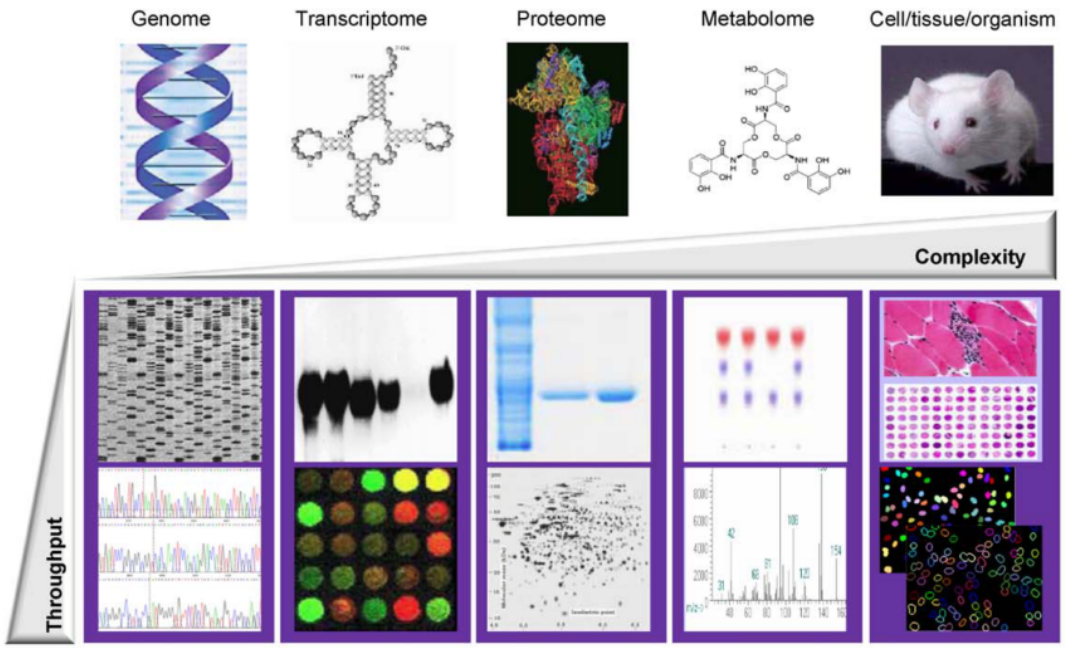


Figure 11. High-throughput approaches allow for detailed analysis of complex phenotypes. Technological development has allowed a deeper understanding of the gene networks expressed during specific biological processes, through the study of DNA sequences, transcriptomes, proteomes and metabolomes. The development of high –throughput technologies such as powerful high-throughput microscopes allowed the detailed study of complex phenotypes and generation of extensive descriptive data but more importantly to a functional insight into how specific cellular features are regulated by intricate networks of genes (Zanella et al., 2010).

1.2.1 siRNA technology for regulating gene expression

The concept of RNAi was first propagated in the early nineties, with the first miRNA being described in *Caenorhabditis elegans* (Carthew and Sontheimer, 2009). miRNAs are small polyadenylated RNA molecules transcribed by the RNA Pol II that, when processed through the interfering RNA (RNAi) pathway, have the capacity of regulating the expression of endogenous mRNAs, ultimately leading to translational repression or target degradation (Bartel et al., 2004). The final outcome of miRNA targeting depends on its complementarity with the 3'-end of the

mRNA. Because miRNAs are only partially complementary, most mRNAs end up being regulated at the translational level (Bartel et al., 2004).

Soon after the discovery of miRNAs another class of double stranded small RNAs (dsRNA) was described to efficiently target gene expression through the RNAi pathway (Fire et al. 1998). Although discovered through exogenous injection of C. elegans, these 20-25 nucleotide long RNA molecules were found to arise endogenously as anti-sense transcripts of specific mRNAs (Carthew and Sontheimer, 2009; Fire et al., 1998; Golden et al., 2008). However, in mammalian cells they originate mainly externally from viral infections (Jackson and Linsley, 2010). Since they arose against very specific targets, siRNAs are therefore highly complementary to their pair mRNAs and extremely efficient in targeting them for cleavage-mediated degradation (Tomari and Zamore, 2005). The observation of RNAi in several animal models led to the fine-tuning of synthetic siRNAs transduction of mammalian cells and booming of the RNAi technology in human research (Elbashir et al., 2001a; Elbashir et al., 2001b).

The RNAi pathway encompasses multiple steps for full processing of mature miRNA and siRNAs (Rao et al., 2009b). Inside the cell the dsRNA is first cleaved into a smaller double stranded RNA molecule with a 3'-end overhang (21-22 nucleotides). This process takes place in the cytoplasm and is mediated by Dicer, an RNA Pol III-related endonuclease in a complex with TRBP (Tat RNA-binding protein) or PACT (PKR activating protein) (Rao et al., 2009b). Although this step does not appear strictly necessary it improves the silencing properties of the siRNA (Murchison et al., 2005). The double stranded siRNA/Dicer/TRBP complex subsequently associates with Ago2, an endonuclease from the Argonaute family that cleaves the passenger strand of the double stranded molecule (Kim et al., 2007; Matranga et al., 2005; Rand et al., 2005). The degradation of the passenger strand converts the pre-RISC (RNA-induced silencing complex) into the holo-RISC (Kim et al., 2007). Once the holo-RISC complex is loaded, the single stranded RNA guides Ago2 towards target mRNAs leading to their degradation due to its endonucleolytic activity (Hutvagner and Zamore, 2002; Yekta et al., 2004). The argonaute family of proteins comprises 3 additional members able to bind the RISC complex: Ago1, 3 and 4, none of them with endonuclease-like activity (Hock and Meister, 2008). Instead, complexes containing these argonaute proteins bind to partially complementary sites located in the 3' UTRs of target mRNAs leading to

translation inhibition or deadenylation (removal of poly(A) tail) of the mRNA and consequent destabilization, most probably occurring in P-bodies (Hock and Meister, 2008).

Due to the transient nature of the siRNA, there was a need of creating a method to achieve sustained RNAi. Brummelkamp and colleagues generated the first vector-based RNAi technology for mammalian cells and since then the field has been improving (Brummelkamp, 2002). shRNAs are inserted into the cells via viral infection and subsequently expressed via RNA Pol II or III-mediated transcription, forming a hairpin like stem-loop structure. While in the nucleus the hairpin is processed by a complex containing the RNase III enzyme Drosha and the double-stranded RNA-binding domain protein DGCR8 in order to produce a stem-loop with a 2 nucleotide- 3' overhang (Lee et al., 2003; Zhang et al., 2002). Exportin 5-mediated translocation into the cytoplasm occurs, where, through association with the previously described DICER/TRBP/PACT complex, the shRNA is processed into a linear double stranded RNA and loaded into an Ago2 containing pre-RISC (Rao et al., 2009b; Yi et al., 2003).

Second generation shRNAs (shRNA-mirs) have been created making use of the increasing knowledge of miRNA biogenesis (Dickins et al., 2005; Silva et al., 2005; Zeng et al., 2005). Specifically these shRNA-mirs were designed on top of the endogenous miRNA mir30 backbone, and their processing has been biochemically characterized as to allow for prediction of the mature shRNA product, which consists of a 22 bp sequence (Silva et al., 2005). Additionally, these vector based shRNAs include a strong promoter, originally the promoter of the RNA Pol III target U6 snRNA, however have the advantage of allowing incorporation of alternative Pol II promoters, including inducible ones (Dickins et al., 2005). Finally, these vectors included a 60 bp barcode sequence for shRNA tracking (Silva et al., 2005).

Although mechanistically identical in terms of target silencing, siRNA and shRNA technologies present some differences inherent to the nature of the precursor molecules, conferring advantages or disadvantages such as specific and nonspecific off-target effects due to partial complementarity with additional mRNAs and stimulation of the immune system, respectively (Falschlehner et al., 2010; Rao et al., 2009b). Indeed, both siRNA and shRNA sharing identical sequences have been shown to silence overlapping off-target transcripts, due to partial

complementarity lying of the so called “seed region”, the first 1-8 nucleotides of the siRNA molecule (Jackson et al., 2006b). However, shRNAs have advantages such as the durability of the effect and possibility of an inducible application and for yielding less off-target effects (Klinghoffer et al., 2010; Rao et al., 2009a). Indeed, while siRNAs are directly introduced into the cells, second-generation shRNAs are produced by the cells through the miRNA pathway, undergoing polyadenylation. For this reason shRNAs tend to be more stable and efficient (Li et al., 2006b; Schwarz et al., 2003; Siolas et al., 2005). Additionally the lack of stability renders siRNAs more susceptible to cytoplasmic degradation and production of smaller molecules likely to silence off-target mRNAs (Rao et al., 2009b). Another advantage of shRNAs is their sustained ability to silence target mRNAs, whereas siRNAs achieve it transiently. In spite of the advantages of using the vector-based approach, siRNAs confer specific advantages as well. The easiness of use is very attractive when compared to shRNA-based strategies, which take longer. Additionally, shRNAs use the endogenous miRNA pathway, which may lead to over saturation of components of the RNAi pathway, such as the exportin 5, affecting the efficacy of endogenous miRNA processing (Grimm and Kay, 2007; Grimm et al., 2006). In fact both RNAi approaches have been successfully and widely used to identify novel regulators of apoptosis, senescence and cancer (Acosta et al., 2008; Berns et al., 2004; Bishop et al., 2010; Luo et al., 2009; MacKeigan et al., 2005; Meacham et al., 2009; Schlabach et al., 2008; Wuestefeld et al., 2013; Zender et al., 2008). Of note, several studies are in progress to assess the use of RNAi in cancer therapy and have been reviewed elsewhere (Guo et al., 2013).

In conclusion, the RNAi technology is nowadays a powerful tool that allows for the functional characterization of the effect of genes in mammalian cells.

Chapter 2. Material and methods

2.1 Cell lines and Tissue culture methods

HEK-293T and IMR-90 cells were obtained from the American Type Culture Collection (ATCC). Both cell types were cultured in Dulbecco's modified Eagle's Medium (DMEM) from Invitrogen and supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Sigma) as well as 1% antibiotic-antimycotic solution (Invitrogen), hereafter referred to as "complete", and grown in an humid incubator at 37 ^{o}C and 5% CO_2. Treatment with trypsin (Invitrogen) was used to detach cells from culture plates. Inducible ER:RAS and ER:MEK cells, were treated with 100nM 4-Hydroxytamoxifen (4-OHT) (Sigma) prepared in DMSO 1 day after plating in order to induce senescence (Acosta et al., 2008; Barradas et al., 2009). Cell Viability and cell number were estimated by flow cytometry. To that end, 50 µl of cell suspension were diluted in 150 µl of Guava ViaCount reagent (Millipore) and applied to the Guava Cytometer (Millipore) for cell measurements.

2.2 Plasmids

pLNC-ER:RAS and pLNC-MEK:ER were used to generate the inducible RAS^{G12V} and MEK1 over expressing cells, respectively and pLXSN used as the empty vector. pGIPZ-based shRNA vectors were purchased from Sigma. For a complete list of plasmids, respective resistances and short hairpins sequences used see Table A2 and Table A3, respectively.

2.3 Transformation of competent E. coli and plasmid DNA purification

In order to amplify plasmid DNA 1 µl of plasmid was transferred into a tube together with chemically competent DH5αTM. The mix was incubated on ice for 30 min, then brought to 42 ^{o}C for 1 min and placed on ice again. The bacteria were then diluted in antibiotic free LB and grown for an hour at 37 ^{o}C with constant shaking. 50-100 µl of the competent bacteria were then plated onto an agar plate with appropriate selective antibiotic and grown overnight at 37 ^{o}C. A single colony

was then picked 16 hours after and grown overnight in 250 ml of antibiotic supplemented media. LB was used for plasmids with ampicillin or kanamycin resistance and low salt LB for plasmids encoding a Zeocin resistance gene. Double selection with Ampicilin and Zeocin was used for pGIPZ plasmids. Approximately 16 h after the bacteria cultures were centrifuged at 6000 rpm for 10 min. The supernatant was discarded and the pellet (bacteria) was collected for further processing. The HiSpeed® Plasmid Purification kit from QIAGEN was used to extract the plasmid DNA. Briefly, it consisted of ressuspending the bacteria pellet in Buffer P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA) supplemented with 100 µg mL^{-1} RNase A. For alkaline lysis of bacteria and denaturing of DNA Buffer P2 (200 mM NaOH, 1% SDS (*w*/*v*)) was added and after mixing neutralization occurred with P3 (3.0 M KAc, pH 5.5) that allowed the precipitation of detergent, proteins and genomic DNA, easily discarded from the solution by centrifugation and filtration. Buffer P3 facilitated the renaturing of the plasmid DNA that was subsequently passed though a column containing a resin with binding properties. The plasmid DNA bound to the column and was washed from impurities with buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (*v*/*v*)). Elution of plasmid DNA occurred with Buffer QF (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% isopropanol (*v*/*v*)) and addition of isopropanol to the eluate led to precipitation of the molecules. After binding the DNA to a new column, it was washed with ethanol and the plasmid DNA was eluted with buffer TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The concentration was determined by measuring the absorbance at 260 nm (A_{260}) in a NanoDrop® ND-1000 UV-Vis spectrophotometer.

2.4 Production of Retrovirus and infection of target cells

HEK293T cells (packaging cells) were seeded at least one day prior to transfection in order to achieve an approximate confluence of 80%. A transfection mix containing 1 ml of plain DMEM, 20 µg of expression plasmid, 2 µg of the VSV-G plasmid (encoding the G protein of the Vesicular Stomatitis Virus, envelope gene), 8 µg of gag-pol plasmid (encoding capsid proteins (gag) and the reverse transcriptase and integrase proteins (pol)) and 75 µl of linear 25 kDa linear polyethylenimine (PEI; 1 mg/ml (w/v), Polysciences) was prepared. 30 min post

preparation, the mix was added to the HEK293T freshly fed with 9 ml of complete DMEM, with extreme care not detach target cells. Approximate transfection efficiency was assessed by monitoring, after 24 h, the fluorescence of control cells transfected with a reporter plasmid expressing mCherry fluorescent protein. In order to concentrate the viral content, the media of the packaging cells was changed to 6ml of fresh complete DMEM 24 h post transfection. In parallel, virus-recipient IMR90 cells were plated at a density of 10^6 cells per 10 cm dish. 48 h post transfection the virus containing supernatant was collected and filtered through a 0.45 µm pore filter (Anachem). A round of infection consisted of replacing the media of target cells by the viral supernatant supplemented with 4 µg/ml of Polybrene and feeding the transfected HEK293T cells with 6 ml of fresh complete DMEM to continue virus production. 3 rounds of infection as described were performed per plasmid, taking place 3 hours apart from each other. The cells were left incubating with the viral media of the 3rd round overnight, to maximize infection efficiency, and after approximately 16 h the viral media was replaced by fresh complete DMEM and the cells were grown until confluent. To assess infection efficiency, the fluorescence of mCherry cells was monitored using the Guava Cytometer or the IN Cell Analyzer 2000 and compared to that of uninfected cells (negative control). Selection of infected cells was performed for approximately 5 days in media supplemented with 1 µg/ml Puromycin or approximately 2 weeks in complete media supplemented with 400 µg/ml G418 (Neomycin).

2.5 Production of Lentivirus and infection of target cells

HEK293T cells (packaging cells) were seeded at least one day prior to transfection in order to achieve an approximate confluence of 80%. The transfection mix consisting of 1 ml of plain DMEM, 10 µg of expression plasmid, 2 µg of the VSV-G plasmid, 8 µg of the psPax2 plasmid (packaging) and 75 µl PEI was prepared and added to the cells 30 min post preparation. Approximate transfection efficiency was assessed by monitoring the expression of the Green Fluorescent Protein (GFP) encoded by the pGIPZ plasmids. 24 h post transfection the media of packaging cells was replaced by 6 ml of fresh complete DMEM and IMR90 cells were plated for infection as before. The target cells were infected, 24 h post

seeding with a 1:4 dilution of the filtered viral supernatant supplemented with 4 µg/ ml Polybrene. 4 hours after infection, the media of the infected IMR90 cells was replaced with fresh complete DMEM and the cells were grown until confluent. To assess infection efficiency, GFP expression by infected cells was monitored using the Guava Cytometer or the IN Cell Analyzer 2000 and compared to that of uninfected cells (negative control). Selection of infected cells was performed as previously described.

2.6 Cell growth assays

2.6.1 BrdU

2 x 10^3 cells/ well were seeded onto a 96 well plate in duplicate or triplicate. The day before washing the cells with PBS and fixing the plate with 4% paraformaldehyde (PFA) (w/v) for approximately 20 min, the cells were treated with 5-Bromo-2′-deoxyuridine (BrdU, 50 µM) for 16-20 h. The BrdU incorporation by the target cells was visualized by immunofluorescence.

2.6.2 Growth curves

5 x 10^5 cells were plated per 10 cm dish and cultured for approximately 7 days after which cell number was estimated. Cells were trypsinized, centrifuged and ressuspended in Guava ViaCount reagent as previously described. Cell count was assessed by flow cytometry using the Guava flow cytometer (Millipore). The plating-count cycle was repeated every ±7 days until control cells reached replicative senescence.

2.6.3 Colony formation assays

15 to 25 x 10^4 cells/well were seeded per 6-well plate and 50 to 100 x 10^4 were plated per 10 cm dish. Once plates containing control cells were confluent all experimental plates were washed in PBS and fixed with 0.5% Gluteraldehyde (w/v) in PBS for 20 min. Fixed cells were then washed with water and stained with 0.2% crystal Violet (w/v).

2.7 Senescence Associated-β-galactosidase (SA-β-gal) Assay

Cells were plated in 10 cm dishes and in the case of IMR90 ER:RAS cells, induced with 100 nM 4-OHT 16-24 h post seeding. 6 days post induction cells were trypsinized, counted and plated onto 6 well plates at a density of $6x10^4$ cells/ well. After 48 h plates were washed with PBS and fixed with 0.5% Gluteraldehyde (w/v) for 15 min. To adjust the pH, cells were washed two times with PBS supplemented with 1 mM $MgCl_2$; pH6 (PBS/ $MgCl_2$), and then incubated with X-Gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside [Boehringer], 0.12 mM $K_3Fe[CN]_6$, 0.12 mM $K_4Fe[CN]_6$, 1 mM $MgCl_2$ in PBS at pH 6.0). Incubation occurred at 37 ^oC, generally overnight, with moderate shaking to avoid the formation of crystals. Once the cells were stained the solution was discarded and the plates were washed with PBS and stored at 4 ^oC in the dark until further use. To quantify SA-β-gal positive cells, an optic microscope was used (Olympus CKX41, supplied with a DP20 digital camera). Several representative pictures were taken and percentages were calculated from at least 100 cells.

2.8 Small interfering RNA (siRNA) Reverse Transfection

Each 96 well plate (master plate) contained 0.1 nmol of lyophilized siRNA per well (QIAGEN). siRNAs were ressuspended in RNAse free water and subsequently aliquoted across daughter plates, so that each contained 3.6 µl of a 1 µM siRNA suspension per well. Aliquoting procedure was performed using an automated handling system (Biomek). Before transfection, siRNAs were incubated for 30 min with 17.5 µl of a 3.5% Hiperfect solution (v/v) (QIAGEN) in plain DMEM. $2x10^3$ cells were then reverse transfected per well upon addition of 100 µl of a cell suspension containing $2x10^4$ cell/ml in complete media, leading to a final siRNA concentration of 30 nM per transfection. The cells were left to seed overnight and transfection media was replaced by complete media 16-18 h after transfection, supplemented with 4-OHT in the case of IMR90 ER:RAS cells. Media was changed every 3-4 days and cells were fixed 4 to 5 days post induction with 4-OHT for studying most markers or 8 days for analyzing IL8 expression. BrdU

anaylsis was performed as previously described. Transfection efficiency was evaluated by assessing fluorescence of a control siRNA, *siGlo* (Dharmacon), with fluorescent properties.

2.9 Protein analysis

2.9.1 Immunofluorescence

Cells seeded onto 96 well plates were washed with PBS and fixed with 4% PFA (w/v) for 20-30 min, washed again and kept in PBS at 4 ^{o}C until immunofluorescence. To permeabilize cells, the plates were treated with 0.2% Triton X-100 (v/v) in PBS for 5 min and then washed in PBS and blocked for 1 h with a blocking solution containing 1% BSA (w/v) (Sigma) and 0.2% Fish Skin Gelatin (v/v) (Sigma) in PBS. Primary antibodies were diluted in blocking solution and incubated for 30 min. For BrdU detection, a treatment with 0.5 U/ml DNAse and 1 mM $MgCl_2$ was performed in parallel to anti-BrdU antibody incubation. The plates were then thoroughly washed with PBS at least 3 times and then incubation with an Alexa-conjugated secondary antibody in blocking solution occurred for 30 min. After washing 3 times with PBS the nuclei were stained with 1 µM 4',6-diamidino-2-phenylindole (DAPI) for 15 min. Cells were kept in PBS until acquisition to avoid dehydration. For a comprehensive list of antibodies and dilutions used, see Table A1.

2.9.2 High Throughput Microscopy (HTM) and High Content Analysis (HCA)

To visualize the immunofluorescence staining High Throughput Microscopy (HTM) was performed using the IN Cell Analyzer 1000 or 2000 (GE Healthcare). The high throughput microscope was set to take pictures of several fields per well of a 96 well plate, for 1,2 or 3 wavelengths (in the case of a co-staining procedure) until images of at least 1000 cells were acquired (unless otherwise indicated). To process and quantify the expression of the probed epitopes, the InCell Investigator software (v1.7) was used. Cells were identified and nuclei segmented with basis on DAPI staining. Top-hat segmentation was used defining a minimum of 100 µm^2 for the nuclear area. A collar around the nucleus of 1 µm (or larger, for cytoplasmic

markers) was created to define the cell area. An average intensity numerical value was assigned per cell (for a given nuclear or cytoplasmic marker) by the software, and individual cell values were plotted on a chart. By defining a threshold intensity value, it was possible to categorize the cells into “positive” or “negative” for each marker and calculate the average percentage of positive cells per well for the probed protein. All sample images were analyzed under the same threshold filter (**Figure 12**). Alternatively the average nuclear or cytoplasmatic intensity (or the co-variance of the raw intensity value) was used. All antibodies were thoroughly analyzed to assure specificity of staining.

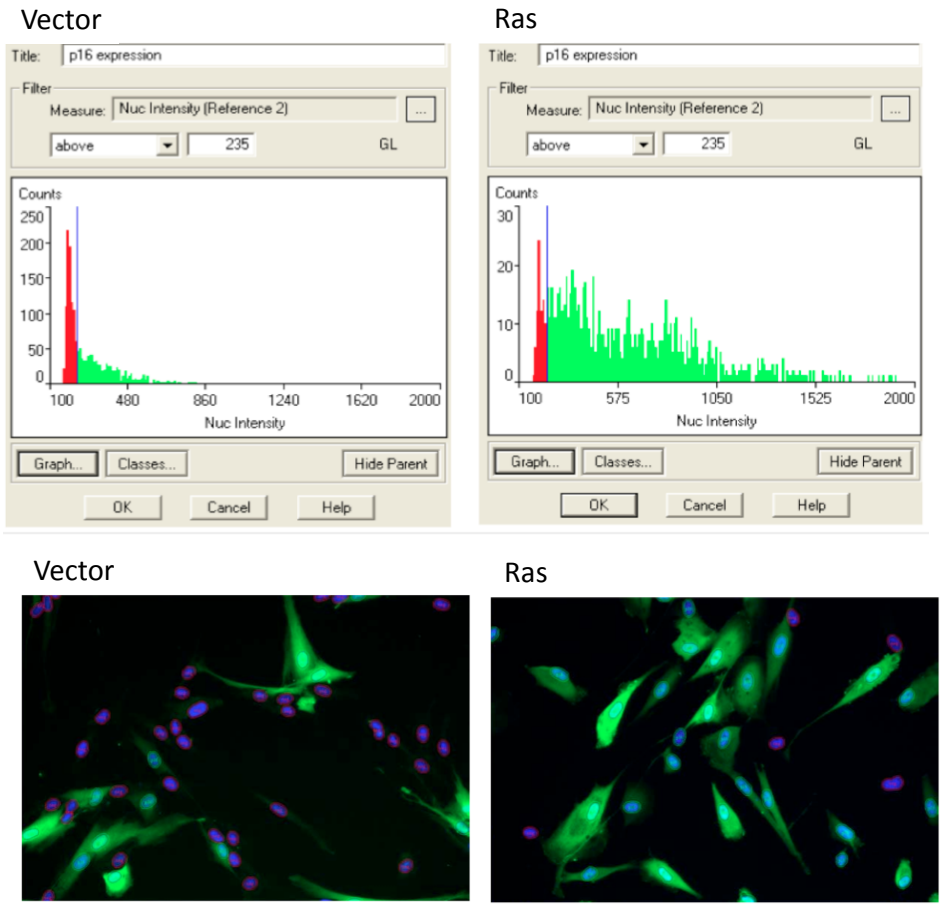


Figure 12. High content analysis for quantifying protein expression by immunofluorescence. p16 expression in IMR90 cells expressing constitutive RAS are shown as an example. Cells were seeded in 96-well plates, fixed at an appropriate time point and stained by immunofluorescence (IF) for p16 and DAPI (nucleus). Several IF images were subsequently acquired with the IN Cell Analyzer until 1000 cells are counted. For expression quantification, p16 intensity (Nuc intensity Reference 2) for individual cells is plotted on a histogram and a threshold filter is created to distinguish between cells expressing high (green labeled nuclei) or low levels (red labeled nuclei) of p16 and therefore calculate the percentage of p16 positive cells. The same as shown in here was used for calculating the expression of additional senescence markers, in cells undergoing different types of senescence as detailed in the results chapter (Image adapted from Banito et al., 2009).

2.9.3 Whole cell extracts

For whole cells extracts, confluent plates of cells were washed in cold PBS, scraped and pelleted by centrifugation. Cells were ressuspended in RIPA buffer (50 mM Tris pH8, 150 mM NaCl, 1% Triton X 100, 0.5% Na-Doc, 0.1% SDS, 1 mM EDTA) supplemented with protease inhibitors and incubated on ice for 30 min with occasional vortexing. Extracts were then centrifuged for 15 ^{o}C at top speed and the supernatant was transferred into a new clean tube (whole cell extract). Alternatively, cell pellet was ressuspended in 1x Laemmli buffer (60 mM Tris-Cl pH 6.8, 2% Sodium-dodecyl-sulphate (SDS, w/v), 10% glycerol (v/v), 5% β-mercaptoethanol (v/v), 0.01% bromophenol blue(w/v)) pre-heated at 95 ^{o}C. The cell suspension was boiled at 95 ^{o}C for 5 min, then passed through a 26 gauge needle 5 times and incubated at 95 ^{o}C for 5 min again.

2.9.4 Cellular Fractionation

Confluent plates of cells were trypsinized and passed through a 40 µm pores mesh (Falcon) to avoid clumps, pelleted by centrifugation, ressupended in cold PBS and pelleted again to wash off serum contaminants. To collect the cytoplasmic fraction the cells were ressuspended in a Hypotonic Solution (Nuclear extract Kit, Active motive) supplemented with *Phos-stop* (Roche), protease inhibitors (Roche), DTT and PMSF and incubated for 15 min on ice. The suspension was sampled throughout the procedure and visualized with Trypan blue (Sigma) under an optical microscope. The plasma membrane was then broken open with detergent (Nuclear extract Kit, Active motive) as suggested by the supplier. The suspension was subsequently centrifuged for 5 min at 3500 rpm and the supernatant (cytoplasm) transferred into a fresh tube. The pellet (nuclei) was ressupended in Hypotonic Solution and sampled to assess nuclear integrity. Another centrifugation took place to clean pellet from cytoplasmic contaminants. The nuclei were then ressupended in a buffer (Buffer B) containing EDTA and EGTA supplemented with *Phos-stop*, protease inhibitors and DTT and incubated on a shaking platform at 4 ^{o}C for 1-16 h. To collect the nucleoplasm the broken nuclei were centrifuged at 4000 rpm for 5 min, and the supernatant transferred into a clean tube (nucleoplasm). To clean from soluble contaminants, the pellet

(chromatin) was ressuspended in Buffer B and centrifuged again under the same conditions. The chromatin was then subjected to a DNAse treatment (2 U/µl DNAse I; 20 mM Tris7.5; 10 mM $MgCl_2$) for an hour on ice to release the proteins from the DNA.

2.9.5 Protein quantification

To quantify the protein content of whole cell lysates extracted with RIPA buffer or extracts resulting from cellular fractionation, the DC Protein Assay (BioRad) was used according to supplier directions. For proteins extracted with Laemmli buffer the RC DC Protein Assay (BioRad) was used due to the presence of substances incompatible with the use of the DC Protein Assay alone. Both assays were performed on a 96 well plate and absorbance was read at 660nm (A_{660}) on the Bio-Rad 680XR microplate reader. Several dilutions of BSA were used to plot a calibration curve.

2.9.6 Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Equal amount of protein extracts were diluted with 3x Laemmli buffer, boiled for 5min at 95^{o}C and loaded either onto a gradient Mini-PROTEAN® TGXTM Precast Gel (BioRad) or, in the case of samples for Mass Spectometry, onto a Criterion^{TM} TGX^{TM} Precast gel and ran in an appropriate electrophoresis tank. Electrophoresis was performed in running buffer (25 mM Tris, 190 mM Glycine, 0.1% SDS (w/v)) at 150 volts until an appropriate separation of the SeeBlue® Plus2 Pre-stained protein standard (Novex®) was achieved. In the case of LC–MS/MS the gel was subsequently stained with ProtoBlue Safe (National Diagnostics) for 16 h and extensively washed with distilled water for protein bands detection.

2.9.7 Western Blot

Proteins were subsequently transferred from the polyacrilalmide gel onto a Polyvinylidene (PVDF) membrane previously activated with methanol. Protein transfer occurred in transfer buffer (25 mM Tris, 190 mM Glycine, 20% methanol (v/v)), at constant 100V for an hour. Transfer efficiency was visualized by briefly staining the membrane with Ponceau S (Sigma). Membranes were blocked overnight with 5% fat milk (w/v) in PBST (PBS supplemented with 0.05% Tween 20 (v/v) (Sigma)) and primary antibodies were diluted in 1% fat milk (w/v) in PBST and incubated at room temperature for 2 hours. After primary antibody incubation, membranes were extensively washed with PBST and subsequently probed with a Horseradish Peroxidase conjugated (HRP) secondary antibody (Invitrogen) for 1 hour at room temperature. After extensive washes with PBST, membranes were transferred onto a cassette and probed with Enhanced Chemiluminescence (ECL) reagent (Amersham/GE Healthcare) and finally developed.

2.9.8 Identification of proteins by Mass Spectromerty

Mass spectrometry and peptide quantification analysis was performed by Pedro Cutillas and Peter Faull, in the Biomolecular Mass Spectrometry and Proteomics Laboratory (CSC). Gel bands were reduced with dithiothreitol, alkylated with iodoacetamide and then subjected to overnight trypsin digestion. The peptide extracts underwent a second round of trypsin digestion to minimize the number of missed cleavages. Peptide mixtures were analyzed using an UltiMate 3000 Rapid Separation LC, coupled to a LTQ-Orbitrap-Velos mass spectrometer (ThermoFisher Scientific). Separation was performed in a 3 hr gradient using a 50 cm Acclaim pepmap C18 column (ThermoFisher Scientific). Protein identification and quantification was performed using Maxquant v1.2.0.13, with the embedded Andromeda 1.2.0.0 search engine and the IPI_Human_v3.37 database. Generally a count of 3 or more peptides was used as significance threshold as well as a Macot score of 90. Relative enrichment of each protein per fraction was calculated and used to create a heat map. For that, protein abundance was first normalised

by dividing protein intensity for one protein by the sum of all protein intensities in a given condition (fraction). The normalised protein intensity (abundance) for technical replicates (triplicates) were then averaged. Normalised protein abundance (average) for a given protein was then divided by the maximum value for that protein across the conditions (fractions) being compared. These values were then coloured coded on a scale of 0-1 with 1 (dark blue) being the maximum normalised protein abundance condition.

2.10 RNA expression analysis

2.10.1 RNA Purification

Cells were plated on the same day and on collection day were washed with cold PBS, scraped off the plate and transferred into a fresh tube. 800 µl of TRIzol® Reagent (LifeTechnologies/ Thermo Fisher Scientific) were added to the cell pellets and the mixture was vortexed and frozen at -80 ºC overnight for proper homogenization. Once thawed 150 µl of chloroform was added and the mix was vortexed and centrifuged for 15 min for phase separation. The top RNA-enriched phase was then transferred into a fresh RNAse free tube, with extreme care not to perturb the interphase containing DNA. 70% ethanol was then added on a 1:1 ratio to the RNA suspension, mixed and then processed and extracted with the RNeasy Mini Kit (QIAGEN). Brieftly, the ethanol containing mixture was passed through a RNA binding silica-based membrane by brief centrifugation. The RNeasy column was first washed with 700 µl of RW1 buffer by centrifugation and then twice with 500 µl of the ethanol containing buffer RPE. Residual ethanol was then removed through centrifugation at top speed for 1 min. The RNA was finally eluted by passing RNAse free water through the column and collected into a fresh tube. RNA concentration was measured at 260 nm (A_{260}) using a NanoDrop® ND-1000 UV-Vis spectrophotometer.

2.10.2 Complementary DNA (cDNA) synthesis

All samples were processed in parallel using the Superscript II™ Reverse Transcriptase Kit. In summary, 2 µg of RNA of each sample were first mixed with Random Hexamers (50ng/µL N8080127, Invitrogen) and 10 mM dNTP mix (18427, Invitrogen) and heated at 65 ^{o}C for 5 min and subsequently chilled on ice. After a brief centrifugation step were added to the mix 5x First-Strand Buffer, 0.1 M DTT, 200 units of the SuperScript II Reverse Transcriptase (18064, Invitrogen) and RNAse free dH_2O. The contents were centrifuged again and then inserted into a thermocycler and cDNA conversion occurred by bringing the mixture to 25 °C for 10 min first and then 42 °C for 50 min. The reaction was inactivated by heating at 70 °C for 15 min.

2.10.3 Quantitative RT-PCR (RT-qPCR) Analysis

RT-qPCR reactions were performed on the CFX96TM Real-Time PCR Detection System (BioRad) using SYBR Green PCR Master Mix (Applied Biosystems). Gene expression data was normalized to Ribosomal protein S14 (RPS14) (a list of RT-qPCR primers used is given in Table A4

Chapter 3. Introducing the Model: RAS-induced senescence in primary fibroblasts

In 1995 Littlewood and colleagues described a c-Myc inducible model, taking full advantage of the estrogen receptor (ER) ligand-binding domain properties. By coupling the c-Myc gene to a mutated form of the ER ligand-binding domain, unable to respond to endogenous tamoxifen, they were capable of achieving ER:c-Myc over expression, by treating the cells with 4-OHT, to which endogenous ER is insensible to (Littlewood et al., 1995). Since then, the same technology has been used for other oncogenes such as the oncogenic H-RAS^{V12}, which has been widely used as a model of OIS (Acosta et al., 2013a; Barradas et al., 2009; Tarutani et al., 2003).

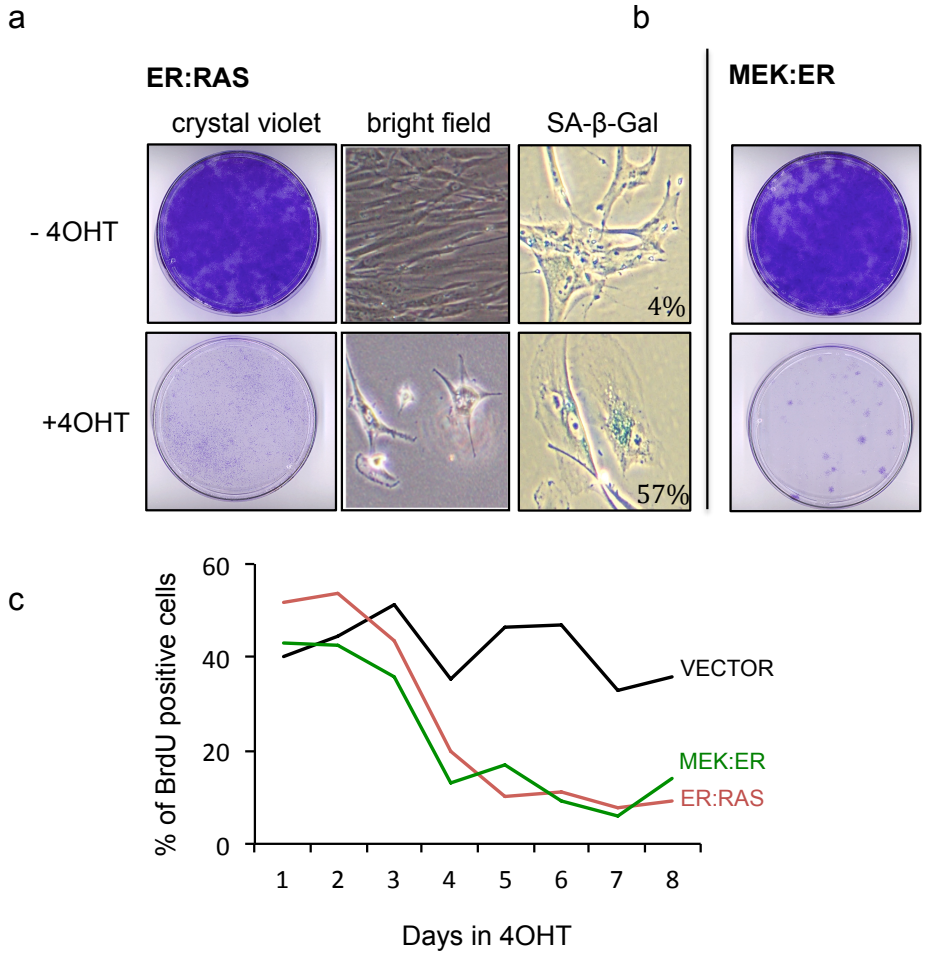


Figure 13. Acquisition of senescence features by primary fibroblasts upon H-RAS^{V12} activation with 4-OHT. a) HRAS^{V12} overexpression leads to decreased cell number as assessed by crystal violet staining, altered morphology and increased SA-β-Gal activity . b) Crystal violet staining showing cell cycle arrest upon MEK:ER overexpression. c) The decreased cell number corresponds to a decreased proliferation rate displayed by oncogene overexpressing cells.

The 4-OHT inducible ER:RAS oncogene was stably expressed in young IMR90 fibroblasts and, upon induction, cells exhibited senescence associated features: a stable cell cycle arrest was noticeable 4-5 days post induction with 4-OHT, IMR90 cells became flat and enlarged and expressed SA-β-galactosidase under suboptimal pH (**Figure 13a, b, c**). Expression of MEK:ER led to a cell cycle arrest similar to that of H-RAS^{V12} overexpressing IMR90 cells (**Figure 13b, c**).

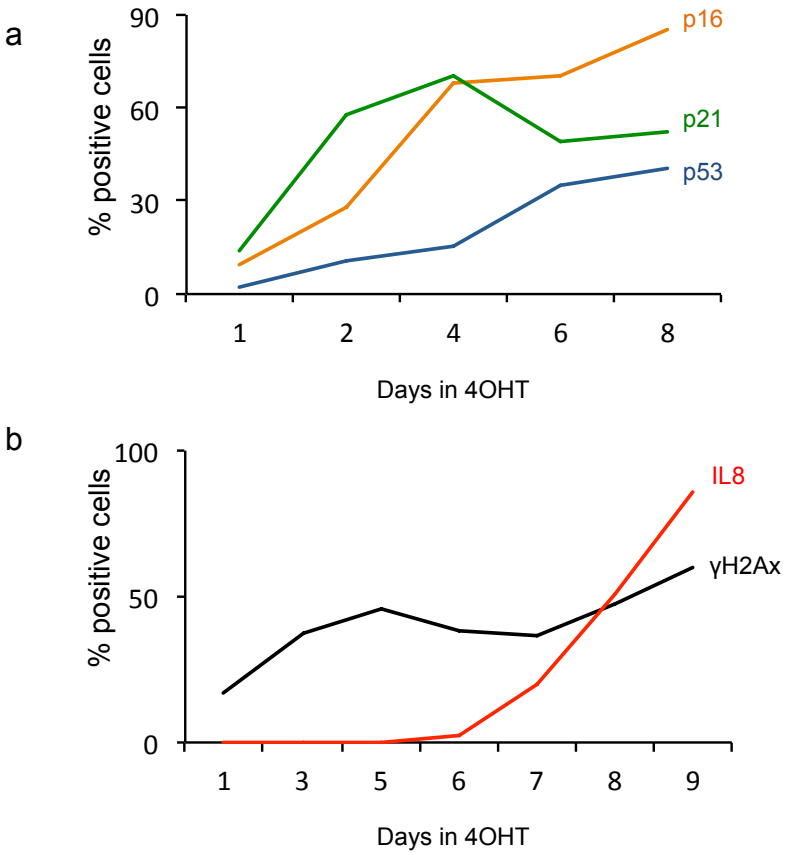


Figure 14. Expression of senescence-associated genes by IMR90 ER:RAS. IMR90 ER:RAS were induced with 4-OHT and sampled at the indicated time points. After fixation cells were stained by immunofluorescence and several pictures were taken using the IN Cell Analyzer 1000. Percentage of positive cells was calculated using the In Cell Investigator software. At least 500 cells were counted. a) ER:RAS overexpression activates tumour suppressor pathways. b) Induction of RAS expression with 4-OHT leads to accumulation of DNA damage, as measured by immunofluorescence against γH2Ax and production of proinflamatory cytokines (IL8).

Another hallmark of senescent cells is the activation of tumour suppressor pathways. As expected, treatment with 4-OHT led to the engagement of p16/Rb and p53/p21 pathways in IMR90 ER:RAS, which can be seen as soon as day 2 during the time course (**Figure 14a**). Additionally, by measuring the expression of markers such as γH2Ax or 53BP1, we could visualize the accumulation of DNA

damage along time, with the production of proinflammatory cytokines (SASP), such as IL8, escalading later on at around day 7 (SASP) (**Figure 14 b**).

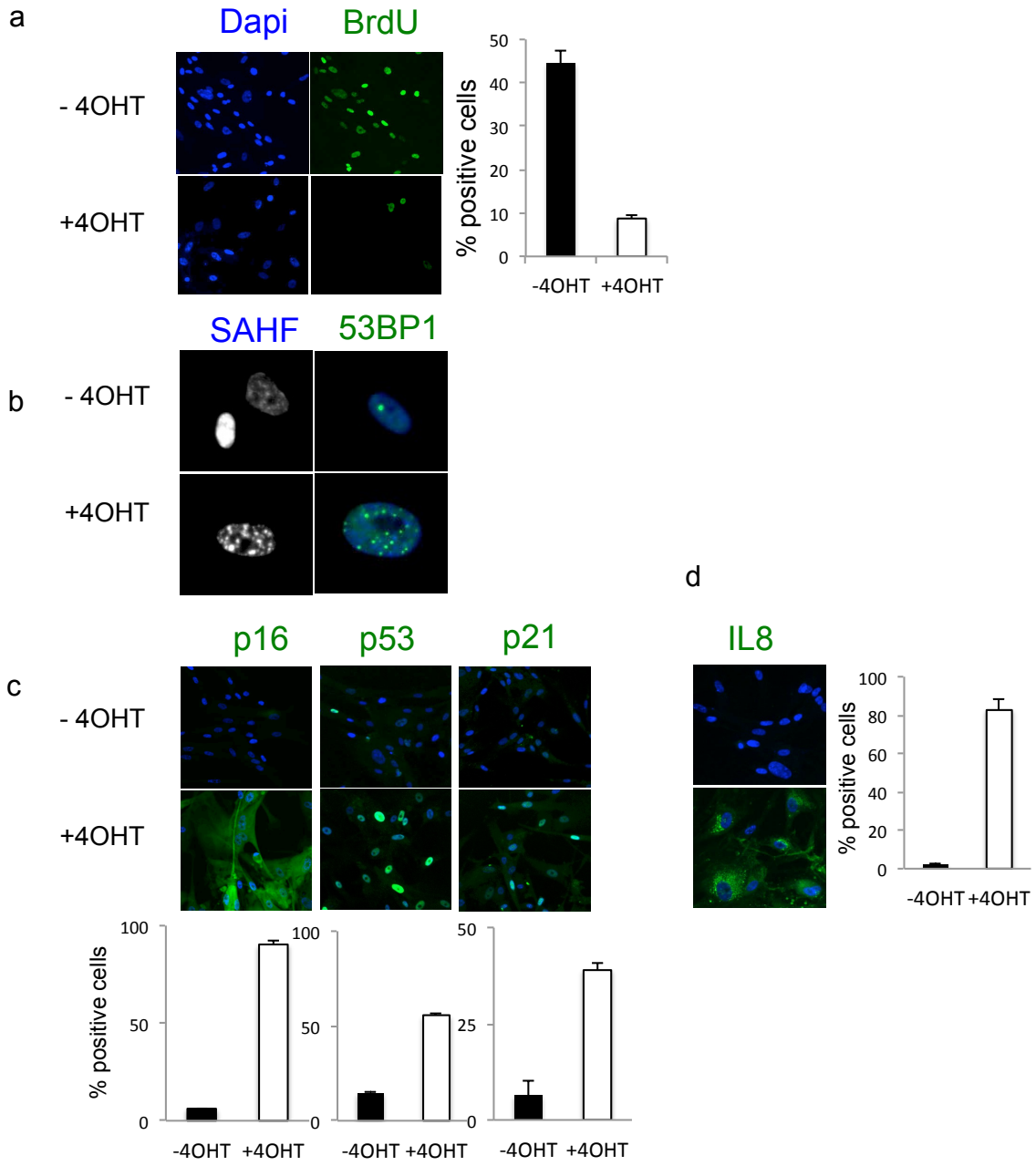


Figure 15. Representative immunofluorescence (IF) images and respective quantification data collected by HCA. IMR90 ER:RAS cells were fixed at day 6 and day 8 (for the SASP). a) Treatment of IMR90 ER:RAS with 4-OHT led to decreased BrdU incorporation as assed by IF using an anti-BrdU antibody. b) DAPI staining of senescent cells revealed the formation of heterochromatin foci (SAHF) (left) and increased DNA damage was assessed by IF against 53BP1. c) RAS overexpression led to increased expression of the tumour suppressor genes p16 and p53/ and p21. d) IF at day 8 post 4-OHT treatment revealed increased levels of IL8 (SASP)

The use of the IN Cell Analyzer allows for the high content analysis of senescence-associated markers. Once cells were stained with specific antibodies, thoroughly tested for specificity, the usage of a high throughput microscope permitted the quantification of the examined markers providing, in parallel, information about protein localization, thus consisting of a very powerful tool for protein analysis.
IMR90 ER:RAS cells were treated with 4-OHT and fixed for further analysis. Percentage of positive cells per marker was calculated from at least 1000 cells, unless otherwise indicated. Immunofluorescence combined with HCA allows the study of several aspects of the senescent phenotype: cell cycle arrest, heterochromatin rearrangement (SAHF), assessed by DAPI staining and DNA Damage Response (DDR) accumulation as depicted by increased number of 53BP1 foci (**Figure 15b**). The engagement of tumour suppressor pathways can also be visualized by IF (**Figure 15c**), as well as the production of proinflammatory cytokines (SASP), such as IL8 (**Figure 15d**).

In conclusion, the IMR90 ER:RAS model of OIS consists of a very robust system to study senescence triggered by activated oncogenes, as it recapitulates, in very short time, the major hallmarks of oncogene-induced senescence from activation of tumour suppressor pathways, the SASP and DDR to SAHF formation. This combined with specific antibodies and a powerful microscope and software permits the collection, in a relatively short time, of a large amount of data relative to the senescence-associated markers, of both qualitative and quantitative nature.

Chapter 4. Cataloguing the subcellular proteome of cells undergoing OIS

In order to catalogue and identify proteins differentially expressed, as well as changing subcellular localization during oncogene-induced senescence we conducted a proteomics analysis of cells undergoing OIS. To this end, growing and senescent human fibroblasts were fractionated into cytoplasm, nuclear soluble and insoluble fractions and the distribution of proteins in the different fractions was analyzed by mass spectrometry.

4.1 Subcellular fractionation of cells undergoing OIS

IMR90 ER:RAS cells were treated with 4-OHT for 9 days in order to induce oncogene-induced senescence. As a control IMR90 fibroblasts, infected with the empty vector (LSXN), referred to as IMR90 vector, were cultured in parallel and treated also with 4-OHT (hereafter referred to as “growing”). Confluent plates were collected for each condition and subjected to subcellular fractionation. Senescent and growing cells were tripsinized and filtered through a 40μm pore cell strainer to avoid clumps and washed several times in cold PBS. To purify the cytoplasm, cells were incubated with a detergent-based buffer, vortexed and centrifuged to pellet intact nuclei. The supernatant, containing cytoplasmic proteins, was collected and nuclei were disrupted via incubation in an EDTA-EGTA solution with continuous vortexing. The nuclear soluble fraction was separated from the nuclear insoluble pellet by centrifugation, and was treated with DNAse.

To assess the purity of the subcellular extracts of both senescent and growing fibroblasts, we performed immunoblotting and probed the collected fractions (nuclear insoluble, soluble, cytoplasm and whole cell extracts) with antibodies against proteins known to specifically localize to these subcellular compartments under analysis (**Figure 16a**).

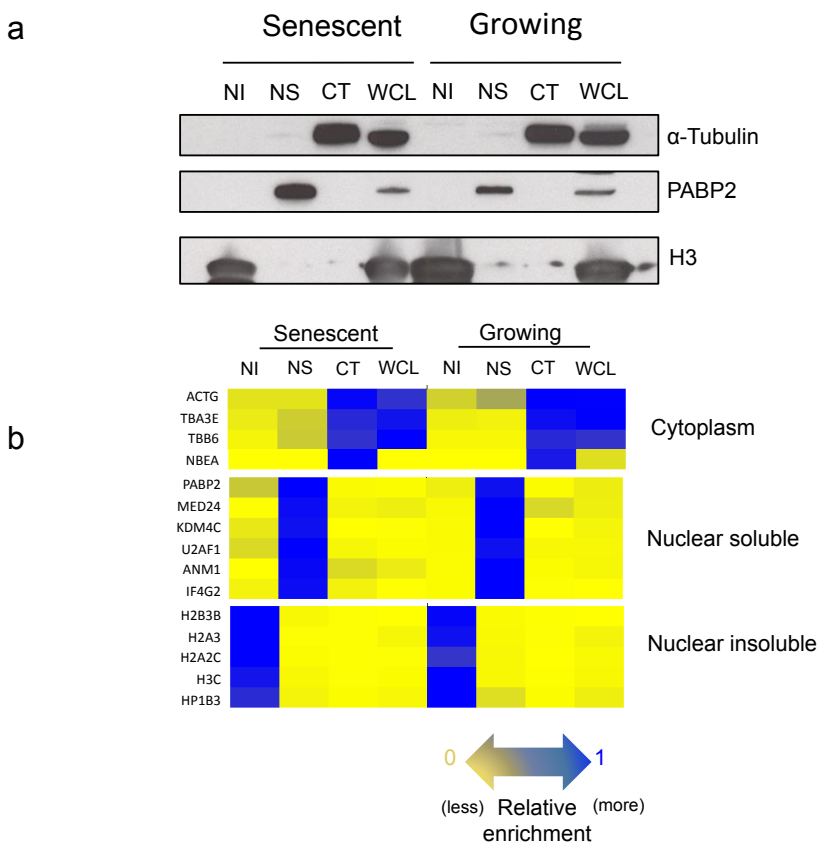


Figure 16. Subcellular fractionation of senescent and growing human primary fibroblasts. IMR90 ER:RAS and LXSN cells were treated with 4-OHT for 9 days after which they were pelleted and subjected to subcellular fractionation. **a)** Western blot against α-Tubulin, PABP2 and Histone H3, specific markers of the cytoplasm (CT), nuclear soluble (NS) and nuclear insoluble (NI) fractions, respectively. **b)** Heat-map for the same fractions analyzed by Mass Spectrometry. Displayed proteins localize to a specific fraction, suggesting the success of the fractionation technique. Values for relative protein enrichment per fraction range from zero (bright yellow) to 1 (dark blue). (WCL= whole cell lysates)

Alpha-tubulin, histone H3 and PABP2 were used as markers of the cytoplasm, nuclear insoluble (chromatin) and nuclear soluble fractions, respectively (**Figure 16a**). For further confirmation of the quality of the fractionation, we analyzed the relative enrichment per fraction of specific proteins, detected by mass spectrometry (**Figure 16b**). Cytoskeleton proteins such as Tubulin (TBA3E and TBB6) and actin (ACTG) and also a lysosomal trafficking regulator (NBEA) were enriched in the cytoplasm (**Figure 16b**). The detection of several variants of histones H2A, H2B and H3 as well as the heterochromatin protein HP1B3 on the chromatin samples suggested the purity of the fractions. As a nucleoplasm marker we looked into the proteins specifically enriched in the nuclear soluble fraction in our proteomics analysis. Several proteins with such a profile were identified (**Figure 16b**). As an example, we performed western blot against PABP2 and

observed that this protein was detected in the soluble fraction of both senescent and growing cells and also on the total extracts (**Figure 16a**). Altogether these experiments suggested the accuracy of the fractionation.

4.2 Changes in protein expression during OIS

Mass spectrometry analysis detected approximately 4,000 proteins. Relative protein enrichment per fraction was calculated and is shown on a heat map (**Figure 17**). Overall, the pattern of protein expression in the cytoplasm and nucleoplasm (nuclear soluble fraction) of senescent cells overlapped with that observed for the same fractions in proliferating cells, with only a few distinct groups of differentially expressed proteins (**Figure 17**

In contrast to the overall preserved pattern of expression of cytoplasmic and nuclear soluble proteins, a clearly distinct pattern of nuclear insoluble proteins between senescent and growing cells was detected (**Figure 17**). This preliminary result suggests a major rearrangement of the chromatin protein landscape during oncogene-induced senescence. These observations prompted us to perform a more detailed investigation of the protein expression changes in OIS. To this end, we focused mainly on two aspects: the study of proteins changing subcellular localization and the identification of the main clusters of proteins with altered expression during senescence.

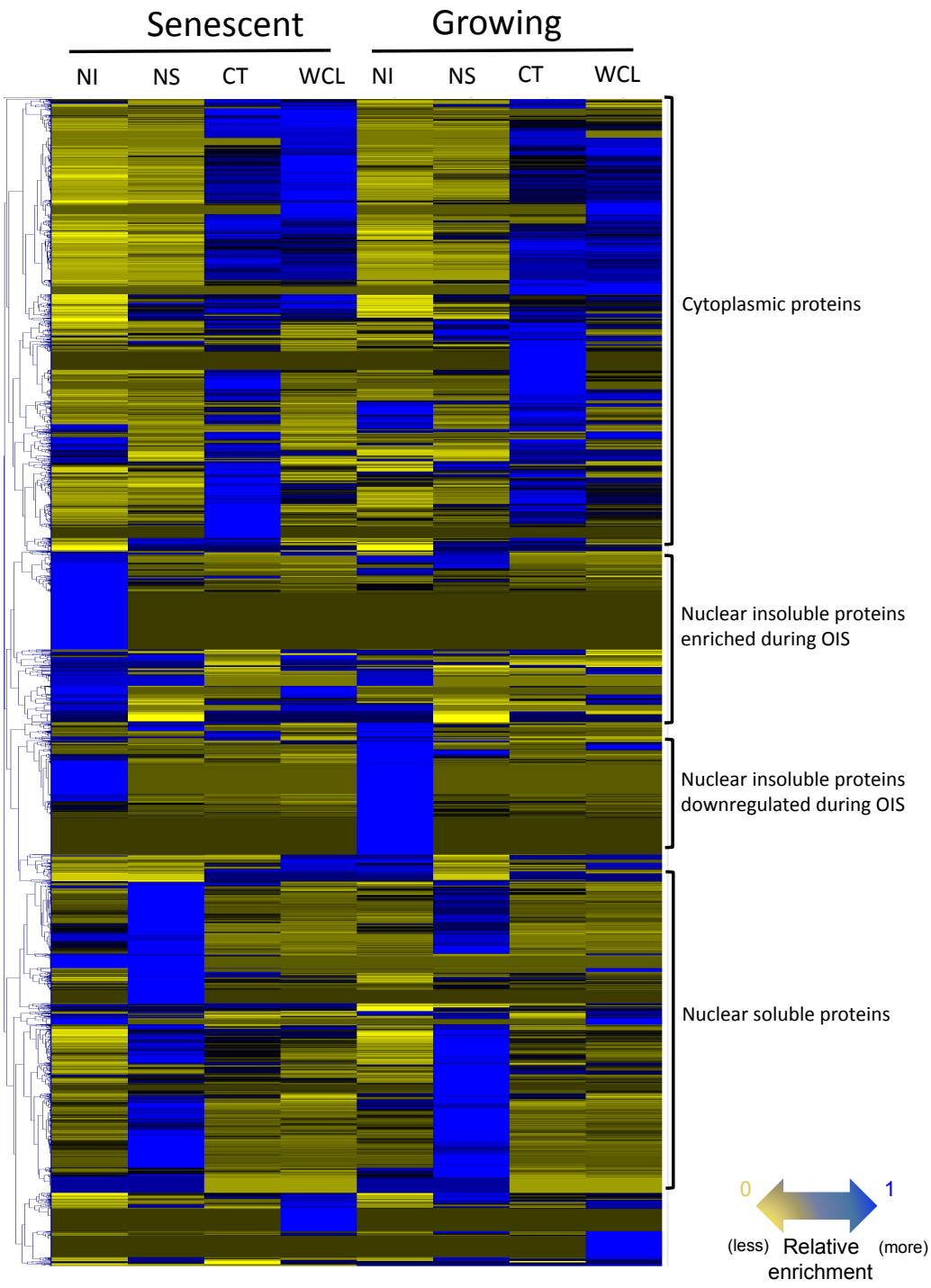


Figure 17. Mass spectrometry analysis of proteins from fractionated senescent and growing cells. Heat map of the relative enrichment per fraction of the 4000 proteins (aprox.) detected in the proteomics analysis. Senescent and growing cells display a similar pattern of enrichment for both cytoplasmic and nucleoplasmic (nuclear soluble) proteins. Conversely, the nuclear insoluble fractions of senescent and growing cells display major differences in terms of protein enrichment.

4.3 Proteins changing subcellular localization in senescence

Mass spectrometry analysis performed on fractionated cells allows the identification of proteins changing subcellular localization whilst not changing the overall levels of expression. This is as an advantage when compared to analysis of whole cell lysates, where the overall expression of proteins is considered and only events involving *de novo* synthesis of proteins or degradation are likely to be detected.

To identify proteins changing localization in senescence we compared the pattern of protein distribution along fractions in senescence with that observed in growing cells via calculation of the Pearson correlation (R) of the relative enrichments. The Pearson correlation is a measure of the strength of the linear relationship between two variables that can be positively correlated (R=1), not correlated (R=0) or negatively correlated (R=-1).

Since we were interested in proteins changing subcellular localization, we filtered all correlation values with an R>0.8 out. From these we selected only the proteins for what at least 3 peptides were detected and presented a Mascot score of >90. The Mascot score relates to the probability that the observed match is a random event (the higher the score, the lower the probability). A group of 287 proteins matched these criteria. We analyzed the expression of these proteins in both samples and grouped them according to their expression pattern in senescence and growing cells.

4.3.1 Factors shuttling from the nuclear soluble to the insoluble fraction during OIS

A group of proteins showed a pattern of distribution consistent with that of a re-localization from the soluble to the insoluble nuclear fraction during senescence (**Figure 18a**). While some of these proteins completely shifted from the soluble to the insoluble fraction, as for instance the arginine and glutamate-rich protein, ARGL1, others were mildly expressed in the nucleoplasm in growing cells (*i.e.* Leucine-Rich Repeats And IQ Motif Containing - LRIQ1). Within the group of proteins that migrate to the chromatin during senescence, we identified EP300, a

transcriptional co-activator previously shown to negatively regulate senescence (Yan et al., 2013). In order to understand the processes these proteins were involved in, we performed gene ontology (GO) analysis using DAVID (http://david.abcc.ncifcrf.gov/). This group of proteins was significantly associated with chromatin and nuclear related processes (**Figure 18b**). Additionally, proteins of this group were involved in DNA damage and repair. That was the case of RD23B and SSRP1 and SP16H, two proteins belonging to the FACT complex, that have been previously related with senescence (Safina et al., 2013). Interestingly, another protein belonging to a complex with a role in senescence was identified - SMCA5, a subunit of the SWI/SNF complex (Khursheed et al., 2013; Tu et al., 2013). Altogether, these results suggest that a subset of nucleoplasmatic proteins is likely recruited to the chromatin during senescence. The identification of known regulators of senescence in the group of proteins changing subcellular localization reinforces the idea that such changes might reflect a functional role.

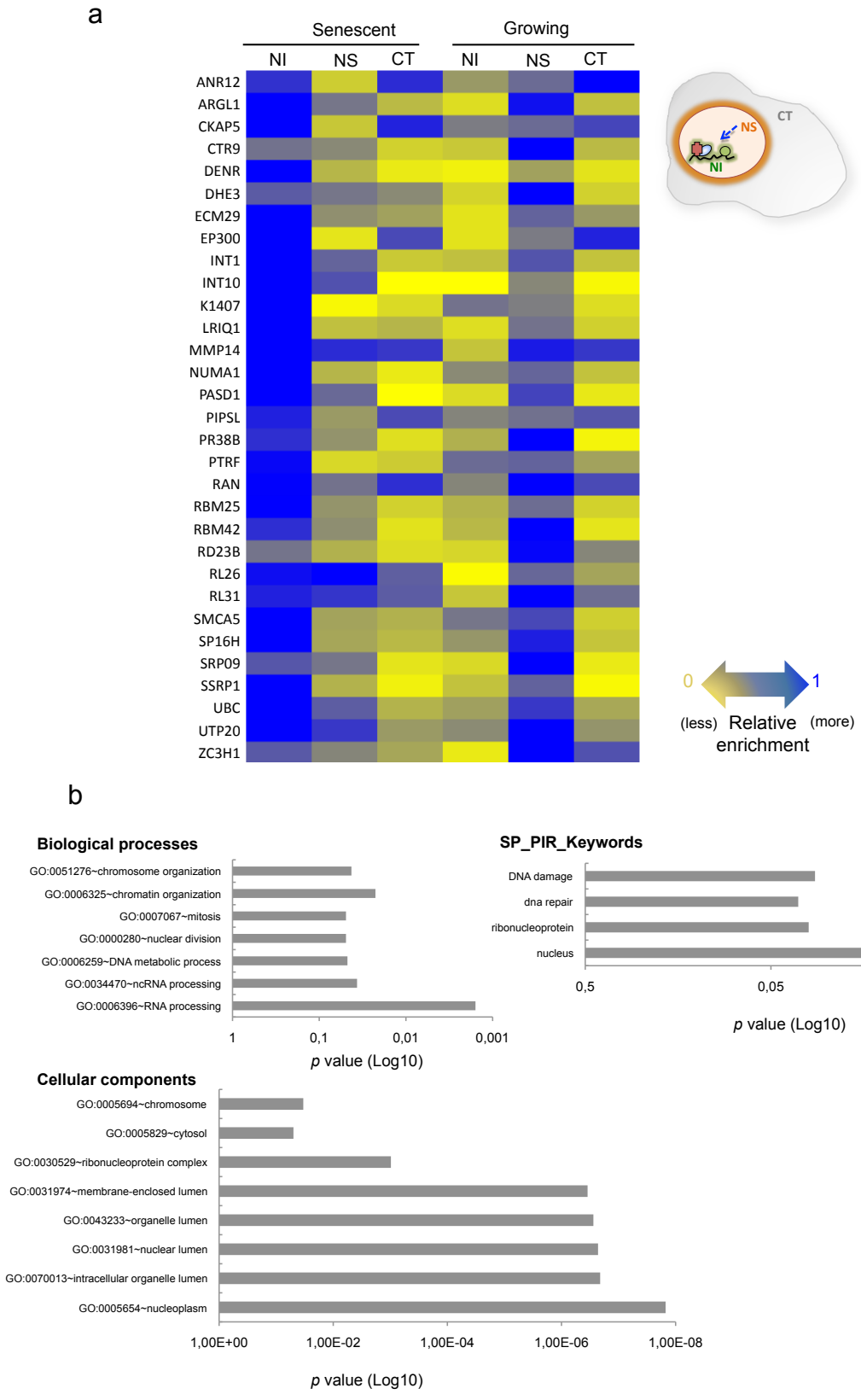


Figure 18. Several proteins that accumulate in the nuclear soluble fraction in proliferating cells move to the chromatin upon induction of senescence. a) Enrichment heat map for nuclear soluble proteins accumulating on the chromatin upon induction of senescence. b) GO annotations for proteins in a) show that proteins are involved in processes such as chromatin remodeling, RNA processing and DNA damage.

4.3.2 Proteins shuttling from the chromatin to the nuclear soluble fraction during OIS

Gene expression is a dynamic process that requires a fine balance between the recruitment and release of transcriptional activators and repressors from the DNA. In this sense it seemed appealing to us to investigate not only the proteins being recruited to the chromatin, but also the factors released from the chromatin upon induction of senescence (**Figure 19**). Gene ontology analysis suggested that this group of proteins was involved in RNA localization, processing and transport. Interestingly, three related proteins clustered together within this group, all showing a pattern consistent with a re-localization from the chromatin to the soluble pool of proteins in the nucleus (**Figure19a**). The Far Upstream element-Binding Proteins, FUBP1,FUBP2 and FUBP3 are known to have RNA binding properties and to be involved in mRNA translation, stabilization and processing and are overexpressed in cancer correlating with increased proliferation and migration of cancer cells. (Davis-Smyth et al., 1996; Malz et al., 2009; Weber et al., 2008; Zhang et al., 2013; Zhang and Chen, 2013). Interestingly, a role for FUBP1 in regulating p21 expression has been reported (Rabenhorst et al., 2009). Altogether, these results suggest that senescent cells might shift their RNA processing activity and that some of these proteins could potentially control senescence via regulation of mRNA stability.

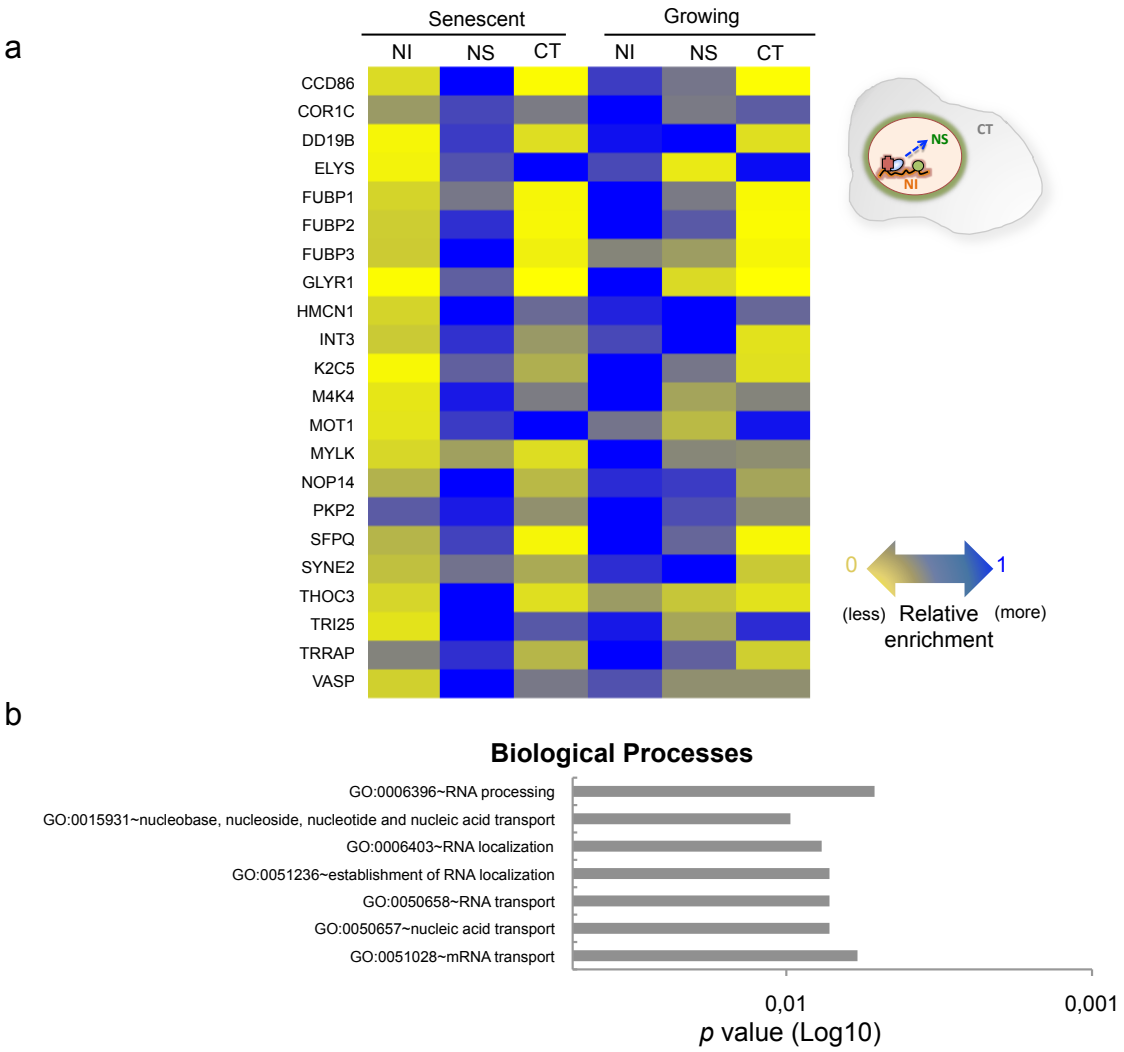
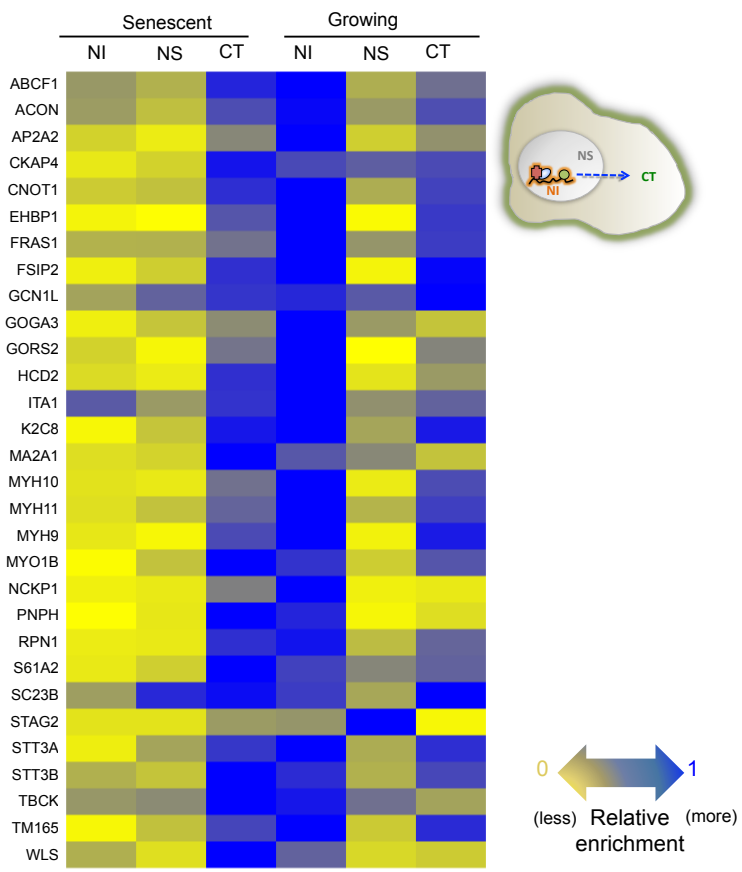


Figure 19 Proteins moving from the chromatin to the nuclear soluble fraction upon induction of senescence. a) Heat map for proteins that localize to the chromatin on proliferating cells and tend to accumulate on the soluble nuclear fraction during senescence. b) GO terms for proteins shown in a) relate with RNA procesing and transport.

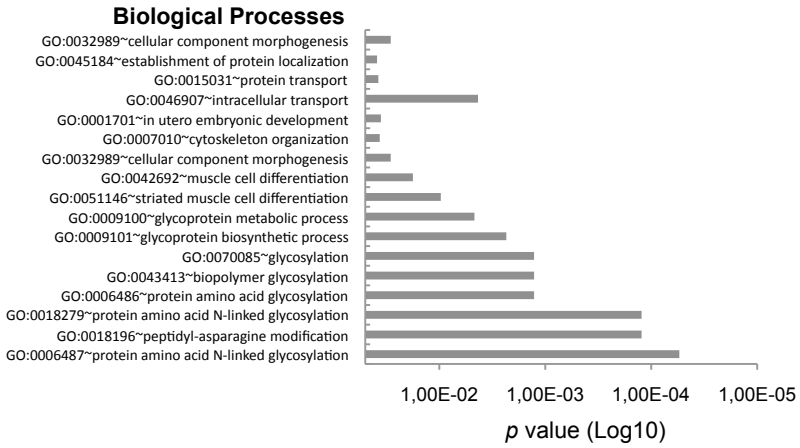
4.3.3 Proteins moving from the chromatin into the cytoplasm during OIS

We next focused on a group of proteins that localized to chromatin in proliferating cells, but relocalized to the cytoplasm during senescence (**Figure 20a).**

a


Senescent
Growing
NI
NS
CT
NI
NS
CT
ABCF1
ACON
AP2A2
CKAP4
CNOT1
EHBP1
FRAS1
FSIP2
GCN1L
GOGA3
GORS2
HCD2
ITA1
K2C8
MA2A1
MYH10
MYH11
MYH9
MYO1B
NCKP1
PNPH
RPN1
S61A2
SC23B
STAG2
STT3A
STT3B
TBCK
TM165
WLS
NS
NI
CT
0
1
(less) Relative (more)
enrichment

b


Biological Processes
GO:0032989~cellular component morphogenesis
GO:0045184~establishment of protein localization
GO:0015031~protein transport
GO:0046907~intracellular transport
GO:0001701~in utero embryonic development
GO:0007010~cytoskeleton organization
GO:0032989~cellular component morphogenesis
GO:0042692~muscle cell differentiation
GO:0051146~striated muscle cell differentiation
GO:0009100~glycoprotein metabolic process
GO:0009101~glycoprotein biosynthetic process
GO:0070085~glycosylation
GO:0043413~biopolymer glycosylation
GO:0006486~protein amino acid glycosylation
GO:0018279~protein amino acid N-linked glycosylation
GO:0018196~peptidyl-asparagine modification
GO:0006487~protein amino acid N-linked glycosylation
1,00E-02
1,00E-03
1,00E-04
1,00E-05
p value (Log10)

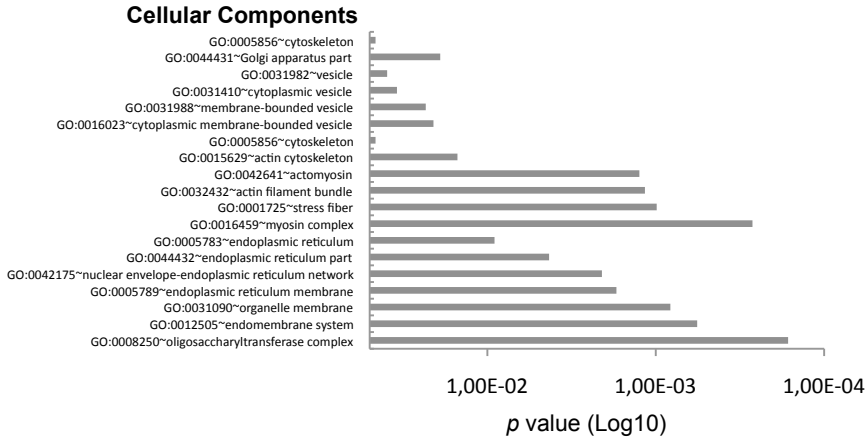

Cellular Components
GO:0005856~cytoskeleton
GO:0044431~Golgi apparatus part
GO:0031982~vesicle
GO:0031410~cytoplasmic vesicle
GO:0031988~membrane-bounded vesicle
GO:0016023~cytoplasmic membrane-bounded vesicle
GO:0005856~cytoskeleton
GO:0015629~actin cytoskeleton
GO:0042641~actomyosin
GO:0032432~actin filament bundle
GO:0001725~stress fiber
GO:0016459~myosin complex
GO:0005783~endoplasmic reticulum
GO:0044432~endoplasmic reticulum part
GO:0042175~nuclear envelope-endoplasmic reticulum network
GO:0005789~endoplasmic reticulum membrane
GO:0031090~organelle membrane
GO:0012505~endomembrane system
GO:0008250~oligosaccharyltransferase complex
1,00E-02
1,00E-03
1,00E-04
p value (Log10)

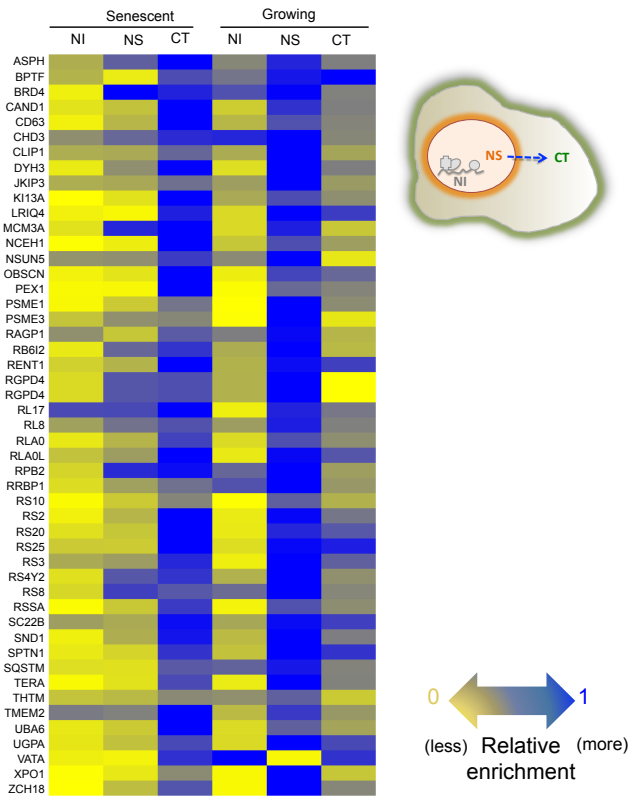
Figure 20. Proteins moving from the chromatin to the cytoplasm during senescence. a) Heat map for proteins that mainly accumulate in the chromatin on proliferating cells and acquire a cytoplasmic localization during senescence. b) GO terms for proteins in a) show proteins relate with functions associated with protein transport and processing, as for instance glysoslation.

GO annotations associated with this cluster included protein transport and protein glycosylation (**Figure 20b**). Glycosylation is a post-translation modification that takes place mainly in the endoplasmic reticulum and Golgi apparatus. Here we report the apparent release of proteins involved in glycosylation from the chromatin as cells undergo senescence. Histone glycosylation has been previously described, therefore the localization and release of these proteins from the chromatin could offer another level of regulation of gene expression (Fujiki et al., 2011; Sakabe et al., 2010).

4.3.4 Proteins shuttling from the nuclear soluble fraction to the cytoplasm during OIS

Next we analyzed the proteins moving from the soluble nuclear fraction to the cytoplasm (**Figure 21**). Most of the factors shuttling from the nucleoplasm to the cytoplasm were either ribosomal proteins (*e.g.* RS2) or ribosome binding proteins (*e.g*. RRBP1), possibly reflecting an increased protein synthesis, known to occur partly in the cytoplasm. Congruently, most GO terms associated with mRNA and protein transport and localization, as well as with translation elongation (**Figure 21b**). Consistently with this, cellular components associating with this group encompassed ribosome and ribosomal subunit (**Figure 21b** - lower panel). In addition to proteins associated with translation, also factors associated with protein catabolism, (*i.e.* proteosomal-mediated degradation of proteins) moved from the nuclear soluble fraction to the cytosol during OIS (**Figure 21**).

a


Senescent
Growing
NI
NS
CT
NI
NS
CT
ASPH
BPTF
BRD4
CAND1
CD63
CHD3
CLIP1
DYH3
JKIP3
KI13A
LRIQ4
MCM3A
NCEH1
NSUN5
OBSCN
PEX1
PSME1
PSME3
RAGP1
RB6I2
RENT1
RGPD4
RGPD4
RL17
RL8
RLA0
RLA0L
RPB2
RRBP1
RS10
RS2
RS20
RS25
RS3
RS4Y2
RS8
RSSA
SC22B
SND1
SPTN1
SQSTM
TERA
THTM
TMEM2
UBA6
UGPA
VATA
XPO1
ZCH18
NS
CT
NI
0
1
(less) Relative (more)
enrichment

b

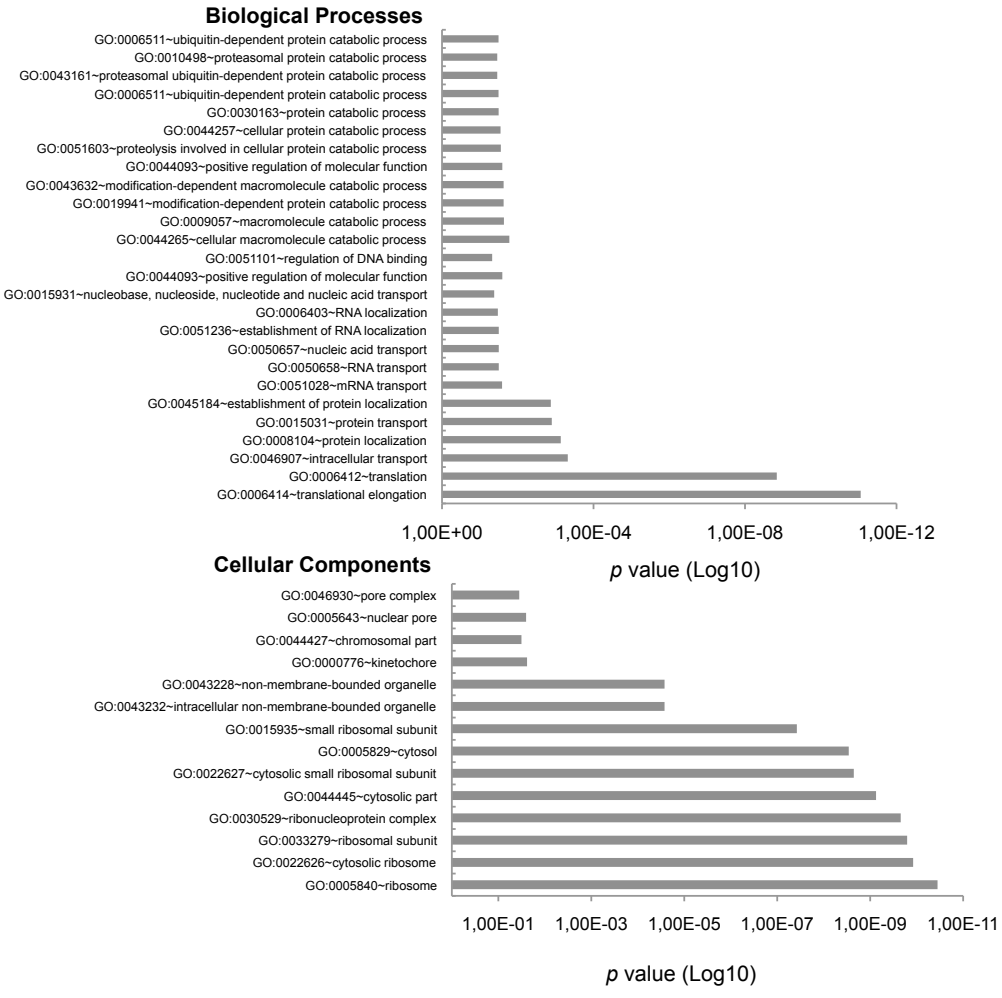

Biological Processes
GO:0006511~ubiquitin-dependent protein catabolic process
GO:0010498~proteasomal protein catabolic process
GO:0043161~proteasomal ubiquitin-dependent protein catabolic process
GO:0006511~ubiquitin-dependent protein catabolic process
GO:0030163~protein catabolic process
GO:0044257~cellular protein catabolic process
GO:0051603~proteolysis involved in cellular protein catabolic process
GO:0044093~positive regulation of molecular function
GO:0043632~modification-dependent macromolecule catabolic process
GO:0019941~modification-dependent protein catabolic process
GO:0009057~macromolecule catabolic process
GO:0044265~cellular macromolecule catabolic process
GO:0051101~regulation of DNA binding
GO:0044093~positive regulation of molecular function
GO:0015931~nucleobase, nucleoside, nucleotide and nucleic acid transport
GO:0006403~RNA localization
GO:0051236~establishment of RNA localization
GO:0050657~nucleic acid transport
GO:0050658~RNA transport
GO:0051028~mRNA transport
GO:0045184~establishment of protein localization
GO:0015031~protein transport
GO:0008104~protein localization
GO:0046907~intracellular transport
GO:0006412~translation
GO:0006414~translational elongation
1,00E+00
1,00E-04
1,00E-08
1,00E-12
p value (Log10)
Cellular Components
GO:0046930~pore complex
GO:0005643~nuclear pore
GO:0044427~chromosomal part
GO:0000776~kinetochore
GO:0043228~non-membrane-bounded organelle
GO:0043232~intracellular non-membrane-bounded organelle
GO:0015935~small ribosomal subunit
GO:0005829~cytosol
GO:0022627~cytosolic small ribosomal subunit
GO:0044445~cytosolic part
GO:0030529~ribonucleoprotein complex
GO:0033279~ribosomal subunit
GO:0022626~cytosolic ribosome
GO:0005840~ribosome
1,00E-01
1,00E-03
1,00E-05
1,00E-07
1,00E-09
1,00E-11
p value (Log10)

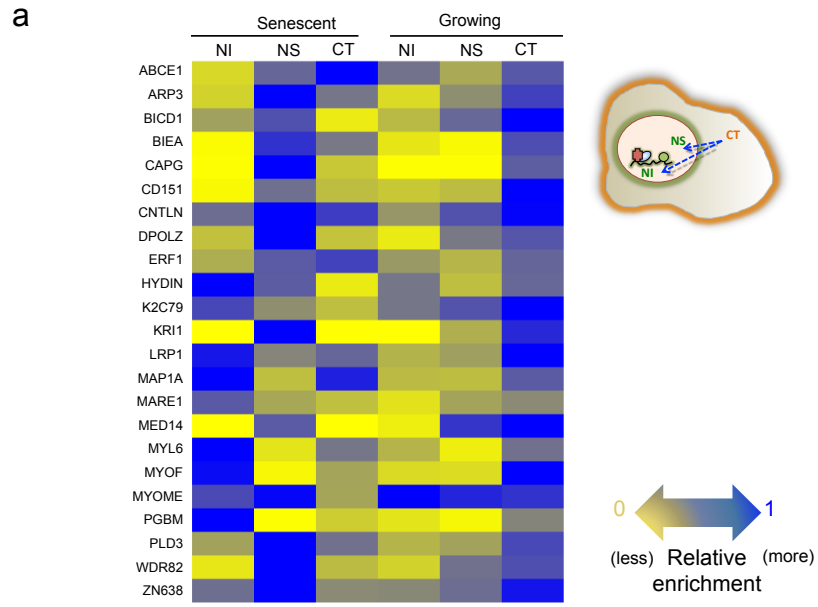
Figure 21. Soluble nuclear proteins migrating to the cytoplasm during OIS. a) Heat map showing proteins that shuttle from the nuclear soluble fraction to the cytoplasm upon induction of senescence. b) GO annotations for proteins shown in a). GO annotations associate with anabolic (RNA translation and protein transport) and catabolic processes (ubiquitin, proteosomal degradation of proteins).

Genes involved in fatty acid degradation (process that occurs in the peroxisome) are also present in this cluster (*i.e*. PEX1) and seemed to preferentially localize to the cytoplasm upon induction of senescence, suggesting the overall engagement of catabolic pathways in senescence.

4.3.5 Proteins moving from the cytoplasm into the nucleus during OIS

Finally, we looked at the proteins shifting from the cytoplasm to the nucleus (**Figure 22**). Upon activation by phosphorylation or other post-translational modification, many proteins translocate from the cytoplasm to the nucleus in order to regulate gene expression. That is the case for instance of the SMAD2 and SMAD3 proteins upon TGFβ-mediated phosphorylation (Nicolas et al., 2004). Therefore, investigating nuclear import of proteins during senescence seemed extremely appealing from the functional point of view.

Our data identified 23 proteins that shifted from the cytoplasm to the nucleus during senescence. These were mostly associating with processes related with cytoskeleton and organelle organization and biogenesis (**Figure 22**). For example, ARP3, MAP1A and CAPG are proteins with actin-binding properties and seem to accumulate in the nucleus during senescence (**Figure 22**). Since alteration of the cell shape (as the cells become enlarged and flat) is one of the key features of the senescent phenotype, would be interesting to understand the functional implications of this observation.



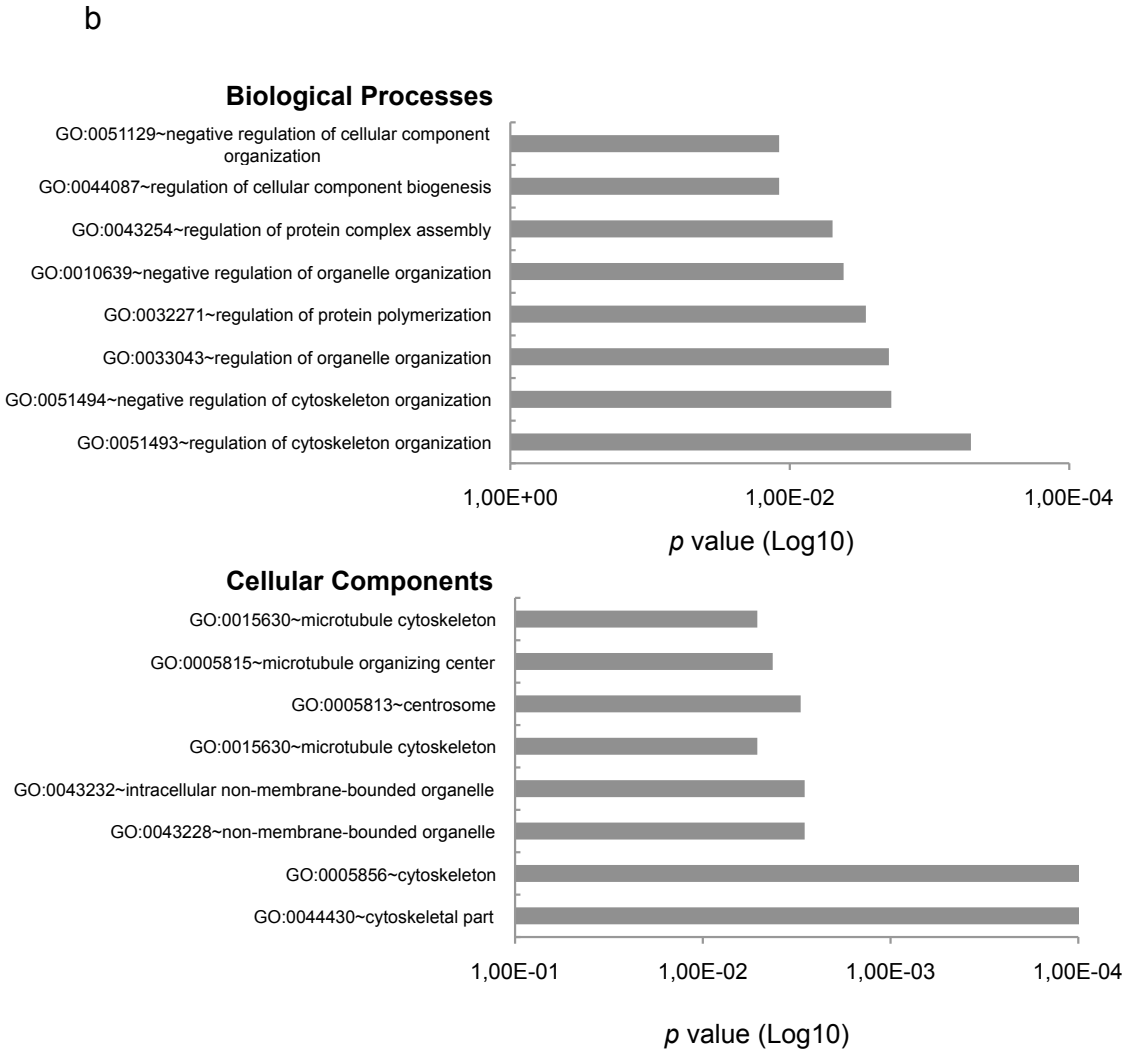


Figure 22. A group of cytoplasmic proteins relocalizes to the nucleus as cells undergo senescence. a) Heat map shows that proteins with a cytoplasmic localization in proliferating cells accumulate in the nucleus during senescence. b) GO terms for proteins in a) show these proteins are associated with cytoskeleton and and organelle organization processes.

4.4 Clustering analysis identifies 3 groups of proteins differentiating senescent from proliferating cells

In order to further investigate changes in protein expression taking place during oncogene-induced senescence we performed clustering analysis, to unravel the proteins, or group of proteins, behind such changes. To this end, Enrico Petretto and Xiaolin Xiao from the Integrative Genomics and Medicine lab (CSC MRC) used a Higher-Order Generalized Singular Value Decomposition (HO-GSVD) - based algorithm, termed C3D method, Cross-Conditions Cluster Detection- to analyze these data (**Figure 23**) (Xiao et al., 2014).

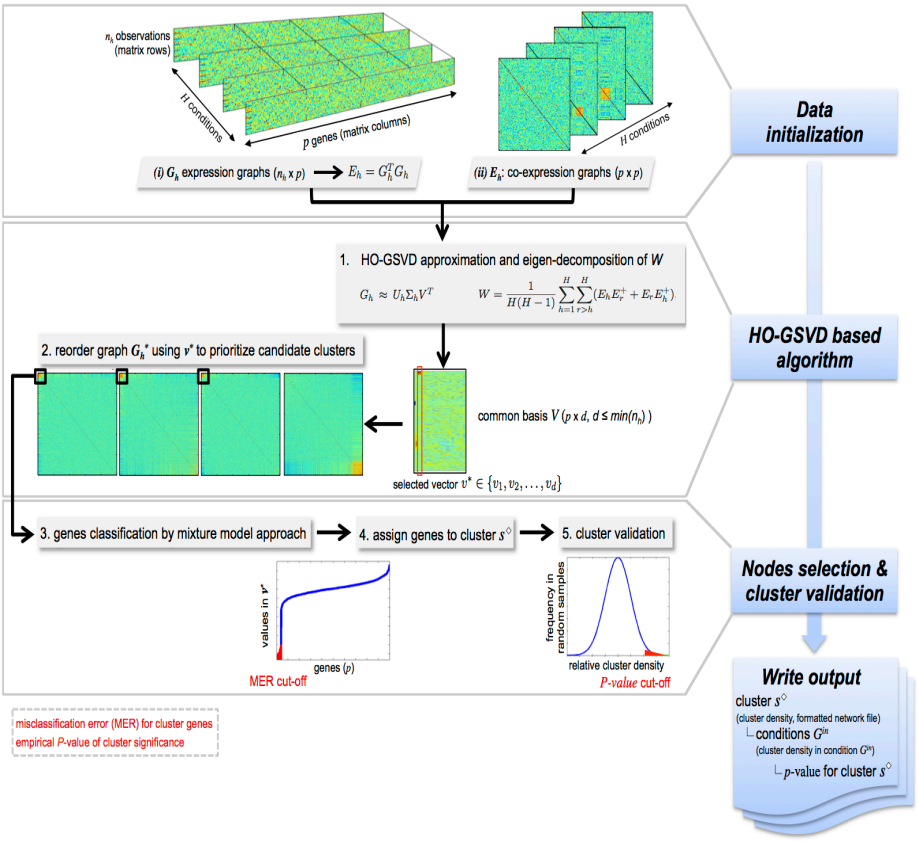


Figure 23. The C3D method. Proteomics data was processed using a High-Order Generalized Singular Value Composition algorithm (HO-GSVD), the C3D method (Cross-Conditions Cluster Detection). This method comprises and initial phase of data initialization, followed by HO-GSVD and finally node selection and cluster validation (Adapted from Xiao et al., 2014).

In summary, they used an algorithm that decomposes the input datasets and identifies the common and differential correlation structures (vectors) between two

conditions, in this case senescent vs growing. After the data is initialized, each vector is then used to reorder the input data matrices so that the candidate ‘‘common’’ (or ‘‘differential’’) clusters are identified. Once the clusters are identified the genes are classified and assigned to each cluster (**Figure 23**) (Xiao et al., 2014). Upon C3D mediated analysis, several nodes were found as differentially expressed in cells undergoing senescence as compared to the proliferating counterparts.

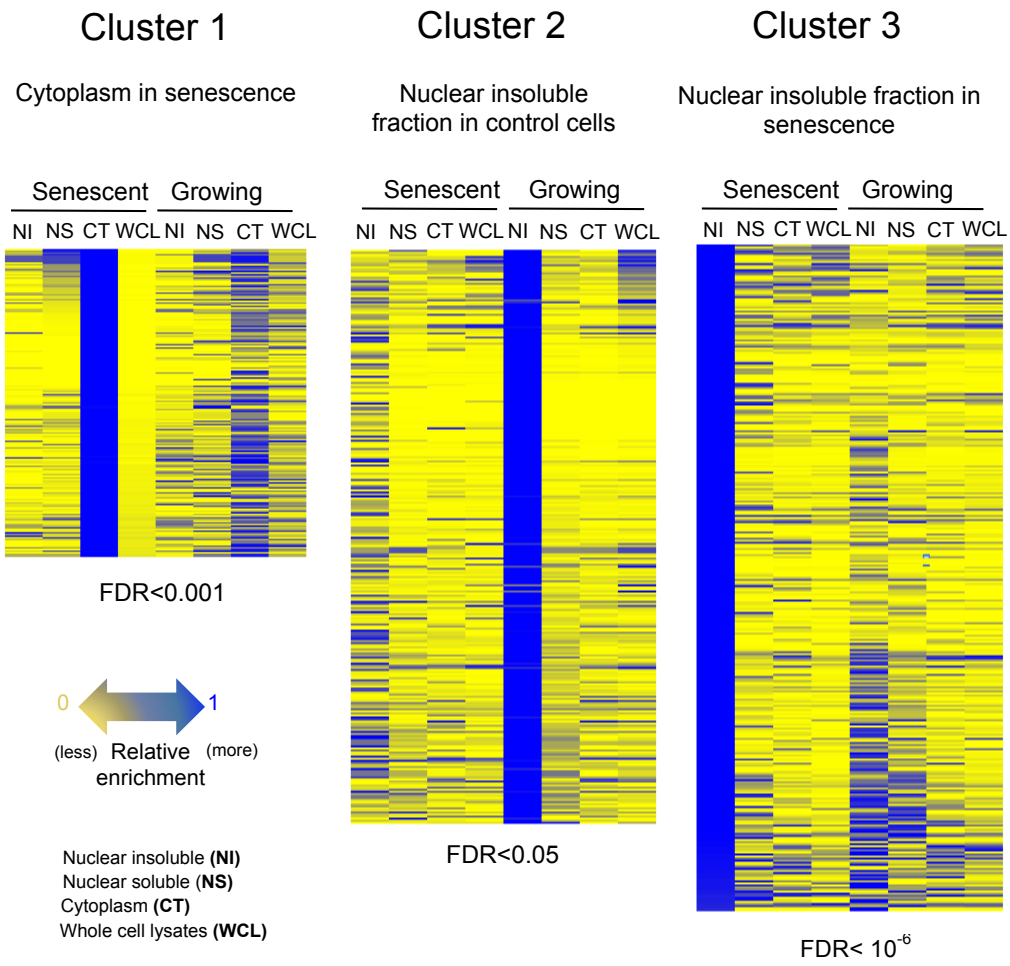


Figure 24. Analysis using the C3D method identifies 3 main protein clusters. The HO-GSVD based algorithm detected 3 main clusters of proteins differentially expressed in OIS. Custer 1 encompasses a group of cytoplasmic proteins expressed in senescence but overall not in the control condition. Custer 2 is a group of proteins enriched in the chromatin of growing cells when compared with senescence. Cluster 3 encompasses proteins enriched in the chromatin during senescence. (NI, nuclear insoluble; NS, nuclear soluble, CT, cytoplasm; WCL, whole cell lysates; FDR, false discovery rate)

We identified three main clusters: a) a group of 185 cytoplasmic proteins enriched during senescence (Cluster 1), b) a group of 278 proteins specifically enriched in

the chromatin of growing cells (Cluster 2) and c) a group of 324 proteins associated with the chromatin of senescent cells (Cluster 3) (**Figure 24**).
Only proteins showing a false discovery rate (FDR) of <0.05 (*i.e.* less than 5% probability of being a false positive entry in the cluster) were selected for each cluster. A more restrictive filter was used for cluster 1 and cluster 3, with FDR<0.001 and FDR<10^{-6}, respectively (**Figure 24**). As previously mentioned, a peptide count of >3 and a Mascot score of >90 were used to filter for the proteins identified with high confidence. Clustering analysis on non-filtered data did not, however, show any different outcome in the identified clusters and annotations associated with them therefore we used the full dataset for the analysis.

4.4.1 A group of cytoplasmic proteins differentiates senescent from growing cells (Cluster 1)

One of the 3 clusters identified with the C3D method consisted of a group of 185 proteins overexpressed in the cytoplasm of senescent cells (FDR ≤0.001) (**Figure 25**). Gene ontology analysis showed the proteins in cluster 1 were involved in protein transport, metabolism, biosynthesis and glycosylation. Additionally, terms such as vesicle-mediated transport or intracellular protein transport were also associated with this cluster (**Figure 26**). Indeed, there is an increased activity of catabolic pathways associated with proteasome and autophagy-dependent degradation of proteins in senescence what might explain the association of cluster 1 with such GO terms (Salama et al., 2014).

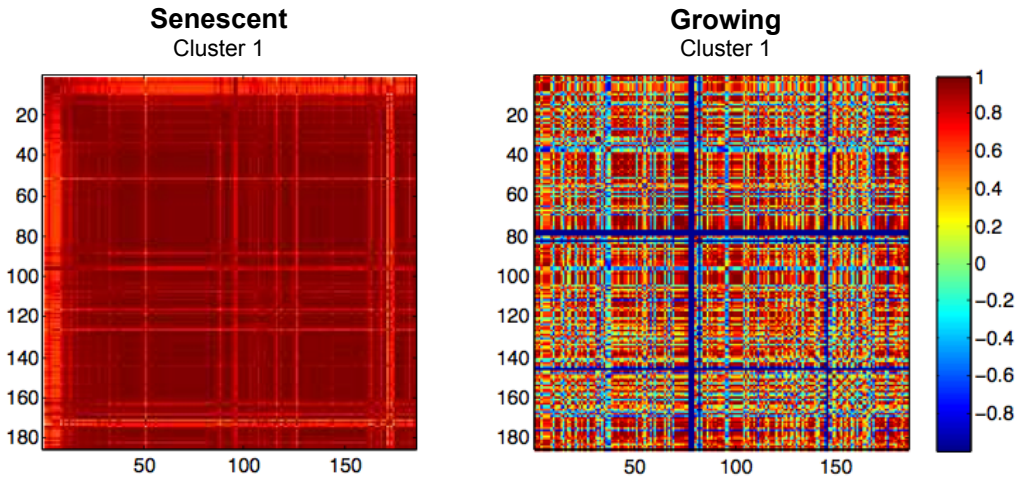


Figure 25. Cluster 1 a is group proteins present in the cytoplasm during senescence. Heat maps show correlations of cluster proteins with senescence (left) and growing (right). This group of proteins are more strongly correlated with senescence than with proliferating cells, therefore defining a relevant cluster distinguishing both conditions.

Moreover, terms linked with ATP-dependent pathways, such as ATP biosynthetic process or ATP metabolic process associated with cluster 1.

We next proceeded with the identification of families of genes enriched in cluster 1. Two functional groups stood out: a group of proteins related with GTPase mediated signaling, including Ras-related proteins, such as RAB13 or RB33B, and an additional group of proteins regulating N-linked glycosylation (**Figure 27**). Interestingly, altered glycosylation has been reported in aging and in plant senescence (Elbers et al., 2001; Fulop et al., 2008; Vanhooren et al., 2011). Whether this is an additional potential hallmark of senescence or simply a reflection the increased turnover and *de novo* protein synthesis would be interesting to address.

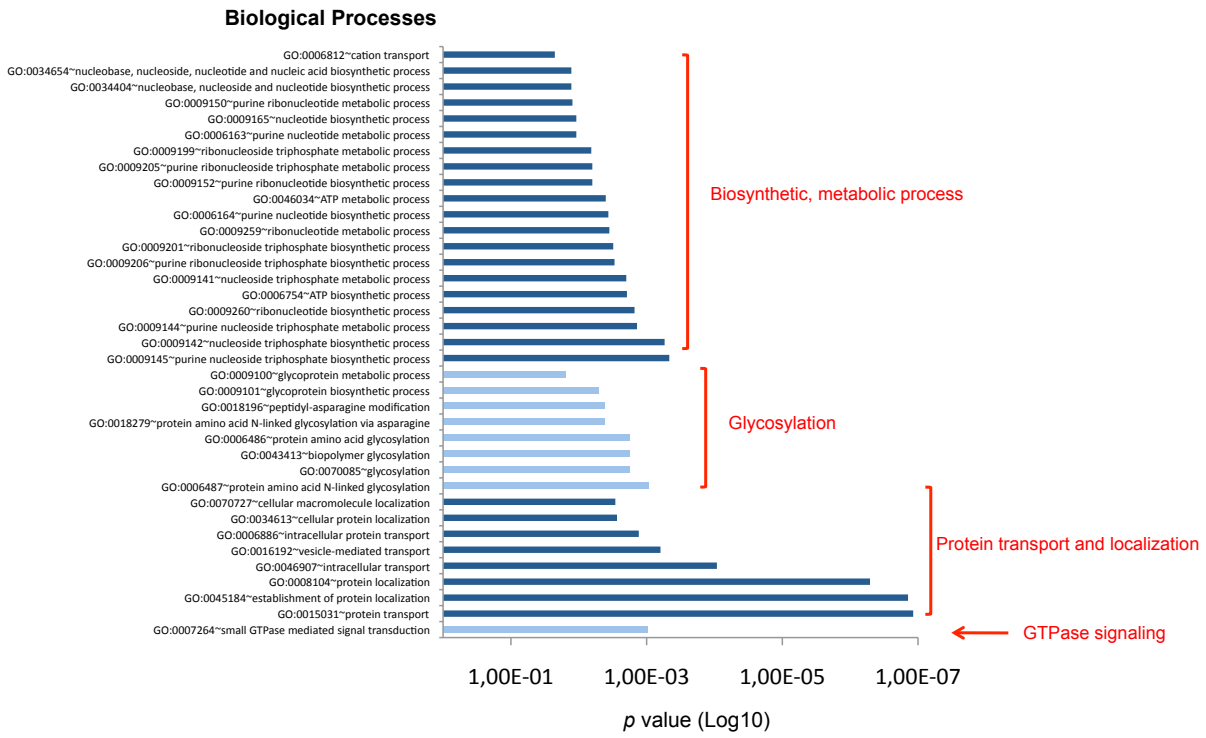


Figure 26. GO annotations for proteins in cluster 1. Gene ontology analysis of cluster 1 revealed proteins in this group are involved in diverse processes that include Rho GTPase activity, protein transport and intracellular localization, protein glycosylation and biosynthesis and metabolism. All terms shown were selected with a *p* value <0.05.

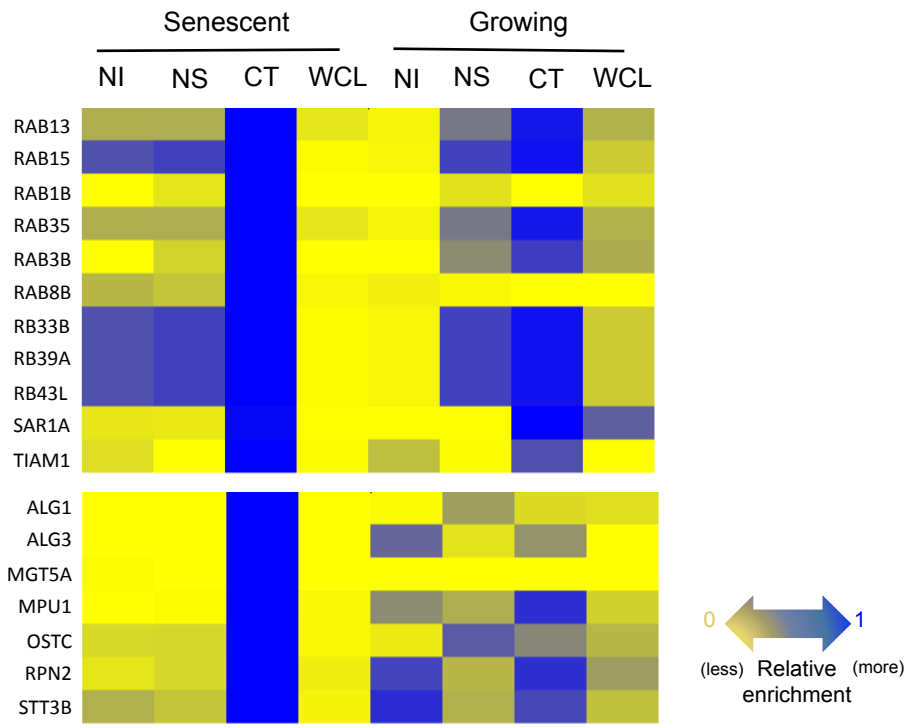


Figure 27. Protein families enriched in cluster 1 include Rho GTPases and factors involved in glycosylation. Proteins involved in RAS signaling (top) and several proteins with a role in glycosylation (bottom) are enriched in the cytoplasm during senscence.

4.4.2 Cluster 2 encompasses proteins enriched in the chromatin of growing cells

An additional group of 278 differentially expressed proteins defined cluster 2, all enriched in the nuclear insoluble fraction of growing cells when compared to the senescent counterparts (**Figure 24**). Indeed, these correlated more strongly with the chromatin of proliferating than senescent cells (**Figure 28**).

Gene ontology analysis revealed that proteins in this cluster were involved in processes such as DNA replication and cell cycle, DNA repair and response to stress (**Figure 29**). Consistent with their localization in the chromatin-bound fraction, cluster 2 proteins were overall involved in chromatin remodeling (**Figure 29**).

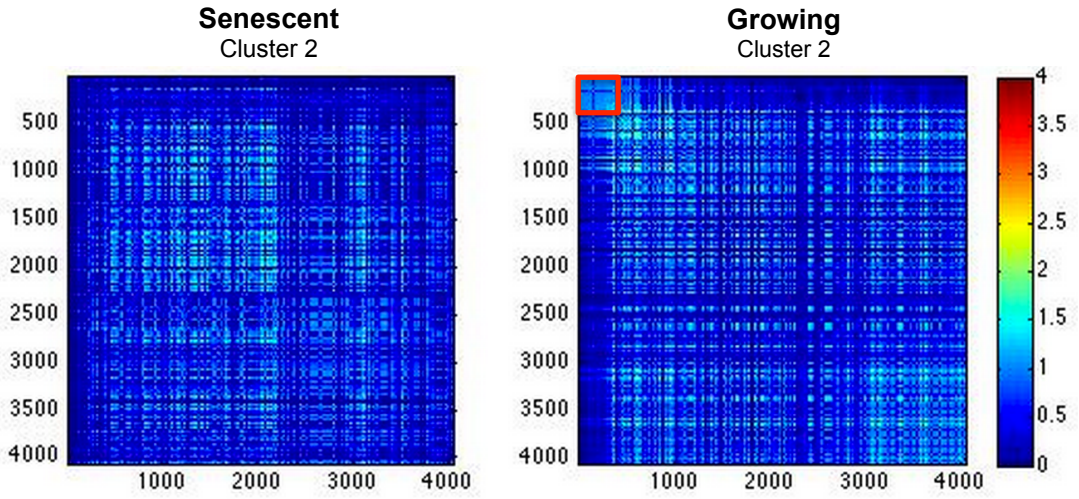


Figure 28. Group of proteins enriched in the insoluble nuclear fraction of growing cells (control) defines cluster 2. Heat maps displaying correlation values for proteins enriched in the insoluble nuclear fraction of control cells showing that these proteins are more strongly positively correlated with the control condition then with senescence, producing a tight cluster of differentially expressed proteins between the two samples.

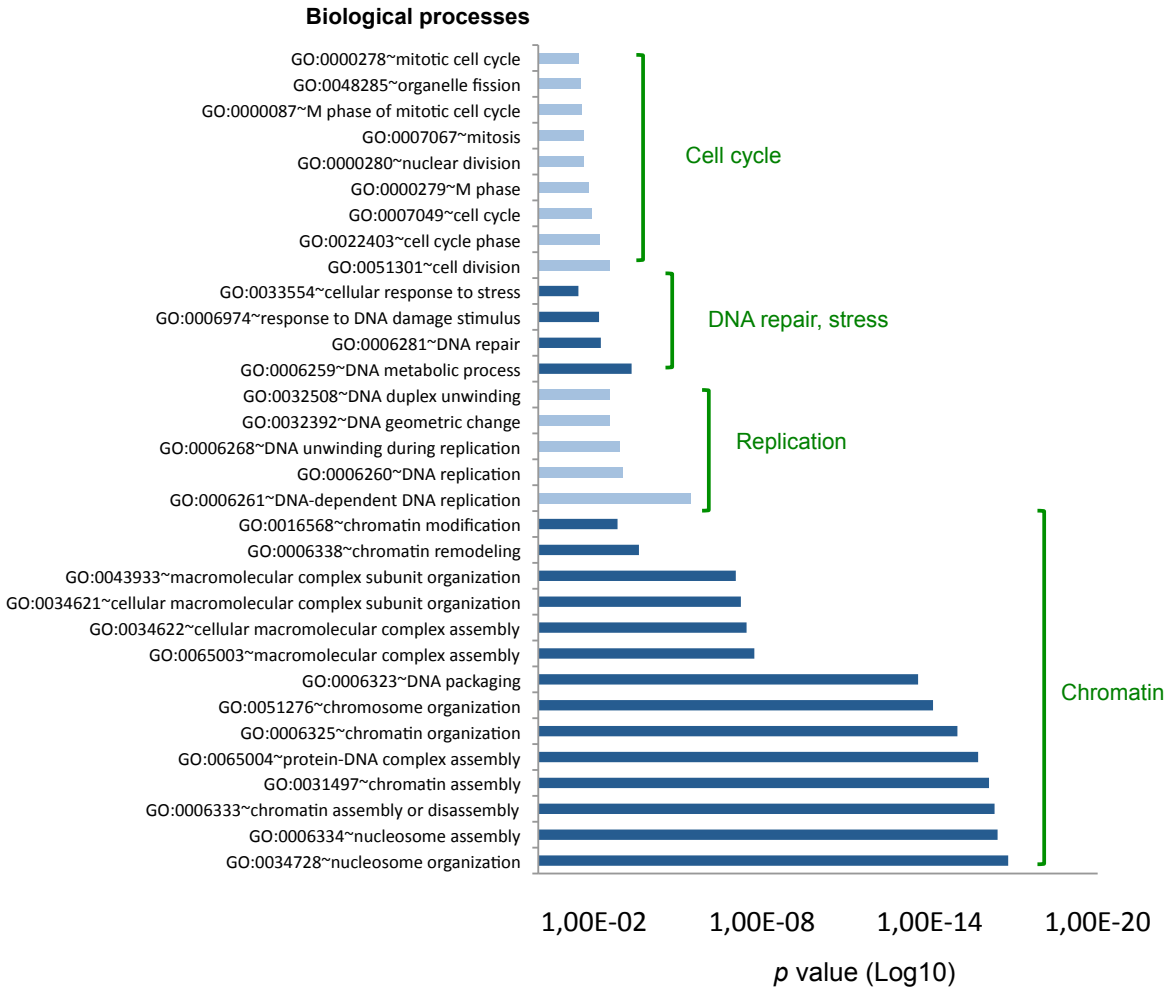


Figure 29. GO annotations associated with cluster 2. Gene ontology generated using the bioinformatic tool DAVID, rendered several groups of biological processes for proteins enriched in cluster 2. These include terms such as replication, DNA repair and cell cycle. However the most enriched terms corresponded to processes related with chromatin, suggesting cluster 2 contains mainly factors involved in chromatin processing.

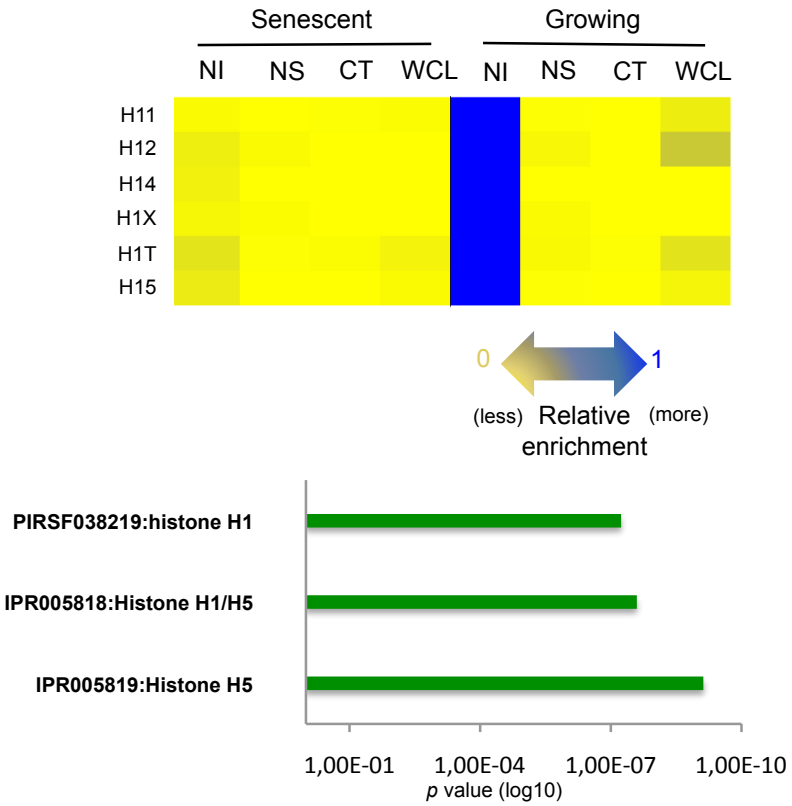


Figure 30. The histone H1 cluster, detected in cluster 2, is depleted from the senescent chromatin. Histone H1 was enriched on the chromatin of proliferating cells as opposed to that of the senescent counterparts. Loss of H1 from the chromatin is thought to contribute to SAHF formation (Funayama et al., 2006).

Within the proteins enriched in cluster 2 we found several variants of the histone H1, suggesting that histone H1 is reduced during senescence (**Figure 30**). This is consistent with previous publications showing that cells undergoing both oncogene-induced and replicative senescence show decreased levels of linker histone H1, most probably due to a post-translational mechanism (Funayama et al., 2006).

Additionally we observed that several members of the high mobility group of proteins (HMGB2 and 3, HMGN1,2, 3 and 4) were depleted from the nuclear insoluble fraction during senescence when compared with cells undergoing proliferation (**Figure 31**).

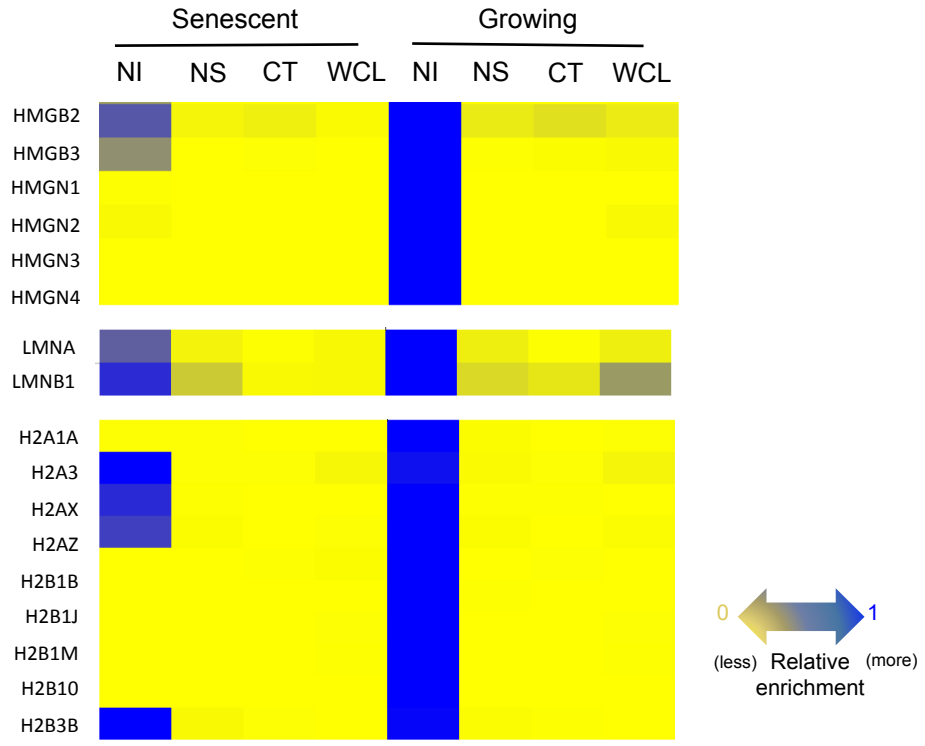


Figure 31. Cluster 2 encompasses members of several families of chromatin remodelers with reduced expression in senescence. Various members of the High mobility group protein family are depleted from the chromatin of senescent cells (top). Lamins A and B, are slightly more enriched in the chromatin of proliferating cells when compared with that of senescent cells (middle). Several variants of the H2A and H2B histone clusters are absent from the senescent chromatin (bottom).

Lamins were also slightly depleted in senescence, specifically LMNA and LMNB1 (**Figure 31**). Lamin B1 loss has been described in cells undergoing both replicative senescence or oncogene-induced senescence (Dreesen et al., 2013; Freund et al., 2012; Sadaie et al., 2013; Shah et al., 2013; Shimi et al., 2011).

Finally, several variants belonging to the H2A and H2B clusters of histones showed reduced expression in senescence, as previously reported (**Figure 31**) (Lopez et al., 2012)

4.4.3 A group of proteins is significantly enriched in the senescent chromatin (Cluster 3)

Another cluster detected with the C3D method consisted of a group of proteins localizing to the nuclear insoluble fraction of senescent cells (**Figure 32**).

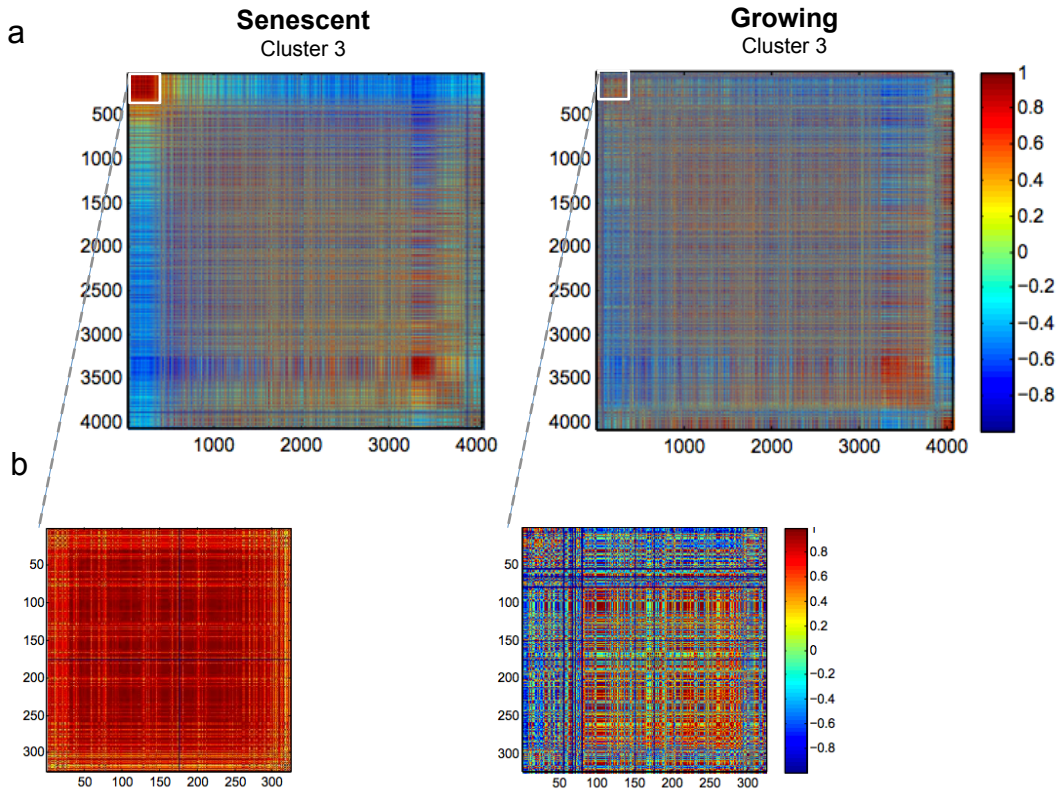


Figure 32. Group of proteins enriched in the senescent chromatin compose cluster 3. Correlation heat map for cluster 3 data **a)** Full set and **b)** top 324 proteins with FDR<10^{-6}, defining cluster 3. Cluster 3 proteins correlated more strongly with senescence than growing, suggesting these proteins are upregulated in the chromatin during OIS.

This cluster contained a group of proteins that strongly correlated with senescence, while being globally anti-correlated or non correlated with the control (**Figure 32a**). From these, three hundred and twenty four (324) proteins presented an FDR<10^{-6} (cluster 3) (**Figure 32b**).

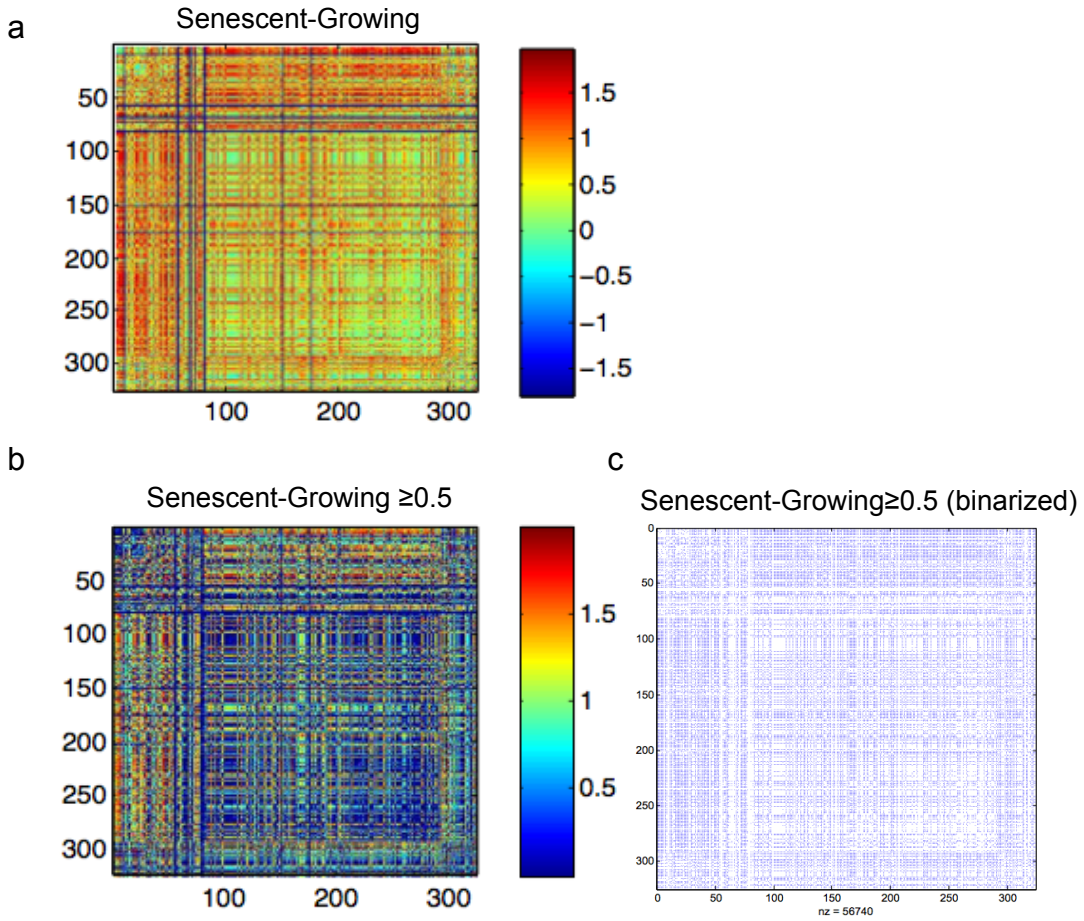


Figure 33. Proteins in cluster 3 strongly correlate with the senescent sample but not with control. Calculation of the difference between protein correlations in senescence and protein correlations in growing cells. **a)** Heat map for correlation differences showing overall positive values, suggesting a higher correlation between proteins in cluster 3 and senescence. **b)** Heat map for positive correlation differences showing most difference values are >0 **c)** Difference matrix plotting difference values of ≥ 0.5 (blue pixels).

Moreover, when the difference between correlation values for senescent and growing cells was calculated for these 324 proteins we found that most correlation values differed by at least 0.5, reinforcing the strong association of proteins belonging to cluster 3 with the chromatin of senescent but not growing cells (**Figure 33a-c**).

We then proceeded with the identification of the proteins and processes associating with cluster 3 by performing a gene ontology analysis. This analysis revealed a significant association ($p<0.05$) with chromatin organization, remodeling and modification (**Figure 34**). The composition of these 2 clusters (1 and 2) with differentially expressed proteins in the chromatin-bound fraction mirrors the profound chromatin remodeling that is known to take place during senescence (Narita et al., 2003; Narita et al., 2006). Additionally, proteins in cluster 3 were involved in cell cycle and DNA repair, which are of conceptual relevance in senescence. Interestingly, members of the SWI/SNF complex (*i.e.* BAZ2A, ARID1A, etc) were also enriched in cluster 3 (**Figure 35a, b**). As members of this complex have been associated with the establishment of senescence, this observation not only provided an internal control but also reinforced the assurance of a successful fractionation. In addition to components of the SWI/SNF complex, other senescence-associated factors were significantly enriched in cluster 3, such as the HMGA2, a member of the high mobility group A (**Figure 35a, b**). This protein accumulates in the chromatin of senescent cells contributing to the formation of the SAHF (Narita et al., 2006).

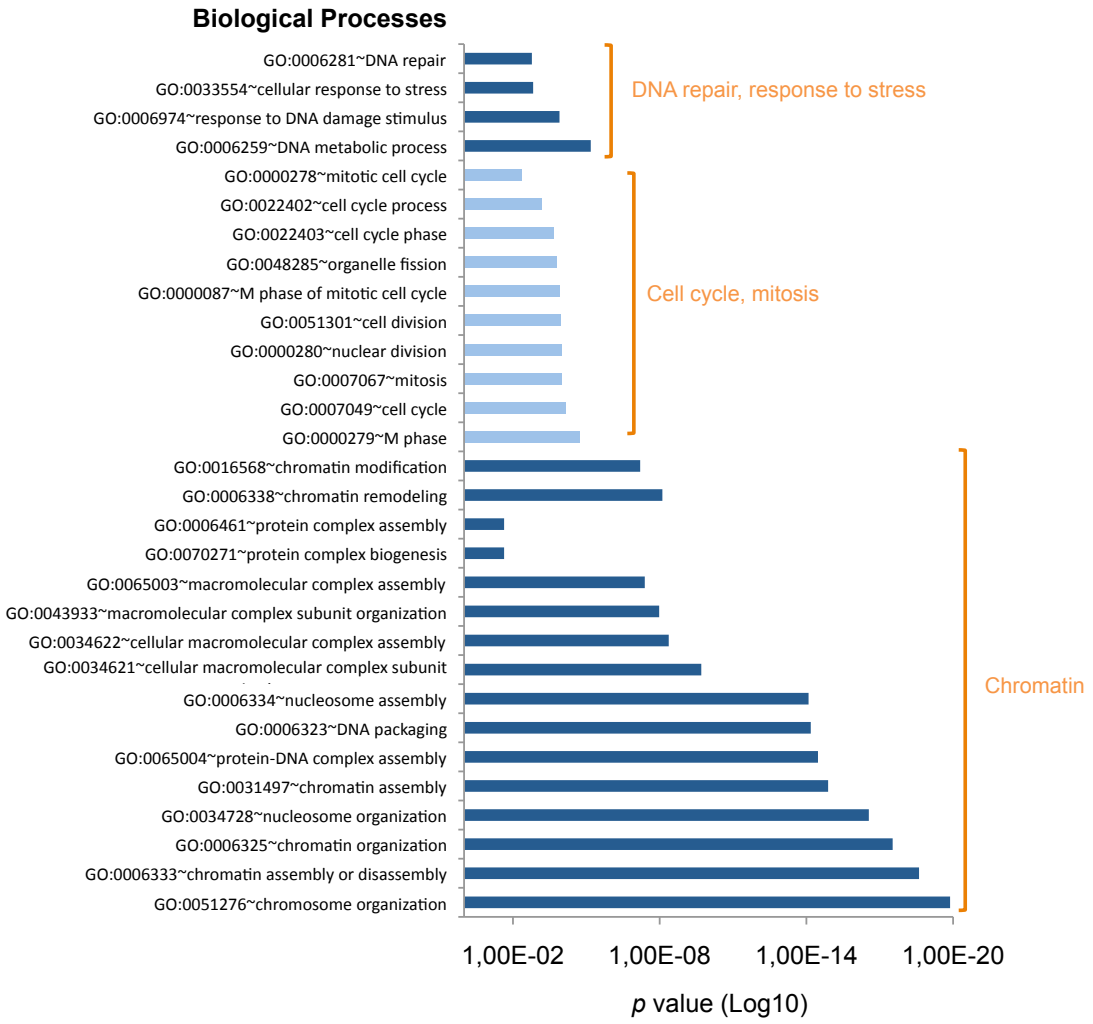


Figure 34. GO annotations associated with cluster 3. Cluster 3 is significantly associated with chromatin-associated processes, such as nucleosome organization or chromatin assembly. Other significant GO annotations relate with cell cycle and mitosis, and DNA repair and response to stress, both senescence related processes.

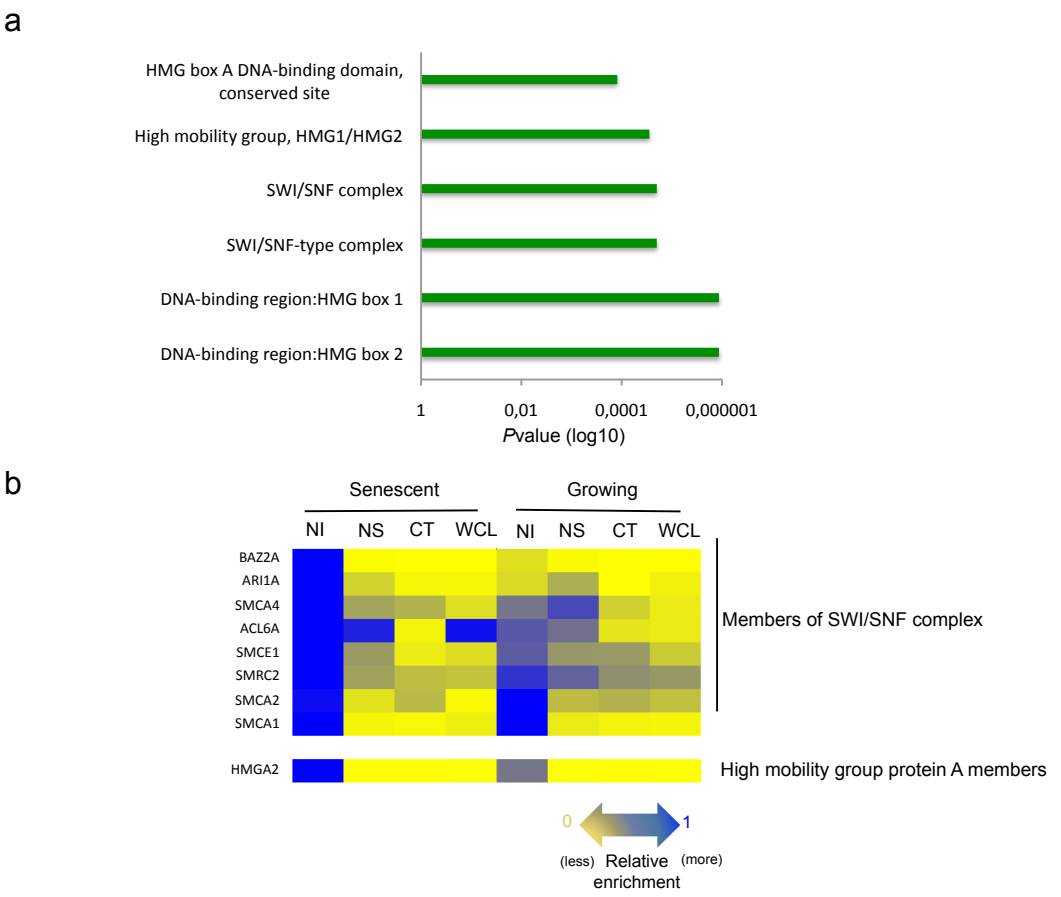


Figure 35. Members of the senescence-associated SWI/SNF complex and high mobility group A proteins are detected in the mass spectrometry analysis and belong to cluster 3. a) GO terms associated with members of the SWI/SNF complex and HMGA proteins are significantly enriched ($p<0.05$) in cluster 3. **b)** Heat map shows the enrichment of several members belonging to the chromatin remodeling complex as well as HMGA2, a high mobility group A protein in the nuclear insoluble fraction of senescent cells, as opposed to growing cells.

Finally, additional families of proteins enriched in the chromatin during senescence in our proteomics data encompassed some histone variants, proteins involved in nuclear export and snRNA processing (**Figure 36**). Specifically, we noted enrichment for the H3 variants, H31T and H32 and histone H4 (**Figure 36**).

Other non-histone proteins accumulating in the nuclear insoluble fraction in senescence were the exportins XPOT and XPO2. These are members of the Nuclear Pore Complex (NPC), involved in the nuclear export of tRNA and importin alpha, respectively (Behrens et al., 2003; Kutay et al., 1997; Kutay et al., 1998; Kuwabara et al., 2001; Tai et al., 2010). Interestingly, also 3 members of the Integrator complex family were found enriched in the nuclear insoluble fraction of senescent cells in our proteomic analysis (**Figure 36).**

The main function of this protein complex is its association with the C-terminal domain of RNA polymerase II and mediation of 3'-end processing of small nuclear RNAs (U1 and U2) (Baillat et al., 2005; Chen and Wagner, 2010).

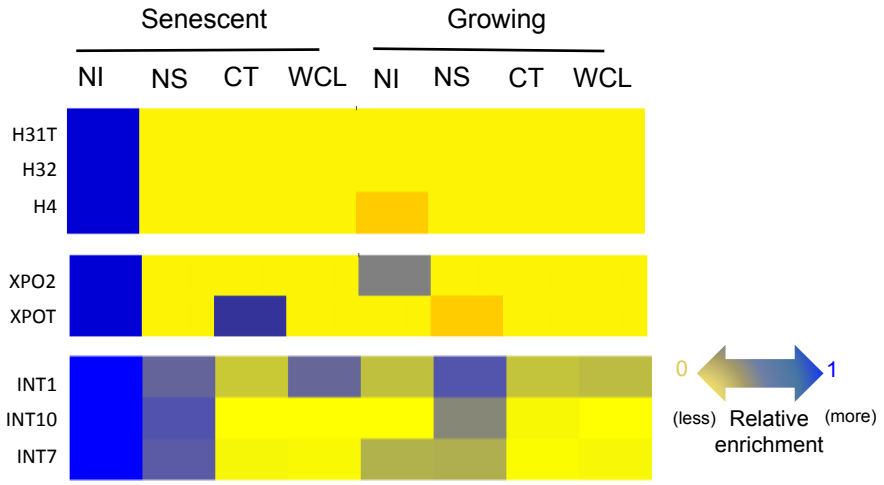


Figure 36. Specific histones, exportins and integrator complex members are enriched in the nuclear insoluble fraction during senescence (Cluster 3). Cells undergoing oncogene-induced senescence show increased levels of the histone H3 variants, H31T and H32, as well as histone H4 in the fraction corresponding to the chromatin. Other non-chromatin protein groups enriched during senescence are the exportins XPO2 and XPOT and the integrator complex members INT1, INT7 and INT10.

Altogether, these results are consistent with a severe chromatin remodeling occurring during senescence (Narita 2003 et al., 2003; Narita et al., 2006). Indeed, the analysis of fractionated cells allowed the identification of the proteins associated with the nuclear insoluble fraction as the most altered fraction as cells undergo senescence. Moreover, the identification of proteins with a functional role in senescence suggests that other proteins present in cluster 3 could also regulate OIS.

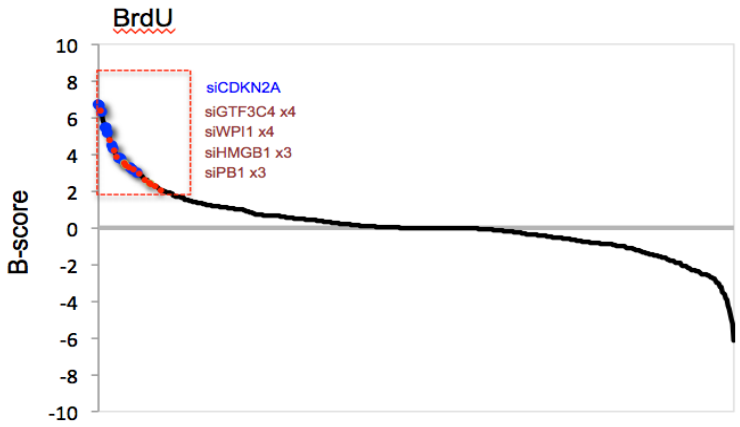
4.5 Functional validation of cluster 3

4.5.1 Several components of cluster 3 regulate OIS

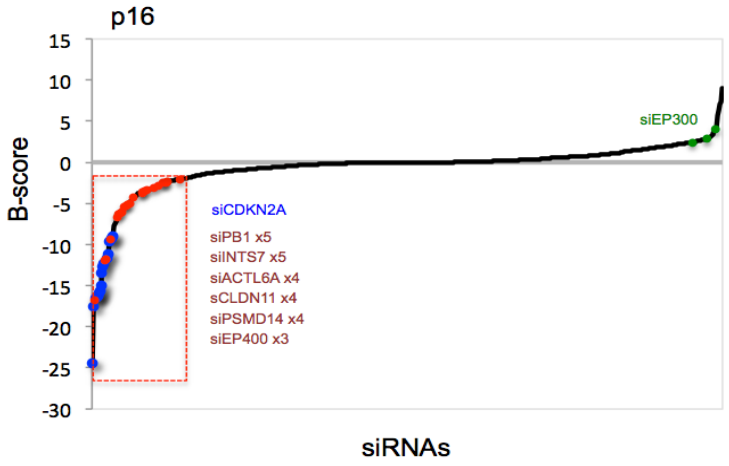
Out of the three main clusters previously defined, Cluster 3 was the one that better distinguished senescence from the proliferating state (FDR<10^{-6}). In addition, the presence of some previously identified regulators of senescence within the cluster (such as SWI/SNF complex members, HMGA2 or EP300) reinforced the idea that cluster 3 could include genes with potential implications for senescence. In order to test the functional relevance for senescence of the proteins identified in cluster 3, we set out to conduct a screen using siRNAs. A more detailed explanation of the screening approach is provided in Chapter 5. A custom library containing 4 independent siRNAs per gene was created targeting a total of 50 genes selected from the proteomics analysis. 25 genes were chromatin-associated factors picked out of cluster 3 and the other 25 genes were not associated with the aforementioned cluster or with chromatin in general (control group). To investigate the potential functional role of these genes in OIS, IMR90 ER:RAS fibroblasts were reverse transfected with the siRNA library and induced to undergo senescence. BrdU incorporation and p16 expression were analyzed by immunofluorescence as readout for senescence at day 4 post treatment with 4-OHT. Expression data was normalized using B-score computation to allow for inter-plate comparison (**Figure 44**). The screen was performed in duplicate.

Next we proceeded with the selection of candidate siRNAs bypassing OIS. For that, we focused specifically on siRNAs displaying a B-score of ≥2 for BrdU and ≤-2 for p16. From these, only genes for which 3 out of the 8 siRNAs analyzed presented such B-scores were selected (**Figure 37**).

a



b



c

siRNAs downregulating p16

Gene	siRNA	B-score
PBRM1	Hs_PB1_4	-5,265
	Hs_PB1_5	-2,838
	Hs_PB1_5	-2,384
	Hs_PB1_6	-11,954
	Hs_PB1_6	-3,389
INTS7	Hs_INTS7_2	-3,444
	Hs_INTS7_2	-3,059
	Hs_INTS7_3	-5,371
	Hs_INTS7_3	-4,216
	Hs_DKFZP434B168_4	-3,256
ACTL6A	Hs_ACTL6A_3	-16,752
	Hs_ACTL6A_3	-6,717
	Hs_ACTL6A_5	-3,691
	Hs_ACTL6A_5	-2,473
PSMD14	Hs_PSMD14_1	-11,742
	Hs_PSMD14_1	-9,389
	Hs_PSMD14_4	-6,14
	Hs_PSMD14_4	-6,043
CLDN11	Hs_CLDN11_5	-2,455
	Hs_CLDN11_6	-3,597
	Hs_CLDN11_8	-6,336
	Hs_CLDN11_8	-2,061
EP400	Hs_EP400_6	-5,185
	Hs_EP400_6	-5,052
	Hs_EP400_8	-2,448

siRNAs increasing BrdU incorporation

Gene	siRNA	B-score
GTF3C4	Hs_GTF3C4_5	3,432
	Hs_GTF3C4_5	2,407
	Hs_GTF3C4_4	3,884
	Hs_GTF3C4_4	4,778
WIPL1	Hs_WIPI1_5	2,054
	Hs_WIPI1_5	3,304
	Hs_WIPI1_4	2,255
	Hs_WIPI1_2	2,6
PBRM1	Hs_PB1_6	6,361
	Hs_PB1_6	4,204
	Hs_PB1_3	2,931
HMGB1	Hs_HMGB1_6	2,408
	Hs_HMGB1_6	3,533
	Hs_HMGB1_4	3,167

siRNAs increasing p16 expression

Gene	siRNA	B-score
EP30 0	Hs_EP300_13	2,413
	Hs_EP300_9	2,829
	Hs_EP300_9	4,001

Figure 37. Screen for the bypass of OIS identifies senescence regulators within cluster 3. IMR90 ER:RAS cells were reverse-transfected with 4 independent siRNA against 25 genes picked out of proteomics cluster 3 and 25 selected randomly from the mass spectrometry data. Cells were induced to undergo OIS and fixed 4 days after treatment with 4-OHT started. BrdU and p16 expression were analyzed by IF and used as readout for the bypass of OIS. **a)** siRNAs against 3 genes from cluster 3 (GTF3C4, HMGB1, PB1) bypassed the cell cycle arrest as measured by BrdU incorporation, whereas only siWIPI1 , from the random pool of genes, scored. **b)** siPB1, siINTS7, siACTL6A and siEP400 (cluster 3) bypassed full induction of p16. siRNA targeting EP300, an inhibitor of OIS, reinforced p16 expression. **c)** Hits were picked with basis on the B-score for individual siRNAs. The cutoff B-Score ≥+2 or ≤-2 was used and only genes for which 3 out of 8 siRNAs scored were selected. Blue, sip16; red, siRNAs bypassing; green, siRNAs reinforcing.

The selective threshold allowed the identification of 4 hits whose knockdown bypassed the cell cycle arrest. From these, three (GTF3C4, PB1 and HMGB1) were genes picked out of cluster 3. Similarly, knockdown of 6 genes prevented p16 induction and 4 of them (PB1, INTS7, ACTL6A, EP400) were part of cluster 3 (**Figure 37a-b**). Altogether, this result suggests a potential causal association between genes in cluster 3 and the onset of senescence, once again reinforcing the importance of the chromatin for the establishment of the senescent phenotype, or at least part of it. Interestingly, some genes belonging to the SWI/SNF complex, specifically ACTL6A and PBRM1, scored in this analysis (**Figure 37**). While we did not concentrate on siRNAs that reinforced OIS, we did notice that siEP300, which has been recently reported to inhibit senescence induced p16 expression (**Figure 37b**) (Yan et al., 2013). Our analysis rendered additional genes with a potential and unreported role in senescence. Knockdown of EP400 and INTS7 involved in histone acetylation and in snRNAs transcription and DNA damage, respectively, partially prevented p16 induction. Additionally, knockdown of GTF3C4 increased BrdU incorporation of IMR90 ER:RAS cells treated with 4-OHT, suggesting potential roles for these genes in regulating senescence.

4.5.2 GTF3C4, identified in cluster 3, regulates senescence

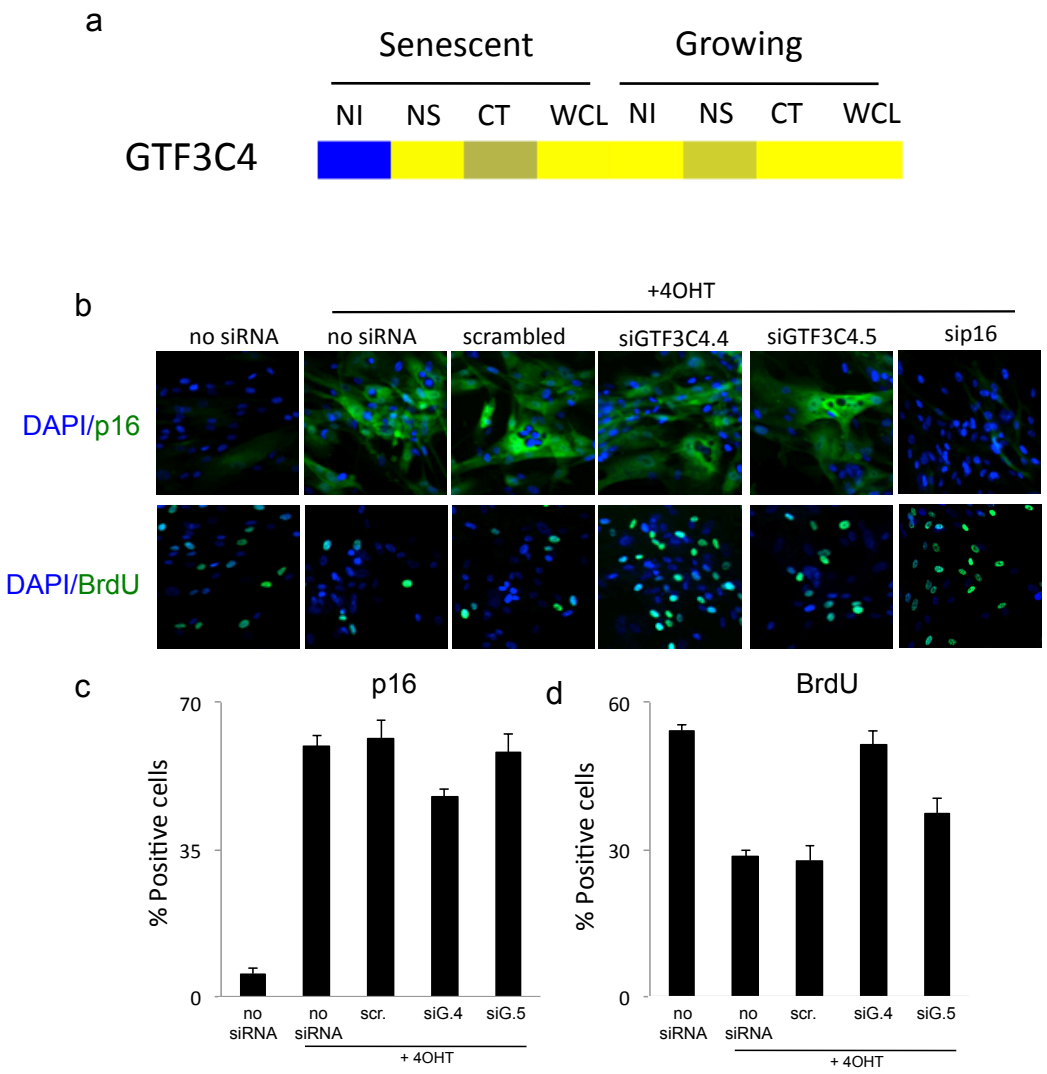


Figure 38. Knockdown of the cluster 3 gene, GTF3C4, bypasses OIS. a) GTF3C4 was detected in the mass spectrometry analysis enriched in the nuclear insoluble fraction of senescent cells. **b)** IF against BrdU and p16 of GTF3C4-depleted IMR90 ER:RAS treated with 4-OHT for 4 days. **c)** Quantification of b) shows an increase in BrdU incorporation while p16 expression is unaffected by any of the siRNAs against GTF3C4.

Amongst the components of cluster 3 we decided to investigate in further detail GTF3C4 (protein name -TF3C4). GTF3C4 (also referred to as TFIIIC90) is a member of the multi-subunit TFIIIC which is involved in RNA polymerase III-mediated transcription of small nuclear RNAs (Dumay-Odelot et al., 2007; Hsieh et al., 1999; Kundu et al., 1999). In our proteomics data, GTF3C4 was

detected to be enriched in the chromatin –bound fraction during senescence (**Figure 38a**).

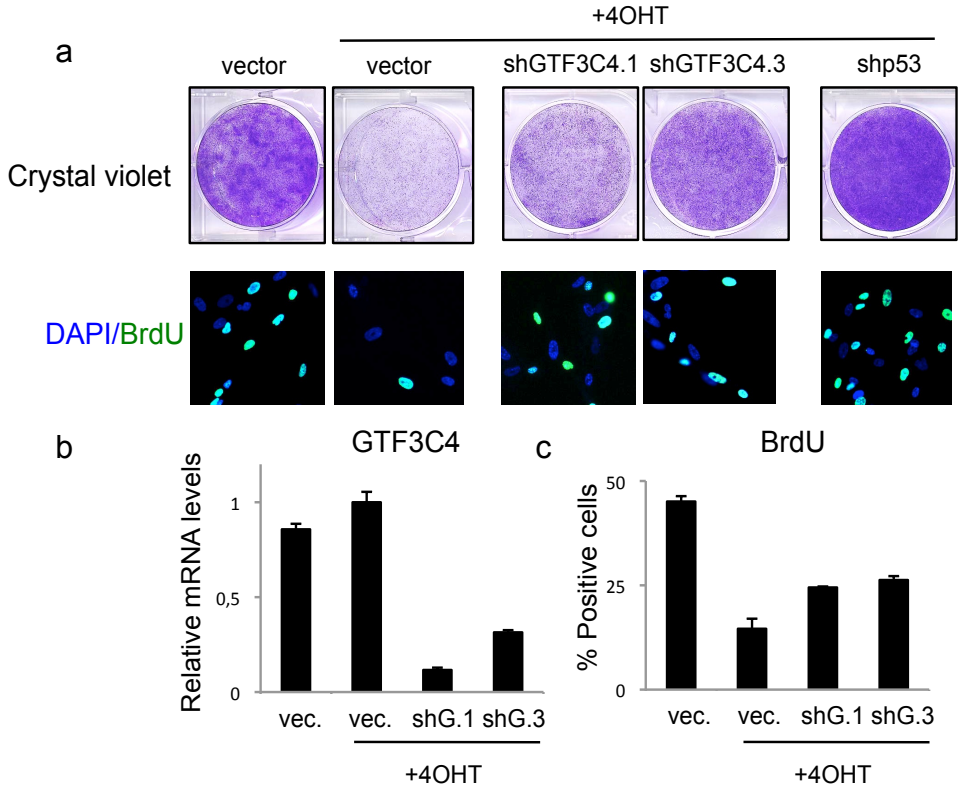


Figure 39. Stable knockdown of GTF3C4 leads to bypass of the stable cell cycle arrest. a) Knockdown of GTF3C4 in IMR90 ER:RAS cells treated with 4-OHT resulted in increased proliferation as assessed by crystal violet staining (top panel) and BrdU incorporation (bottom panel). shp53 was used as a control b) RT-qPCR of GTF3C4 mRNA shows the gene was efficiently knocked down with both shRNAs. c) Quantification of IF against BrdU showed in b.

Knockdown of GTF3C4 resulted in increased BrdU incorporation by IMR90 ER:RAS cells treated with 4-OHT, likely in a p16- independent fashion (**Figure 38b-d).** To confirm these results we infected IMR90 ER:RAS cells with lentivirus expressing several shRNAs against GTF3C4. Two shRNAs efficiently knocked down GTF3C4 (**Figure 39b**). Infected cells were plated at low density, induced to undergo OIS with 4-OHT, and cultured for a few days in order to evaluate the ability of cells expressing shGTF3C4 to form colonies. In agreement with the previous results, knockdown of GTF3C4 led to increased proliferation, as measured by BrdU incorporation and that resulted in increased colony formation along time (**Figure 39a, c**)

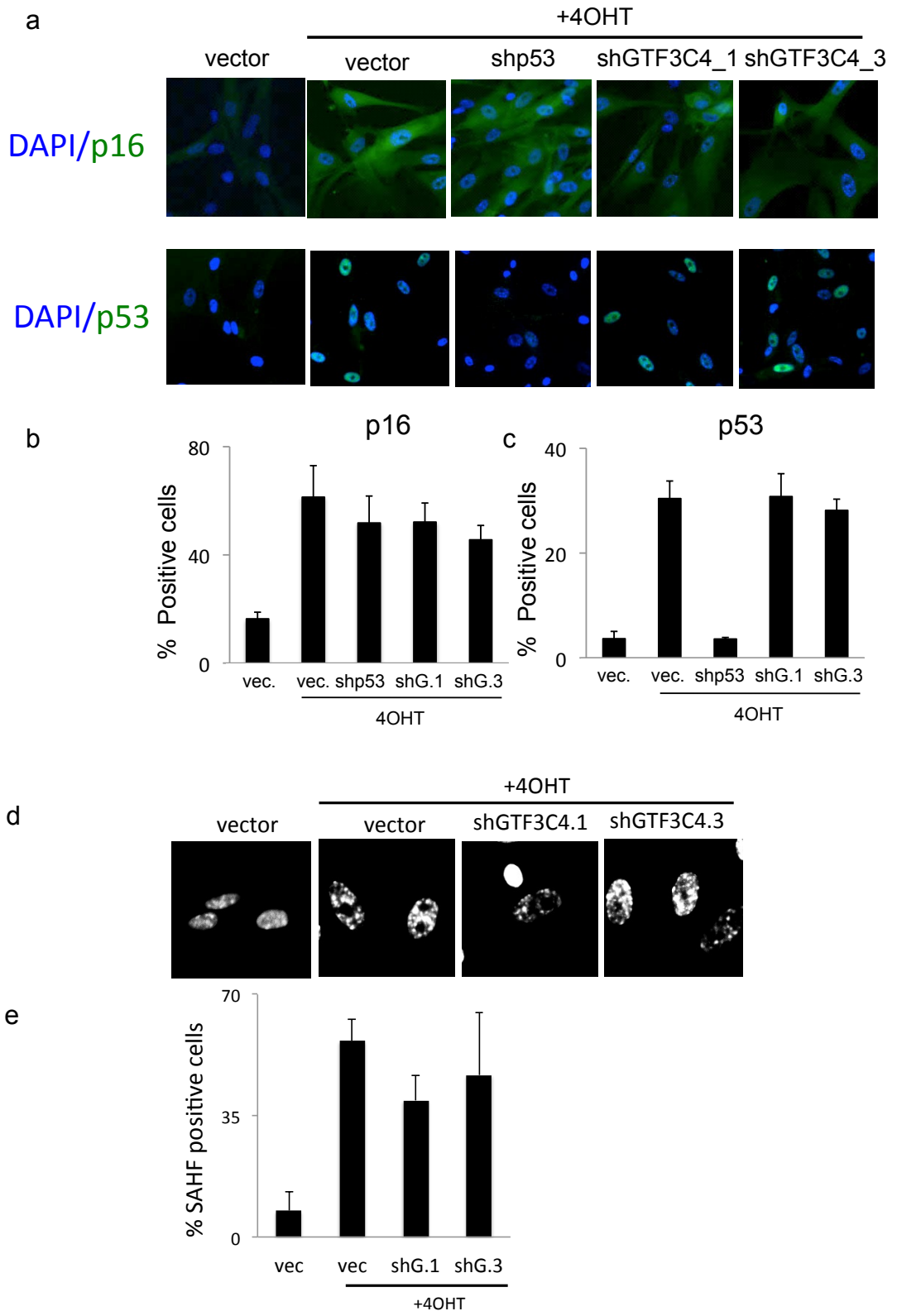


Figure 40. Knockdown of GTF3C4 does not affect p53 and has little impact on p16 expression and SAHF formation. a) Immunofluorescence against p16 (top) and p53 (bottom) on IMR90 ER:RAS infected with shRNAs against GTF3C4. Cells were induced with 4-OHT and fixed at day 6 for IF. b-c) Quantification of the immunofluorescence images collected via high throughput microscopy. d) DAPI stained nuclei revealing the formation of SAHF. e) Quantification of the percentage of cells displaying senescence-associated heterochromatin foci.

Next we sought to investigate whether knockdown of GTF3C4 in oncogene-induced senescence could have implications for the expression of the main tumour suppressor pathways, p16/Rb and DDR/p53 (**Figure 40**).

Upon induction of Ras expression, cells infected with the empty vector underwent senescence, showing increased p16 as well as p53 expression (**Figure 40a**).

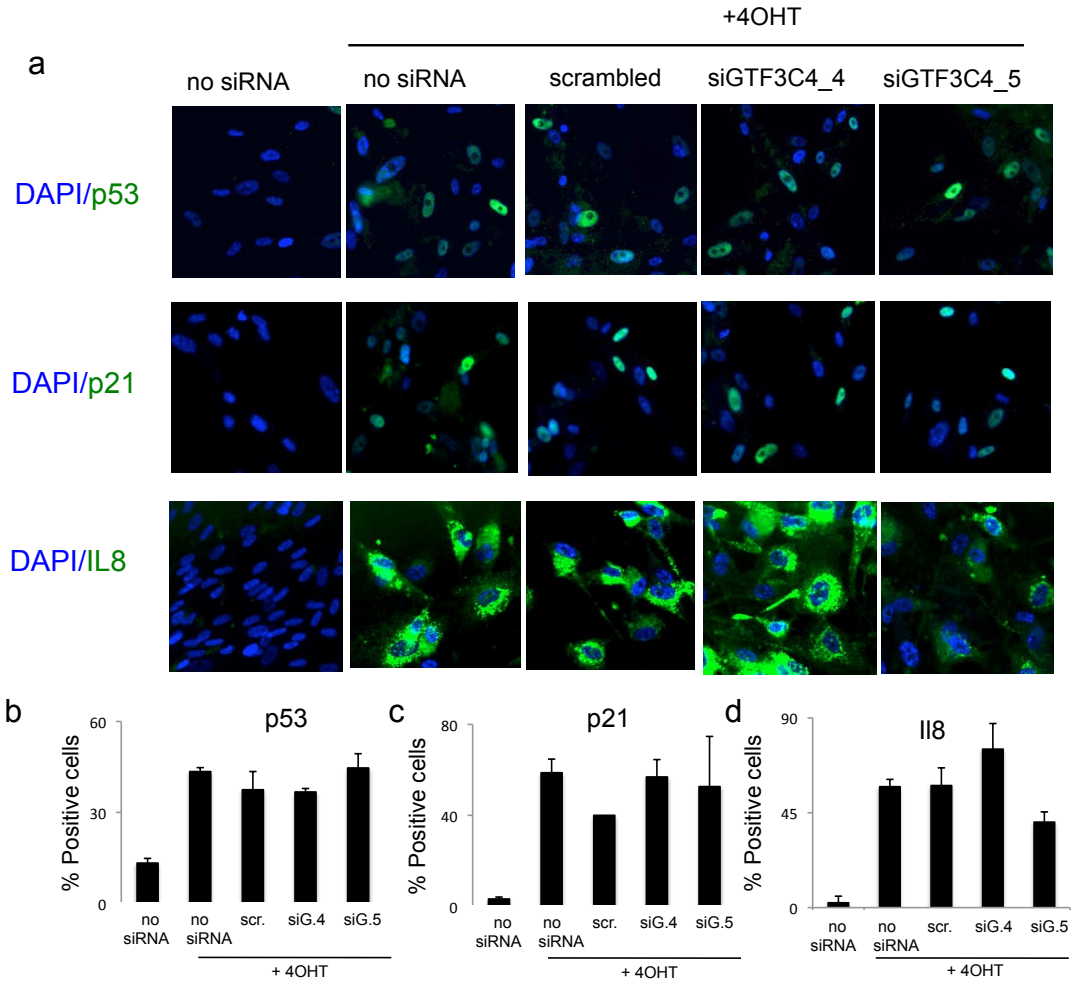


Figure 41. Transient knockdown of GTF3C4 does not affect the integrity of major pathways in OIS. a) IF against p53, p21 and IL8 (SASP). **b-c)** siGTF3C4 do not affect the p53/p21 pathway. **d)** The SASP, as measured by IL8 expression, is not greatly affected upon GTF3C4 knockdown.

Knockdown of GTF3C4 with two independent shRNAs resulted in a slight reduction of p16 expression (**Figure 40a,b**). Knockdown of GTF3C4 had no effect on p53 expression (**Figure 40a,c**). To assess the formation of SAHF we stained nuclei with DAPI. Knockdown of GTF3C4 had a modest impact of the formation of SAHF (**Figure 40d,e**). Additionally, transient knockdown of GTF3C4 had no effect on the expression of other senescence markers such as p21 and the SASP component IL8, as assessed by immunofluorescence (**Figure 41a,c,d**). Also, accumulation of DNA damage seemed unaltered upon GTF3C4 knockdown (data not shown).

In conclusion, these preliminary experiments pinpoint a possible role for GTF3C4 in senescence but additional and more detailed experiments are needed to understand how GTF3C4 controls senescence.

4.6 Discussion and conclusions

4.6.1. Large-scale studies to identify expression changes in senescence

Over the past years, several groups have conducted large-scale studies of gene expression, using microarrays or RNA sequencing (RNA-seq) in order to unravel new gene networks controlling senescence (Shelton et al., 1999; Yoon et al., 2004; Zhang et al., 2003). For instance Purcell et al. recently compared replicative senescence with oxidative stress induced-, chemotherapy- and DNA demethylase-induced senescence through RNA-seq to conclude that not only upregulation of SASP genes is a common feature across different types of senescence, but also that gene expression in senescence induced by demethylase treatment highly overlaps with that observed in fibroblasts undergoing replicative senescence (RS), reinforcing the idea that progressive DNA demethylation has a causal effect in aging (Purcell et al., 2015). Additionally, RNA-seq analysis of young and old fibroblasts suggested a different pattern of long non-coding RNAs (lncRNA) expressed during replicative senescence, allowing the discovery of SAL-RNA1 (XLOC_023166) as a novel negative regulator of senescence (Abdelmohsen et al., 2013). Recently, Nelson *et al*. published an extensive comparison of gene expression between cells undergoing oncogene-induced and replicative senescence using microarrays. They showed that although very similar in nature, replicative and oncogene-induced senescence displayed significant differences in gene expression. Specifically, while both display down regulation of cell cycle genes, up regulation of SASP factors and p16/pRb associated genes and changes in the expression of Wnt signaling effectors, the subset of genes within those categories are different in OIS and RS (Nelson et al., 2014).

While mRNA expression analysis is a valuable tool for identifying expression changes, proteins are the functional effectors of the cell and transcriptome studies do not take into account post-transcriptional modifications. To overcome this, previous studies have used mass spectrometry to investigate protein changes in cells undergoing replicative-, stress- or oncogene induced senescence (Aan et al., 2013; Acosta et al., 2013b; Benvenuti et al., 2002).

For instance, comparison of proteomes of cells undergoing replicative- and stress-induced senescence identified differences in changes of protein expression when compared to normal fibroblasts. Proteins involved in cytoskeleton organization, stress and metabolic pathways, Ca^{2+} regulation and protein folding and degradation were overly differentially regulated in these two types of senescence (Aan et al., 2013). Nonetheless, most of these studies looked for protein changes while analyzing total cell lysates, therefore overlooking changes that do not encompass alterations in total expression levels such as differential post-translational modifications or altered subcellular localization. Recently, collaborative work from Peeper and Altelaar's labs extensively characterized the proteome and phosphoproteome of cells undergoing OIS upon mutant BRAF expression and compared it with that of proliferating cells and cells bypassing OIS (de Graaf et al., 2014). This study yielded several interesting conclusions. For instance, the authors showed that there were major changes in expression related with general oncogene overexpression, shared by both OIS and OIS bypassing cells, and fewer expression changes related with senescence itself. Moreover, they performed proteomics analysis at early and late time points upon oncogene activation and observed differences regarding the proteins (and related processes) that were up and down regulated at each of these time frames, with cell cycle genes changing expression early on and some extracellular matrix components and inflammatory cytokines being specifically upregulated in senescence at late stages of senescence. Additionally, and more importantly, this work unraveled specific changes in protein phosphorylation without major changes in total protein expression. That was the case of the prototypical senescence regulator, RB1, which was hypophosphorylated during OIS and OIS bypass but did not change the total level of expression (de Graaf et al., 2014), thus reinforcing that protein function can be fine-tuned by post-translational modifications.

Aiming for a more specific approach to study senescent sub-proteomes, Acosta et al. performed a SILAC-based approach focusing specifically on the extracellular space to study the SASP (Acosta et al., 2013a). Also, Althubiti et al. characterized the plasma membrane-associated proteome of cells

undergoing senescence upon p21 or p16 overexpression, and identified novel potential senescence selective markers with prognostic value for cancer survival (Althubiti et al., 2014). The present study proposed to use an alternative approach by applying mass spectrometry on fractionated cells in order to specifically identify proteins enriched on particular subcellular compartments (chromatin, nucleoplasm and cytoplasm). “Spatial proteomics”, as it is called, has been previously described and used to characterize subcellular shifts in protein localization across compartments, mainly upon induction of DNA damage (Aslanian et al., 2014; Boisvert et al., 2010; Boisvert and Lamond, 2010). Specifically, these studies made use of the Stable isotope labelling with amino acids in cell culture (SILAC) technique to compare protein abundance between differentially labelled subcellular fractions from different experimental conditions. Detection of subcellular protein localization is of relevance as altered protein localization, more than its overall expression within the cell has been associated with predisposition to several diseases (Gatto et al., 2014). Protein localization is normally analyzed either by immunofluorescence or by combining cell fractionation and protein blotting, which allows only the analysis of known proteins at a low throughput (Boisvert et al., 2010). Thus, spatial proteomics allows not only the determination of spatial organization of specific proteins within the cell but also identification of novel proteins regulating a particular process (Boisvert and Lamond, 2010). Additionally, variations derived from fractionation and MS techniques seem to have only minor effects of the final measured values of protein abundance for what this large-scale approach is highly accurate, also because it allows the measurement of several subcellular specific markers for precise assessment of the fractionation quality (Boisvert and Lamond, 2010). Overall, this method presents clear advantages compared to previous methods: 1- It allows the identification of proteins changing subcellular localization; 2- Is more sensitive to less abundant proteins that tend to occupy very specific subcellular niches. In the present work, human primary fibroblasts carrying a fusion ER:RAS gene were used as a model of oncogene-induced senescence, allowing for the temporally controlled induction of OIS. Mass spectrometry analysis of senescent and growing cells

identified over 4,000 proteins across different subcellular fractions (**Figure 17**). The overall pattern of protein expression in the nucleoplasm and cytosol was generally conserved as cells underwent OIS. Conversely, the nuclear insoluble proteome displayed major differences in protein representation, with clusters of proteins either overexpressed or downregulated in senescence when compared to the proliferating counterpart (**Figure 17**). This observation suggests that, rather than a universal alteration of protein expression, senescence could encompass minor, but no less important, changes in the expression of specific protein groups localizing to the cytoplasm and nucleoplasm, accompanied by a global rearrangement of the chromatin-associated proteome. This result sheds light on the recurring concept of chromatin remodeling as one of the most prominent hallmarks of aging and senescence (Adams, 2007; Dimauro and David, 2009; Narita et al., 2003, Narita et al., 2006). Of note, several proteins identified across the subcellular fractions were under-represented when the whole cell lysates were analyzed. Thus, the approach herein presented allowed the analysis of subcellular proteomes permitting not only the detection of less abundant proteins, that otherwise would be overlooked, but also changes in subcellular localization of proteins. Most importantly, it identified that proteins associated with the chromatin are the most significantly altered when cells undergo oncogene-induced senescence.

4.6.2 Protein groups involved in basic cellular processes change subcellular localization in OIS

Analysis of fractionated cells by performing spatial proteomics allows the identification of proteins changing subcellular localization (Boisvert and Lamond, 2010). In light of this, a group of proteins was shortlisted from the mass spectrometry output data for displaying a pattern of expression consistent with an intracellular shuttling between compartments (**Figure 18-Figure 22**). Proteins were clustered by the relative abundance when comparing subcellular compartments pair-wise, and gene ontology analysis identified the processes those protein groups associated with. Interestingly, most terms corresponded to basic cellular functions, such as mRNA

processing, protein synthesis and degradation or cytoskeleton organization (**Figure 18-Figure 22**). Previous work has drawn attention to alterations of the mRNA processing machinery during senescence (Chandris et al., 2010; Nose and Okamoto, 1980). Specifically, Chandris et al. had reported perturbations of the mRNA processing machinery in senescent fibroblasts, showing a decrease in polyA mRNAs as cells undergo senescence. Interestingly, this was accompanied by a redistribution of the mRNA processing compartments from a widespread nucleoplasmatic pattern, with speckles accumulating along the interchromatin spaces in young cells to an isolated speckled-pattern in senescence (emptying the interchromatin space), reminiscent of that obtained upon treating cells with an RNA polymerase II inhibitor (Chandris et al., 2010). The importance of RNA-binding proteins (RBP) for the stability or decay of senescence-associated genes has been reviewed elsewhere (Wang, 2012). For instance the RBPs AUF1 and HuR have been shown to negatively regulate senescence by promoting p16 mRNA decay and increase the stability of mRNAs encoding cyclins A and B, respectively, therefore promoting cell cycle progression (Guo et al., 2010; Wang et al., 2001). Conversely the RBP CUGBP1 promotes p21 mRNA stability and translation (Iakova et al., 2004). In the present work, a pattern of redistribution of proteins involved in RNA processing from an essentially chromatin-associated to an exclusive nucleoplasmatic pattern was observed in senescence (**Figure 19**). While we cannot interpret these results in light of the current knowledge, it suggests, to a certain degree, a readjustment of the RNA processing machinery during OIS. Interestingly, several Far Upstream element-Binding factors, involved in RNA binding, translation and mRNA processing were identified within this group of proteins relocating from the chromatin into the nucleoplasm (Davis-Smyth et al., 1996; Malz et al., 2009; Weber et al., 2008; Zhang et al., 2013; Zhang and Chen, 2013). FUBP1 and FUBP3 regulate *c-myc* by binding to its promoter and an additional role for FUBP1 in regulating p21 mRNA translation and stability has been reported (Rabenhorst et al., 2009). Whether these results mirror dysfunctional RNA processing or just a remodeling of the transcriptional complexes bound to the DNA in order to tune

gene expression to facilitate senescence cannot be concluded, however would be interesting to explore and understand.

Also, factors involved in both protein synthesis and catabolism change subcellular localization in senescence (**Figure 21**). These proteins accumulate in the nuclear soluble fraction in growing cells acquiring a strict cytoplasmic localization during OIS. While paradoxical, protein synthesis and degradation cooperate to quality control damaged proteins that tend to accumulate as well as to allow the morphological and metabolic changes that take place during senescence (Charmpilas et al., 2014; Narita, 2010; Narita et al., 2011). Most of the protein synthesis-associated factors detected in the cytoplasm during senescence were ribosomal proteins (**Figure 21**). Considering that protein translation occurs partly in the cytoplasm this increased accumulation of ribosomal proteins in the cytosol might reflect an increased protein synthesis during senescence.

Macro-autophagy is the main catabolic event in OIS and occurs in autophagosomes in the cytoplasm (Salama et al., 2014). The results herein presented show that within the group of proteins shuttling from the nucleus to the cytoplasm during senescence some were related with the proteosome. Gamerdinger et al. have described the autophagic degradation of polyubiquitinated proteins during replicative senescence. The polyubiquitin-binding protein SQSTM has been shown to play a role in this process and is found, in our data, to localize in the cytoplasm of senescent cells as opposed to the proliferating counterparts (Gamerdinger et al., 2009). Hence, the shift of ubiquitin-associated processes, from the nucleus to the cytoplasm could mirror the change of the proteolytic pathways in senescence.

Finally, we also observed relocalization of cytoskeleton-related proteins to the nuclei (**Figure 22**). One of the main characteristics of senescent cells is their enlarged and flattened morphology (Serrano et al., 1997). The enlarged senescent phenotype is partly due to the increased expression of vimentin (Nishio and Inoue, 2005; Nishio et al., 2001). Specifically, the cytoskeleton of senescent fibroblasts contained 3 times more vimentin than that of young fibroblasts (Nishio and Inoue, 2005; Nishio et al., 2001). Our proteomics data show that ARP3, MAP1A and CAPG, all proteins with actin-binding properties,

accumulate in the nuclei of senescent cells. Indeed Kwak et al. had reported the nuclear accumulation of globular actin during replicative and Ras–induced senescence (Kwak et al., 2004). There is, therefore a demand in understanding how and why cell shape is altered in senescence, and looking into differential expression or subcellular localization of cytoskeleton-related proteins might provide a starting point.

Overall the aforementioned results show several proteins involved in basic cellular processes change subcellular localization during OIS. Future experiments should validate these observations though immunofluorescence or by immunoblotting of proteins in fractionated cells.

4.6.3 Glycosylation: a novel hallmark of senescence?

Glycosylation, an enzymatic reaction where a polysaccharide is attached to a protein or lipid, is the most common post-translational mechanism for protein modification, contributing to an increased diversity and specialization of target molecules (Lisowska et al., 2008). The mammalian glycome repertoire is very diverse and encompasses between hundreds and thousands of glycan structures, divided in N-glycans, O-glycans, and glycosaminoglycans, depending on the anchoring residue (Ohtsubo and Marth, 2006). We observed an enrichment of factors involved in both protein and lipid glycosylation, in the cytoplasm of senescent cells (cluster1, **Figure 27**). That was the case of the proteins ALG1, ALG3, MGAT5, MPU1, OSTC, RPN2 and STT3B mostly involved in N-linked glycosylation in the endoplasmic reticulum. Interestingly, analysis of intracellular relocalization of proteins during senescence also suggested the shuttling of the factors STT3A, RPN1, MA2A1 and STT3B, involved in N-linked glycosylation, from the chromatin to the cytoplasm during OIS (**Figure 20**). STT3B, detected in both groups, is the catalytic subunit of the N-oligosaccharyl transferase (OST) involved in co-translational and post-translational N-glycosylation of target proteins (Ruiz-Canada et al., 2009).

Protein glycosylation take takes place mainly in the Golgi apparatus and endoplasmic reticulum (ER). For instance, proteins undergoing N-linked

glycosylation are initially modified in the ER (where also the polypeptide chain is produced) and then further processed in the Golgi, two organelles belonging to the cellular endomembrane system (Helenius and Aebi, 2004; Ohtsubo and Marth, 2006). As we did not control for subcellular localization of these organelles it is possible that the observed differences could arise from variations in the fractionation of the endomembrane system of senescent and growing cells. Nevertheless, free nuclear and cytoplasmic glycosylation has also been reported. Thus, further experiments should try to underscore not only whether the observed differences correspond to real subcellular protein shuttling during OIS and what organelles are involved (Fujiki et al., 2011; Funakoshi and Suzuki, 2009).

Altered glycosylation has been associated with several diseases including cancer (Christiansen et al., 2014; Hakomori and Handa, 2002; Lisowska et al., 2008; Ohtsubo and Marth, 2006). Changes in glysosylation have also been reported in aging (Fulop et al., 2008; Vanhooren et al., 2011). For instance, Vanhooren et al. reported altered N-glycosylation and increased fucosylation in aging mice (Vanhooren et al., 2011). Additionally, alterations and accumulation of glycoproteins in the brain during aging has been discussed, suggesting, together with the results herein presented a possible role for glycosylation in senescence (Sato and Endo, 2010).

4.6.4 Deep remodeling of the chromatin-associated protein landscape during OIS

Senescence encompasses major changes at the chromatin level, including epigenetic remodeling of the *INK4/ARF* locus and SAHF formation (Adams, 2007; Dimauro and David, 2009; Narita, 2007; O'Sullivan and Karlseder, 2012). Recent work has given insights into nuclear spatial rearrangement and the formation and structural complexity of the SAHFs (Chandra et al., 2015; Chandra et al., 2012; Cruickshanks et al., 2013; De Cecco et al., 2013; Swanson et al., 2013). These reports showed senescence encompasses major high-order chromatin remodeling events that implicate an overall decondensation, detachment from the nuclear lamina and clustering of

decondensed regions leading to loss and gain of local and distant contacts between genome areas, respectively (Chandra et al., 2015; Chandra et al., 2012; Cruickshanks et al., 2013; De Cecco et al., 2013; Swanson et al., 2013).

The data presented here reinforced the concept of a deep rearrangement of the chromatin during senescence. Indeed, in contrast to the overall conserved protein landscape observed in the cytoplasm and nuclear soluble fraction, the nuclear insoluble fraction of senescent cells showed a completely different pattern of protein expression when compared to that of proliferating cells **(Figure 17)**. Two of the 3 principle clusters identified by the C3D method were composed of proteins enriched in (cluster 3) or depleted (cluster 2) from the senescent chromatin (**Figure 28**, **Figure 32**).

Analysis of the two chromatin-related clusters allowed the identification of senescence-associated proteins. That was the case for several members of the SWI/SNF complex that were enriched in the chromatin during senescence (**Figure 35**). The SWI/SNF complex regulates nucleosome positioning to modulate transcription (Wilson and Roberts, 2011). Members of this complex can induce senescence, are overexpressed in senescent cells and localized to the chromatin in our mass spectrometry analysis (Burrows et al., 2010; Dunaief et al., 1994; Kia et al., 2008).

An additional senescence-associated protein enriched in the chromatin during senescence is EP300 (also known as p300). Moreover, EP300 was excluded from the chromatin in normal cells suggesting intracellular shuttling (**Figure 18).** p300 is a transcriptional co-activator that regulates transcription via chromatin remodeling through its activity as a histone acetyltransferase, ultimately regulating cell growth, differentiation and senescence (Iyer et al., 2004; Prieur et al., 2011; Yan et al., 2013). The inhibition of the HAT activity of p300 causes senescence both in fibroblasts and in melanoma cell lines (Yan et al., 2013). While decreased expression of p300 has been previously reported in cells undergoing Ras-induced senescence, we do not observe such a decrease. This could be due to cell type variations (the previous study used BJ and TIG3 fibroblasts), to the time window chosen for the proteomics analysis or even to the use of an inducible system as opposed to the

constitutive expression of RAS. Also, Prieur et al. showed that the inhibition of the HAT activity of p300 does not affect the chromatin-binding activity of the protein but cells still undergo cell cycle arrest, suggesting p300 might have additional HAT independent functions at the chromatin level of relevance for senescence (Prieur et al., 2011). It is relevant, to mention that additional reports suggest that p300 can positively regulate senescence via p53 acetylation (Avantaggiati et al., 1997; Grossman, 2001; Pedeux et al., 2005).

SAHF formation is one of the main hallmarks of senescence and some of its components are induced as cells undergo senescence (Rai and Adams, 2012; Salama et al., 2014). Consistent with previous reports and with their role in SAHF formation we also observed enrichment of HMGA2 and macroH2A and depletion of several H1 variants from the chromatin during OIS (**Figure 30, Figure 35**,data not shown) (Catez et al., 2006; Funayama et al., 2006; Maehara et al., 2010; Narita et al., 2006; Narita et al., 2003; Sporn et al., 2009; Zhang et al., 2005).

Epigenetic changes during senescence are partly due to a remodeling of the nuclear lamina. Indeed the premature aging syndrome HGPS is characterized by accumulation of mutated LMNA (LAΔ50) leading to altered nuclear shape and heterochromatin loss (Eriksson et al., 2003; Goldman et al., 2004; Huang et al., 2008; Shumaker et al., 2006). Our data shows a considerable decrease in the expression of LMNA in the chromatin fraction during senescence **(Figure 31)**. Previous work has shown that overexpression of wild type lamin A led to premature aging in human fibroblasts (Huang et al., 2008). Moreover, lamin A positively regulates pRb (Johnson et al., 2004). Although this might go at odds with the decreased expression of lamin A in the proteomics data, it is conceivable that are alterations in the levels of expression in general, rather than the directionality of those changes that induces deterioration of the nuclear envelope and consequent aging. Additionally, we also observed a slight decrease in the levels of another nuclear lamina component, lamin B1, in the chromatin of senescent cells (**Figure 31**). Decreased levels of lamin B1 were previously reported in senescence (Barascu et al., 2012; Dreesen et al., 2013; Freund et al., 2011; Sadaie et al., 2013; Shah et al., 2013). This downregulation contributes to the rearrangement of the chromatin marks

H3K4me3 and H3K27me3 (Sadaie et al., 2013; Shah et al., 2013). Despite the global decrease in lamin B1 levels, some lamin B1 clusters persist in H3K27me3 enriched areas suggesting the maintenance of a pool of lamin B1 to control gene expression during senescence (Chandra et al., 2015; Sadaie et al., 2013). Nevertheless, while senescent cells show low levels of *LMNB1,* overexpression of lamin B1 has also been shown to induce senescence (Barascu et al., 2012; Dreesen et al., 2013). It is therefore important to understand the dynamics of lamin B1 levels in senescence.

Chromatin rearrangement during senescence encompasses alterations of the histone pattern as well. Previous work has shown that histone-containing chromatin fragments disseminate into the cytoplasm via a nuclear-to-cytoplasm chromatin blebbing mechanism (Ivanov et al., 2013). Additionally, reduced biosynthesis of H4 and H3, particularly of variants H3.1 and H3.2, was observed during aging of both yeast and human cells and in differentiating and mature rat neurons, and histone overexpression extended yeast lifespan (Corpet et al., 2014; Feser et al., 2010; O'Sullivan et al., 2010; Pina and Suau, 1987). Also, decreased expression of H2A variants during drug-evoked senescence has been reported (Lopez et al., 2012). Conversely, H3.3 and its cleaved form, H3.3cs1, are incorporated into the senescent chromatin by the HUCA complex (HIRA/UBN1/CABIN1/ASF1a) and induce senescence (Adams, 2007; Corpet et al., 2014; Duarte et al., 2014). In this work we observed reduced expression of H2A and H2B variants in the senescent chromatin reinforcing the previous observations for these histones (Lopez et al., 2012). However, we additionally detected an enrichment of both H4 and histone H3 variants H31T and H3.2 in the chromatin of senescent cells, what go at odds with previous reports (**Figure 36**). One possible explanation for this could be differences in the type of senescence, as previous studies used yeast and human cells undergoing replicative senescence (Feser et al., 2010; O'Sullivan et al., 2010). Indeed, while previous reports showed negative correlation between Asf1 levels and replicative senescence, this protein is important for SAHF formation during OIS (Feser et al., 2010; O'Sullivan et al., 2010; Zhang et al., 2005; Zhang et al., 2007). Further analysis is needed to validate the results herein presented

and to better understand the relevance of histone H4 and H3 variants across several types of senescence.

The proteomics analysis allowed the detection of additional protein families enriched in the senescent chromatin: exportins XPO2 and XPOT and members of the integrator complex INT1, INT7 and INT10 (**Figure 36**). Exportins are involved in the transport of macromolecules from the nucleus into the cytoplasm (Okada et al., 2008). While Exportin 2 (XPO2) is involved in the export of importin-α and has a role in apoptosis and cancer, XPOT mediates tRNAs export progression (Behrens et al., 2003; Kutay et al., 1997; Kutay et al., 1998; Kuwabara et al., 2001; Tai et al., 2010). The localization of both proteins in the nuclear insoluble fraction could suggest a role in the regulation of chromatin-associated processes, what has been observed for additional members of this family (*i.e.* XPO7) (Hattangadi et al., 2014). Nevertheless, localization of these nuclear membrane proteins to the chromatin-containing fraction could consist of an artifact of the fractionation procedure, thus further experiments are needed to understand whether these exportins bind the chromatin and what role play in OIS.

Finally, the Integrator is a multiprotein complex that associates with the C-terminal domain of RNA polymerase II large subunit and mediates 3' end processing of snRNAs (Baillat et al., 2005; Chen and Wagner, 2010). Recent work showed that in gamma-irradiated osteosarcoma cells INT7 localized to the sites of DNA damage (Cotta-Ramusino et al., 2011). INTS7 knockdown led to proliferation arrest bypass suggesting a possible role in irradiation-induced senescence (Cotta-Ramusino et al., 2011). Would be relevant to understand how INT7 contributes to senescence and to what extent other components of the complex, as for instance INT1 and INT10 are involved.

4.6.5 Expression meets function: GTF3C4, a novel OIS regulator

In order to investigate the functional relevance of the changes in expression associated with the chromatin, a small siRNA library was created to target a subset of genes detected on cluster 3. Knockdown of PBRM1, HMGB1 and GTF3C4 led to a bypass of the cell cycle arrest, while siRNAs targeting

PBRM1, ACTL6A, INTS7 and EP400 downregulated p16 expression, therefore suggesting a pairing between altered expression and function, which was further confirmed though analysis of one of the hits (**Figure 37).** GTF3C4, (or TFIIIC90) is a member of the multisubunit complex TFIIIC, which is involved in Polymerase III-mediated transcription (Kundu et al., 1999). Specifically, RNA Pol III mediates transcription of small RNA molecules involved in basic metabolic processes such as components of the protein synthesis apparatus (*i.e.* 5S RNA) and components of the splicing, tRNAs and other RNAs of unknown function (Schramm and Hernandez, 2002). At least six subunits of the TFIIIC complex have been well described to date, and work together to recognize and recruit RNA Pol III, via tethering of TFIIIB, to target promoters (Dumay-Odelot et al., 2007; Schramm and Hernandez, 2002). In addition to the previous functions, GTF3C4 is one of the 3 subunits detaining an intrinsic histone acetyltransferase (HAT) activity towards free and nucleosomal H3 which is thought to alleviate chromatin repression and allow assembly of RNA Pol III and pre initiation complex onto gene promoters (Hsieh et al., 1999; Kundu et al., 1999). Whether GTF3C4 regulates transcription of genes other than those transcribed by RNA Pol III, is not known.

We observed that GTF3C4 is associated with the chromatin during senescence (**Figure 38a**) Unfortunately, no appropriate GTF3C4 antibody was found, therefore the subcellular localization has not been confirmed by immunoblotting.

In the present work, GTF3C4 knockdown bypassed the cell cycle arrest and slightly affected p16 expression and SAHF formation during OIS (**Figure 38; Figure 39**). The expression of additional senescent mediators (p53, p21, 53BP1 and IL8) seemed unaffected by GTF3C4 knockdown suggesting that its function in senescence does not encompass the regulation of the DDR signaling or production of the SASP. One hypothesis would be that GTF3C4 could regulate the *INK4/ARF* locus, affecting p15 rather than p16. Interestingly, TFIIIC has been shown to hold RNA Pol III transcription independent functions (Donze, 2012). Noma et al. showed that the complex TFIIIC could bind the DNA adjacent to heterochromatic regions to help

prevent the spread of heterochromatin in yeast (Noma et al., 2006). In 2013, a review by Kirkland et al. elongated on the potential role of TFIIIC as both barrier and enhancer-blocking insulator, therefore contributing for high-order chromatin changes (Kirkland et al., 2013). It is conceivable that GTF3C4, as part of the TFIIIC complex could contribute to the restriction of the SAHF or even for a potential long-range enhancer-mediated promoter regulation in senescence, but all is rather speculative. In fact, GTF3C4 has not yet been directly linked with senescence.

Nevertheless, recent evidence showed that inhibition of ribosomal biogenesis leads to accumulation of free nascent 5S rRNA, that together with ribosomal proteins RPL5 and RPL11, binds to MDM2 (HMD2) and inhibits its interaction with p53, ultimately activating the latter (Donati et al., 2013; Sloan et al., 2013). This is further potentiated by $p14^{ARF}$ that inhibits ribosome biogenesis (Sloan et al., 2013). Moreover, this p53 activation by the 5S RNP complex, consisting of RPL11, RPL5, and 5S rRNA, has been recently shown to mediate both OIS and replicative senescence in MEFs, due to impaired ribosomal biogenesis, thus suggesting that 5S rRNA, a RNA Pol III gene can mediate senescence (Nishimura et al., 2015).

Also, ERK and c-Myc have been show to stimulate RNA Pol III transcription of tRNAs and 5S rRNA, by mediating phosphorylation or by direct binding to TFIIIB, respectively (Felton-Edkins et al., 2003; Goodfellow and White, 2007; Kenneth et al., 2007; Mauger and Scott, 2004). This has been suggested to account for proliferation-independent cell growth and hypertrophic growth, which are typical of senescence (Goodfellow and White, 2007; Mauger and Scott, 2004). However, reports against RNA Pol III genes positively regulating senescence exist. In one hand, DNA damage signaling pathway induces TFIIIB downregulation and consequent Pol III transcription inhibition (Ghavidel and Schultz, 2001; Schultz, 2003). In the other hand, several tumour suppressors including pRb and p53 have been implicated in repressing RNA Pol III transcribed genes either by inhibiting transcription of members of the RNA Pol III transcriptional machinery or by blocking the binding of TFIIIB and TFIIIC2 to target promoters (Bhargava et al., 2013; Cairns and White, 1998; Chesnokov et al., 1996; Dumay-Odelot et al., 2010; Eichhorn and Jackson,

2001; Hsieh et al., 1999). Nonetheless, GTF3C4 containing complex (TFIIIC) does not seem to suffer from p53-mediated repression (Cairns and White, 1998; Eichhorn and Jackson, 2001). Thus, the scenario regarding RNA Pol III genes in senescence is rather complex and possibly context dependent. The observation that it can be involved in proliferation-independent cell growth and that it is involved in p53 activation suggests a role in senescence (Nishimura et al., 2015). However, the relative contribution of the TFIIIC complex, and its subunit GTF3C4, remains to be elucidated. Indeed, we failed to observe an effect on p53 upon GTF3C4 knockdown, suggesting it could mediate other pathways in OIS. Thus, many questions remain: Does it affect other types of senescence or does it depend on RAS activation? Is it involved in other situations of cell cycle arrest (*e.g.* quiescence)? Is it acting in the context of the TFIIIC complex and is it HAT activity necessary? Answering all these questions would be of relevance to better understand the role of GTF3C4 in senescence.

Chapter 5. siRNA screen for the identification of chromatin and nuclear factors regulating OIS

Our mass spectrometry analysis revealed profound changes in the chromatin of cells undergoing OIS. Indeed, two clusters comprising chromatin-associated proteins were differentially expressed in senescence. Additionally, a small siRNA screen for the bypass of OIS revealed that some of these proteins could regulate senescence. That was the case of GTF3C4, here validated as a potential regulator of OIS. These results prompted us to perform a larger siRNA screen with the purpose of identifying novel chromatin-remodeling factors controlling OIS.

5.1 Setting up an siRNA screen for the bypass of OIS

A custom siRNA library (QIAGEN), targeting 456 different chromatin-remodeling factors, was distributed in twenty 96-well plates and each gene was targeted by two independent siRNAs in sister plates. Since wells located on the plate edge are subjected to more adversities than those in the center of the plate (an artifact known as “edge effect”), the siRNA library has been spotted onto the plate excluding the outermost rows and columns (**Figure 42**). Instead, wells situated at the edge of the plate were used for mock control (cells + transfection reagent only) as well as for monitoring the induction of senescence upon treatment of cells with 4-hydroxy-tamoxifen (**Figure 42**). Briefly, cells were reverse-transfected with the siRNAs in triplicate plates and treated with 4-OHT the day after. Cells were fixed 4 to 5 days post induction, and subjected to immunofluorescence using the high throughput microscope IN Cell Analyzer 2000 (**Figure 42**).

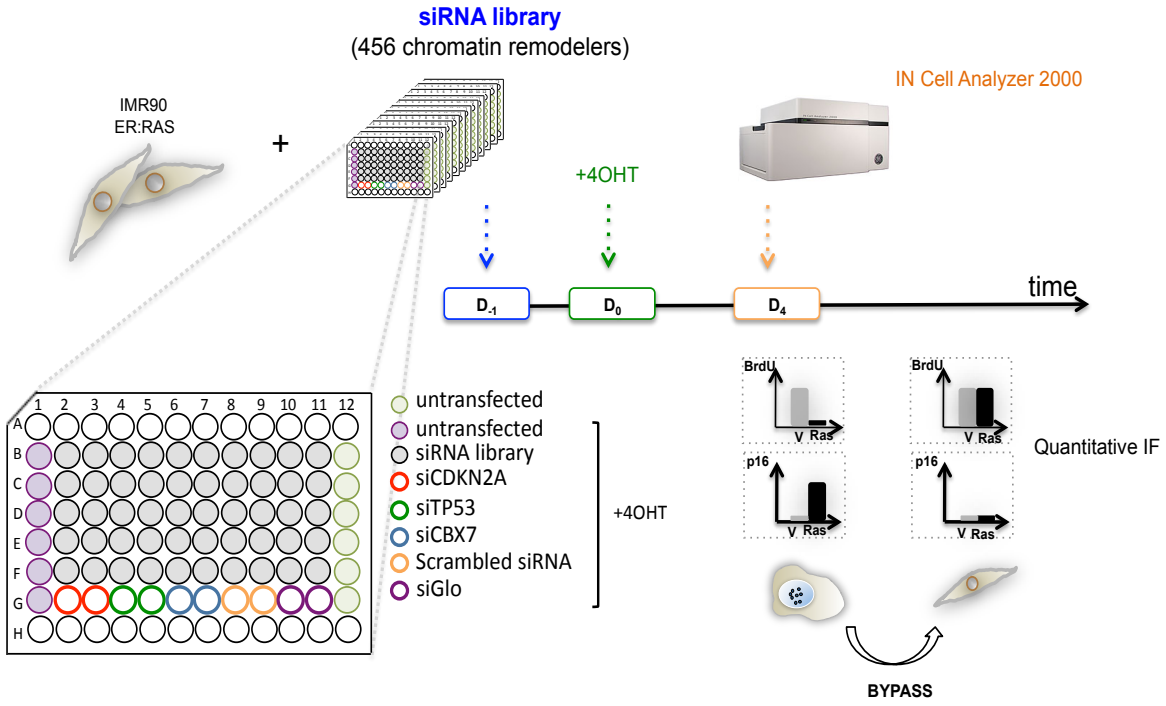


Figure 42. siRNA screen workflow. An siRNA library targeting 456 different genes was spotted onto 96- well plates. Plate edges were avoided, being used for siRNA-free senescent (pink) and proliferating (green) cells (mock control). As proof-of-principle controls, scrambled siRNAs (yellow wells) and siRNAs targeting known regulators of senescence - CDKN2A, TP53, and CBX7 - were spotted (red, green and blue wells). A fluorochrome-combined siRNA was also aliquoted in order to monitor transfection (purple). IMR90 ER:RAS fibroblasts were transfected with the library, treated with 4-OHT the day after and fixed 4 days post Ras induction. Fixed cells were subsequently stained for immunofluorescence to assess BrdU and p16 expression and analyzed by HCA, using the IN Cell Analyzer 2000.

As readout for the screen, we measured BrdU incorporation at day 4 post induction. As previously mentioned, IMR90 ER:RAS cells undergo a stable cell cycle arrest around 4 days post treatment with 4-OHT **(Figure 13).**

In addition to the cell cycle arrest, senescent cells upregulate the expression of cell cycle inhibitors (p16, p21, p53), DDR effectors (γH2Ax or 53BP1) or the SASP (Il8, IL6) **(Figure 13)**. Owing to the functional relevance of INK4a induction for oncogene-induced senescence, as well as the robustness of p16 expression we decided to use it, together with BrdU as a readout marker for the loss-of-function screen **(Figure 14, Figure 15).** Expression of both markers was analyzed by immunofluorescence 4 days after treating cells with 4-OHT, and quantified using the IN Cell Analyzer 2000.

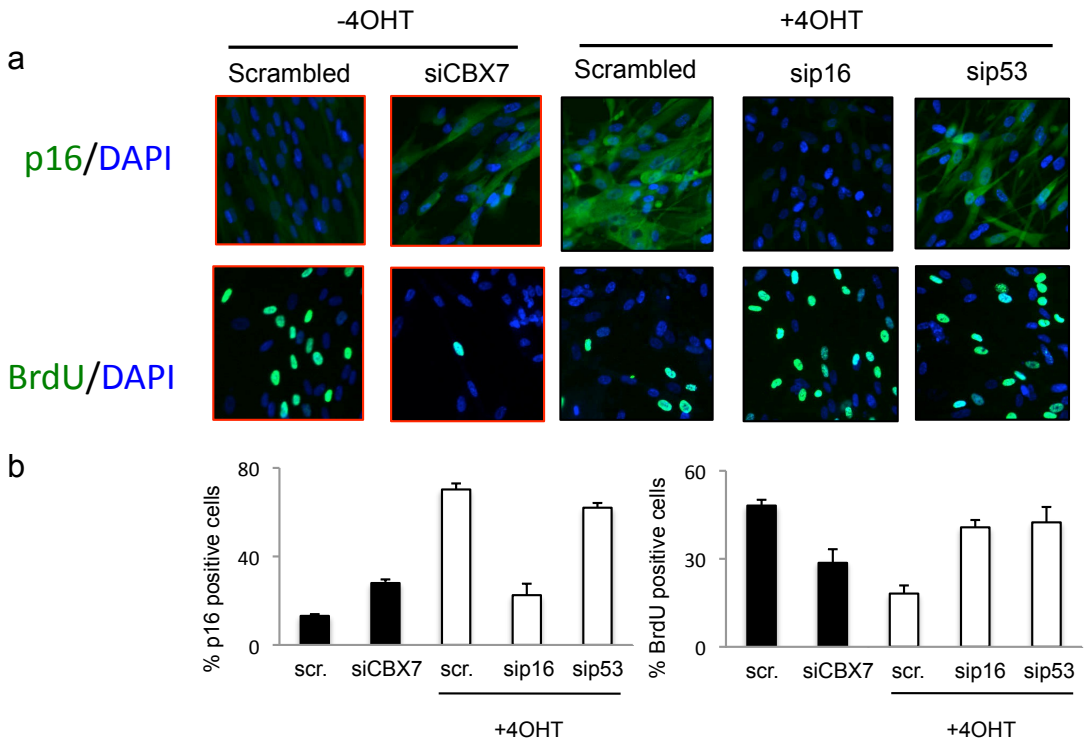


Figure 43. Control siRNAs and readouts for the bypass of senescence at day 4 post induction with 4-OHT. a) IMR90 ER:RAS fibroblasts targeted either with scrambled siRNAs (All Stars, QIAGEN), siCBX7, siCDKN2A and siTP53 were stained with specific antibodies against BrdU and p16, subjected to IF and representative images were collected with the IN Cell Analyzer 2000 automated high-throughput microscope **b)** At least 1000 cells from each condition were counted and the percentage of positive cells was calculated for each marker using the IN Cell Investigator software.

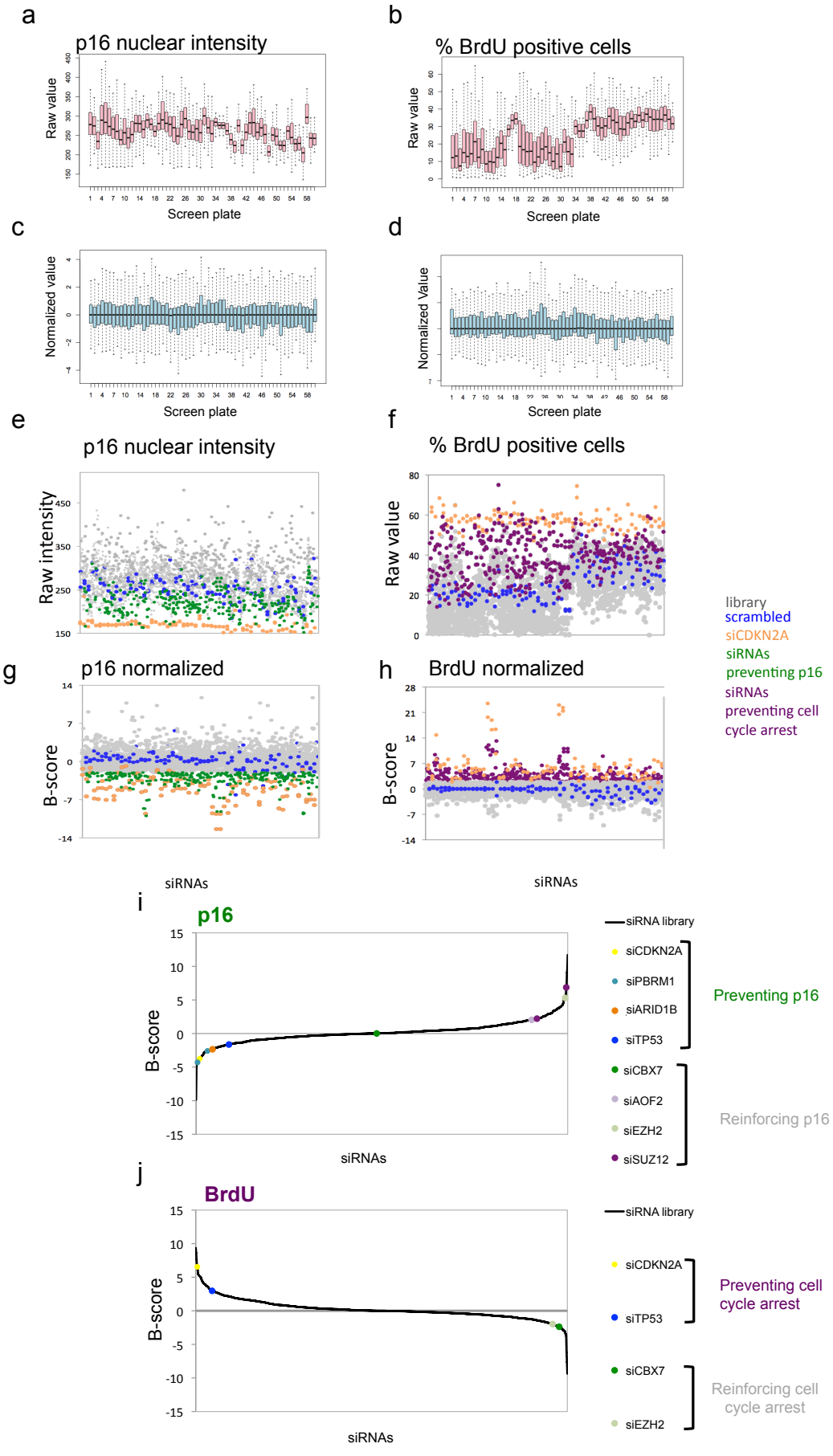
To assess the feasibility of the screen, we used siRNAs against known senescence regulators (CBX7, p16 and p53) (**Figure 42**).

Upon treatment with 4-OHT IMR90 ER:RAS transduced with the scrambled siRNA underwent senescence as measured by increased p16 expression and decreased BrdU incorporation (**Figure 43**). In agreement with its ability to repress the *INK4/ARF* locus, knockdown of CBX7 in non-induced proliferating cells upregulated p16 expression and decreased the percentage of cells incorporating BrdU (**Figure 43**). p16 was efficiently knocked down with siCDKN2A. The downregulation of p16 was accompanied by an increase in the percentage of cells incorporating BrdU at day 4, post treatment with 4-OHT (**Figure 43b**). Similarly, siRNA against p53 led to a bypass of OIS, as measured by the increased percentage of proliferating cells (**Figure 43b**).

The siRNA library consisted of twenty 96-well plates, each containing approximately 60 siRNAs targeting chromatin factors and two replicate siRNAs targeting p16, p53 and CBX7 as well as two scrambled sequences and siGlo, a fluorochrome labeled siRNA, to monitor transfection.
Triplicate plates were used and therefore IMR90 ER:RAS cells were reverse transfected on 60 independent plates. The day before fixing, cells were incubated with BrdU and plates were fixed at day 4 post 4-OHT treatment. Expression of p16 and BrdU was analyzed by immunofluorescence using the IN Cell Analyzer 2000. Expression of readout markers was quantified by high content analysis with the In Cell Investigator software. Raw p16 intensity as well as the percentage of BrdU and p16 expressing cells was calculated for at least 1000 cells per well.

5.2 Primary screen identified a group of potential OIS regulators

Once p16 and BrdU expression was quantified we normalized the data to allow for inter-plate comparison. Percentage of cells incorporating BrdU and p16 expression, as measured by the In Cell Investigator software, were therefore normalized using B-scores, which similarly to Z-scores, use population measurements, in this case the median and median absolute deviation (MAD). Additionally, it incorporates an algorithm that accounts for positional effects (row and column) while being resistant to outliers (Birmingham et al., 2009; Brideau et al., 2003). The B-score was calculated using the software *Web cellHTS2* (http://web-cellhts2.dkfz.de/cellHTS-java/cellHTS2/ (Pelz et al., 2010). To verify the effect that normalization had on the expression of p16 and BrdU, we plotted the average expression of both markers per plate before and after normalization, using the cellHTS2. When the mean raw intensity of p16 and BrdU was calculated per plate we noted inter-plate variability (**Figure 44a,b**). B-score normalization corrected for this inter-plate variability, allowing for more accurate comparison between plates (**Figure 44c-d**).


a
p16 nuclear intensity
Raw value
Screen plate
b
% BrdU positive cells
Raw value
Screen plate
c
Normalized value
Screen plate
d
Normalized Value
Screen plate
e
p16 nuclear intensity
Raw intensity
f
% BrdU positive cells
Raw value
library
scrambled
siCDKN2A
siRNAs
preventing p16
siRNAs
preventing cell
cycle arrest
g
p16 normalized
B-score
siRNAs
h
BrdU normalized
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B-score
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siRNA library
siCDKN2A
siPBRM1
siARID1B
siTP53
Preventing p16
siCBX7
siAOF2
siEZH2
siSUZ12
Reinforcing p16
j
BrdU
B-score
siRNAs
siRNA library
siCDKN2A
siTP53
Preventing cell
cycle arrest
siCBX7
siEZH2
Reinforcing cell
cycle arrest

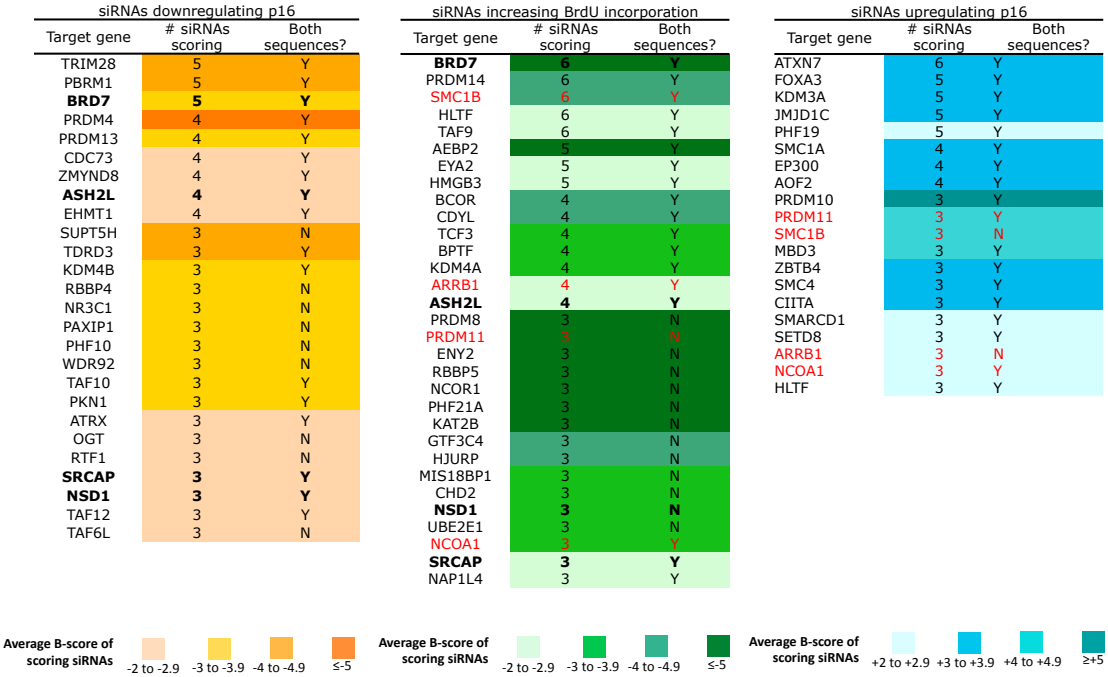
Figure 44. Data normalization and quality control of the screen. A spreadsheet containing raw p16 intensities and the percentages of BrdU positive cells was uploaded onto the Cell-HTS2 website and the B-score was calculated for each individual well (siRNA) per screen plate. a-d) Final web Cell-HTS2 output, showing that upon normalization the inter-plate variability is removed. Shown values correspond to the average of all data points for p16 and BrdU per plate before and after normalization. e-h) Individual siRNA values for raw and normalized p16 expression and BrdU incorporation for all data points across the 60 screen plates. Blue dots represent scrambled siRNA, yellow dots indicate siCDKN2A, green color-codes library siRNAs with a B-score ≤ -2 and purple represent siRNAs showing a B-score of ≥2. i-j) Normalized data ranked by B-score for p16 (i) and BrdU (j), individually. For representational purposes only, triplicates for each siRNA were averaged. Control siRNAs, CDKN2A, TP53 and CBX7 and respective outcome (bypass vs reinforcement) are shown. B-scores for additional library siRNAs targeting known regulators of senescence are shown, therefore working as internal controls.

Indeed, while control siRNAs (scrambled and siCDKN2A) along different plates displayed variable raw intensity of both p16 and BrdU, normalized expression values for each marker were much more comparable across plates (**Figure 44e-h**, yellow and blue dots). Hence, normalization made plates more comparable, allowing siRNAs of different plates to be ranked together and hits selected with basis on how different (how many standard deviations) they were from the median.

For further evaluation of the accuracy of the screen, we investigated the outcome of control siRNAs after normalization. As expected, when we specifically knocked down p16 (siCDKN2A) we observed a decrease in p16 expression as suggested by the resulting negative B-scores (**Figure 44g,i).** Concomitantly, siCDKN2A led to an increased proliferation represented by positive B-score values for BrdU (**Figure 44h,j).** Both, clearly standing out from the overall background B-scores (including those for the scrambled siRNAs) lying around zero, suggesting a successful knockdown and effective bypass of OIS. A similar outcome was observable for sip53 (**Figure 44i-j**). As previously mentioned, CBX7 is a known negative regulator of p16 expression and senescence. Although we did not see an additional upregulation of p16 when this Polycomb gene was knocked down (average B-score settled around zero), siCBX7 did lead to a further decrease in the percentage of proliferating cells, as suggested by the negative B-score value for BrdU. Additional siRNAs targeting senescence regulators were present in the library and affected p16 and BrdU accordingly. Consistent with their role as positive regulators of senescence, knockdown of the SWI/SNF genes

PBRM1 and ARID1B resulted in reduced p16 levels, as indicated by the negative B-score (**Figure 44i, j**) (Burrows et al., 2010; Kia et al., 2008). Likewise, siRNAs targeting the senescence inhibitors SUZ12 and EZH2 scored positively for p16, therefore suggesting a further upregulation of the *INK4/ARF* locus upon knockdown of these genes. Interestingly, knockdown of EZH2 resulted in a negative B-score for BrdU, indicative of an additional decrease in the percentage of proliferating cells, and therefore a reinforcement of OIS in those cells (**Figure 44i, j**) (Agherbi et al., 2009; Bracken et al., 2007; Bracken et al., 2003).

These results suggested that data normalization allowed for inter-plate comparison as intended as well as for the selection of genuine hits, as indicated by the detection of known regulators of senescence impacting on BrdU and p16 expression. Once we assessed the accuracy of the screen, we proceeded with the selection of candidates affecting senescence.



siRNAs downregulating p16

Target gene	# siRNAs scoring	Both sequences?
TRIM28	5	Y
PBRM1	5	Y
BRD7	**5**	**Y**
PRDM4	4	Y
PRDM13	4	Y
CDC73	4	Y
ZMYND8	4	Y
ASH2L	**4**	**Y**
EHMT1	4	Y
SUPT5H	3	N
TDRD3	3	Y
KDM4B	3	Y
RBBP4	3	N
NR3C1	3	N
PAXIP1	3	N
PHF10	3	N
WDR92	3	N
TAF10	3	Y
PKN1	3	Y
ATRX	3	Y
OGT	3	N
RTF1	3	N
SRCAP	**3**	**Y**
NSD1	**3**	**Y**
TAF12	3	Y
TAF6L	3	N

siRNAs increasing BrdU incorporation

Target gene	# siRNAs scoring	Both sequences?
BRD7	**6**	**Y**
PRDM14	6	Y
SMC1B	6	Y
HLTF	6	Y
TAF9	6	Y
AEBP2	5	Y
EYA2	5	Y
HMGB3	5	Y
BCOR	4	Y
CDYL	4	Y
TCF3	4	Y
BPTF	4	Y
KDM4A	4	Y
ARRB1	4	Y
ASH2L	**4**	**Y**
PRDM8	3	N
PRDM11	3	N
ENY2	3	N
RBBP5	3	N
NCOR1	3	N
PHF21A	3	N
KAT2B	3	N
GTF3C4	3	N
HJURP	3	N
MIS18BP1	3	N
CHD2	3	N
NSD1	**3**	**N**
UBE2E1	3	N
NCOA1	3	Y
SRCAP	**3**	**Y**
NAP1L4	3	Y

siRNAs upregulating p16

Target gene	# siRNAs scoring	Both sequences?
ATXN7	6	Y
FOXA3	5	Y
KDM3A	5	Y
JMJD1C	5	Y
PHF19	5	Y
SMC1A	4	Y
EP300	4	Y
AOF2	4	Y
PRDM10	3	Y
PRDM11	3	Y
SMC1B	3	N
MBD3	3	Y
ZBTB4	3	Y
SMC4	3	Y
CIITA	3	Y
SMARCD1	3	Y
SETD8	3	Y
ARRB1	3	N
NCOA1	3	Y
HLTF	3	Y

Figure 45. Primary screen identified 69 novel potential regulators of senescence. Genes for which 3 out of 6 siRNAs were leading to a B-score ≥ +2 or ≤ -2 were selected for each marker. A total of 69 genes were selected: 22 bypassing p16 expression only, 23 exclusively bypassing the cell cycle arrest, 4 bypassing both (bold), 16 reinforcing p16 expression and also 4 reinforcing p16 expression and concomitantly bypassing the cell cycle arrest (red).

To avoid the confounding effect of outliers we analyzed the 3 replicates individually. Therefore, and considering the library contained 2 different siRNAs per gene, we looked for how the 6 independent siRNAs per gene scored both both p16 and BrdU.

As a first step we filtered for individual siRNAs displaying a B-score of ≥+2 for BrdU and/or ≤ -2 for p16. We decided on two deviations from the median as it provided a satisfactory separation of those siRNAs from the background data, for both p16 and BrdU, being permissive enough to allow the detection of control siRNAs within its limits (**Figure 44e-j**). Interestingly, when we compared the distribution of the data points displaying a B-score of ≥2 for BrdU and/or ≤ -2 for p16, before and after normalization, we noted that normalizing allowed for a better distinction of those siRNAs from the general population of siRNAs (including scrambled siRNAs, **Figure 44e-h**).

"Bypass hits" were defined when a B-score of ≥+2 for BrdU or ≤ -2 for p16 was observed for at least 3 of the 6 individual siRNAs. Additionally, we also selected hits for the reinforcement of p16 when a B-score ≥+2 for p16 was verified for 3 out of 6 siRNAs. The final list of "bypass hits" encompassed a group of 22 genes whose knockdown prevented p16 induction, another subset of 27 genes whose silencing increased the percentage of BrdU incorporation and finally a group of 4 hits whose depletion concomitantly bypassed both p16 and BrdU (**Figure 45**). The list of hits whose knockdown reinforced p16 included 20 genes. Four of these genes, however, led to a concomitant bypass of the growth arrest (**Figure 45**). Altogether, sixty-nine genes for the bypass and/or reinforcement of BrdU and p16, respectively, passed the selective threshold and were picked for a secondary screen (**Figure 45**).

5.3 A secondary screen validated 25 genes regulating OIS

In order to validate the primary screen, and refine the candidates, 4 independent siRNAs for all 69 genes were obtained from QIAGEN creating a smaller library to conduct a secondary screen. The screen was conducted under the same conditions as previously described (**Figure 42**).

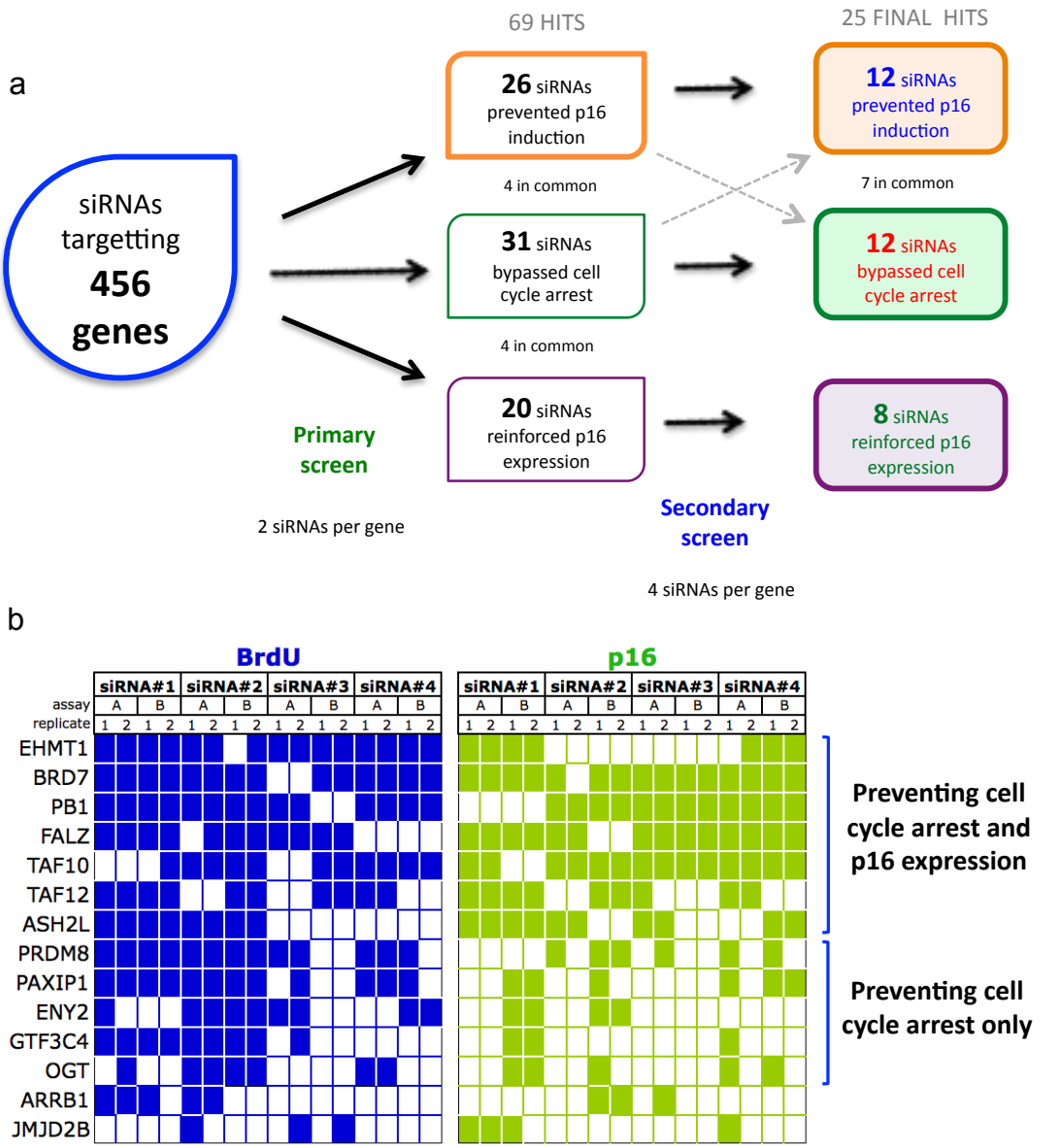


Figure 46. Secondary siRNA screen. A secondary screen was conducted under the same conditions as for primary screen using 4 different siRNAs per gene. Screen was performed twice (assays A and B) using duplicate plates each time. Because of this, each gene was targeted by 16 independent siRNAs. Genes were selected as hits when 6 out of 16 siRNAS led to a 20%-fold increase or decrease in BrdU or p16 expression compared to scrambled, respectively. a) Hit selection workflow: From 456 genes, 69 bypassed the selective threshold set for the primary screen (26 preventing p16 expression, 31 bypassing the cell cycle arrest and 20 reinforcing p16 expression, with some overlapping genes). Upon secondary screen with 4 different siRNAs per genes, only 25 out of these 69 were validated (12 preventing p16 induction and 12 bypassing the cell cycle arrest, with 7 genes in common, while 8 reinforced p16 expression). b) Heat map for hits bypassing OIS. Outcome of all 4 siRNAs per assay (A and B) and per duplicate are depicted. Colored squares represent a 20%-fold difference for BrdU (blue) or p16 (green) for a given siRNA compared to the scrambled sequence of the same plate. A hit was defined once 6 out of the 16 siRNAs led to 20%-fold increase in BrdU or decrease in p16, being that duplicates should score in parallel. All first 12 genes bypassed the cell cycle arrest with 7 (top) of them concomitantly bypassing p16 expression as well. ARRB1 and JMJD2B are shown as negative controls.

The secondary screen was performed twice (two independent runs) in duplicate each time. For selective purposes, we pooled the data from the two screens together, treating both runs, as well as the duplicate plates independently.

For each siRNA we calculated the fold change of the percentage of p16 or BrdU positive cells, always relative to the expression of those markers in cells transfected with the scrambled siRNA on the same plate. Since there were 4 different siRNAs per gene and the screen was performed twice, we analyzed how the 8 pairs of duplicate siRNAs scored.

Hits were defined when at 3 out of the 8 pairs of duplicate siRNAs per gene displayed a 20%-fold increase in BrdU incorporation or 20%-fold decrease in the percentage of p16 positive cells (**Figure 46b**). This yielded a group of 5 genes whose knockdown increased proliferation alone, another subset of 5 hits whose depletion downregulated p16 and other 7 genes concomitantly affecting both p16 and BrdU (**Figure 46a**).

In order to investigate siRNAS upregulating p16 expression we also performed the secondary screen using IMR90 fibroblasts. We found that knockdown of 8 genes induced a ≥20%-fold increase of the percentage of cells expressing p16 (**Figure 46a**).

The secondary screen rendered a functionally diverse group of genes, some of which had already been related with the establishment of senescence. For instance the Bromodomain containing 7 protein, BRD7 has been shown to regulate p53 transcriptional activity and to be essential for the establishment of replicative senescence (Burrows et al., 2010; Drost et al., 2010; Zhou et al., 2004). The previously mentioned transcriptional activator EP300 resurfaced again in the secondary screen as a negative regulator of OIS with siEP300 leading to a reinforcement of both p16 expression and cell cycle arrest (data not shown) (Yan et al., 2013).

Some of the candidates identified belonged to the same family or functional group. That was the case, for instance, of the PR domain containing proteins PRDM4, PRDM8 and PRDM10 and PRDM11, the first two preventing p16 and increasing BrdU incorporation, respectively, and the last two inducing p16; of the histone methyltransferases ASH2L and EHMT1, as well as of the

histone demethylases JMJD1C and KDM1A, whose knockdown reinforced p16 expression (**Figure 46**, data not shown). Another functional group consisted of general transcription factors, represented by TAF10 and TAF12 whose knockdown impaired p16 expression and bypassed the cell cycle arrest (**Figure 46b**).

The knockdown of 7 of the 25 genes, here exemplified by ASH2L, BPTF (also known as FALZ) and EHMT1, led to a downregulation of p16 expression with a concomitant increase in proliferation (**Figure 46b**, **Figure 47**). BPTF is a histone-binding component of the NURF complex (specific to H3K4me3), involved in nucleosome-remodeling while ASH2L and EHMT1 are involved in histone methylation (Li et al., 2006a; Steward et al., 2006; Tachibana et al., 2005).

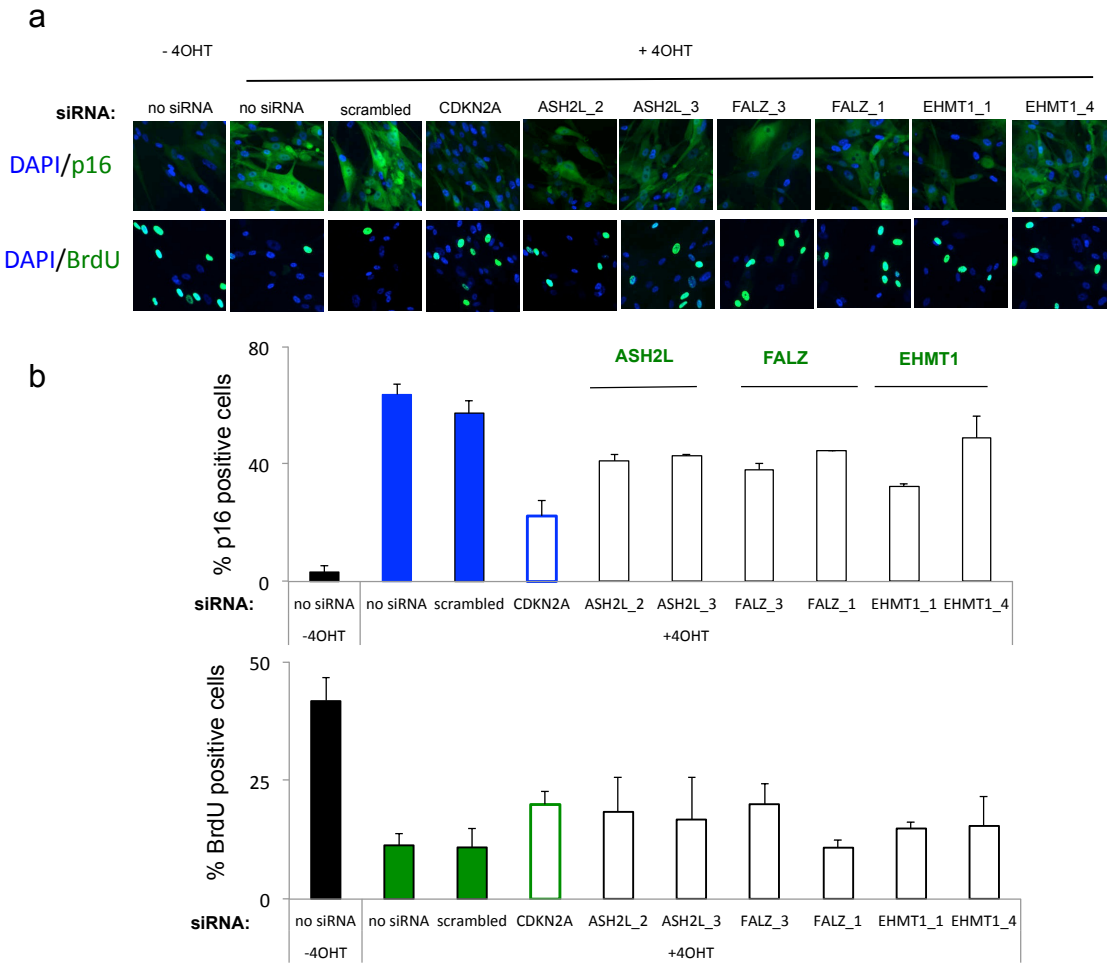


Figure 47. Example of hits preventing p16 induction and partially bypassing the cell cycle arrest. The knockdown of 7 genes led to a decreased p16 expression and increased BrdU incorporation. a) Immunofluorescence of p16 and BrdU, acquired with the high throughput microscope IN Cell Analyzer 2000. b) At least 1000 cells were counted and used to quantify the percentage of p16 and BrdU positive cells. ASH2L, FALZ (= BPTF) and EHMT1 are shown as examples.

Specifically, ASH2L is part of both SET1/Ash2 and MLL1/MLL complexes, involved in generating H3K4me3, whereas EHMT1 regulates the formation of H3K9me and H3K9me2 (Steward et al., 2006; Tachibana et al., 2005). Knockdown of all three genes, with at least 2 different siRNAs, downregulated p16 expression while increasing the proliferation of IMR90 ER:RAS cells treated with 4-OHT. These results suggested a possible role for these genes in the regulation of OIS, potentially via the p16/Rb pathway (**Figure 47**).

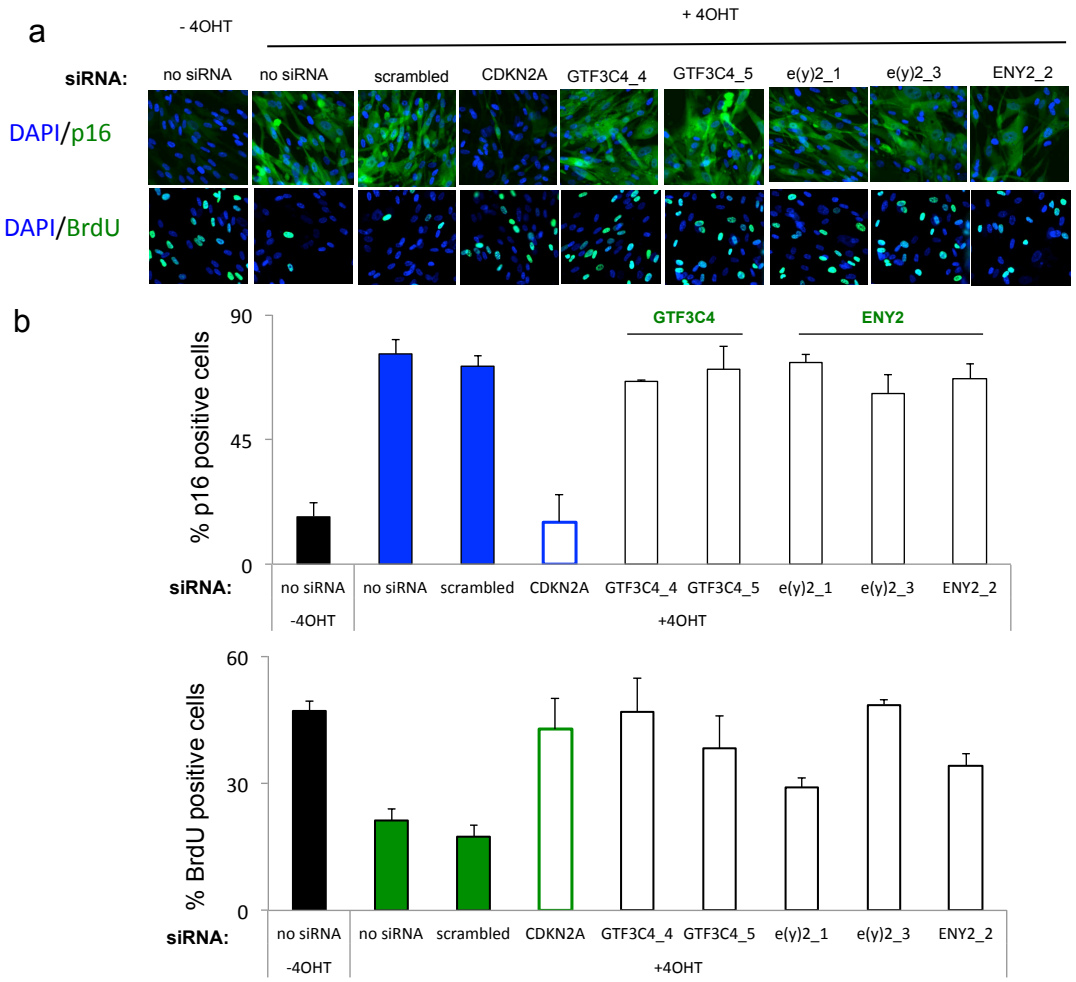


Figure 48. Example of hits bypassing the cell cycle arrest without affecting p16 expression. The knockdown of 5 genes induced a bypass of the cell cycle arrest, ENY2 and GTF3C4 are shown as examples. a) Immunofluorescence images of p16 and BrdU, collected by high content analysis. b) Quantification of the percentage of p16 and BrdU positive cells upon knockdown of ENY2 and GTF3C4.

An additional group of siRNAs increased the proliferation without affecting p16 expression. That was the case of siRNAs against ENY2, GTF3C4, OGT, PAXIP1 and PRDM8 (**Figure 46**). This group of genes includes factors with functions in histone acetylation and deubiquitination, DNA damage, histone

methylation and protein and histone O-GlcNAcylation (Eom et al., 2009; Fujiki et al., 2011; Galan and Rodriguez-Navarro, 2012; Hsieh et al., 1999; Wu et al., 2009). We have already shown, in our proteomics analysis, that GTF3C4 is upregulated during senescence, specifically localizing to the chromatin, and we have reported a potential functional role for this gene in OIS (**Figure 38-Figure 41**), once again reinforced with this experiment (**Figure 48**). For instance ENY2 is a member of the SAGA complex of histone acetyltransferases and its knockdown also increased the percentage of BrdU positive cells (**Figure 48**). This protein is also involved in H2B de-ubiquitination and mRNA export from the nucleus (Galan and Rodriguez-Navarro, 2012).

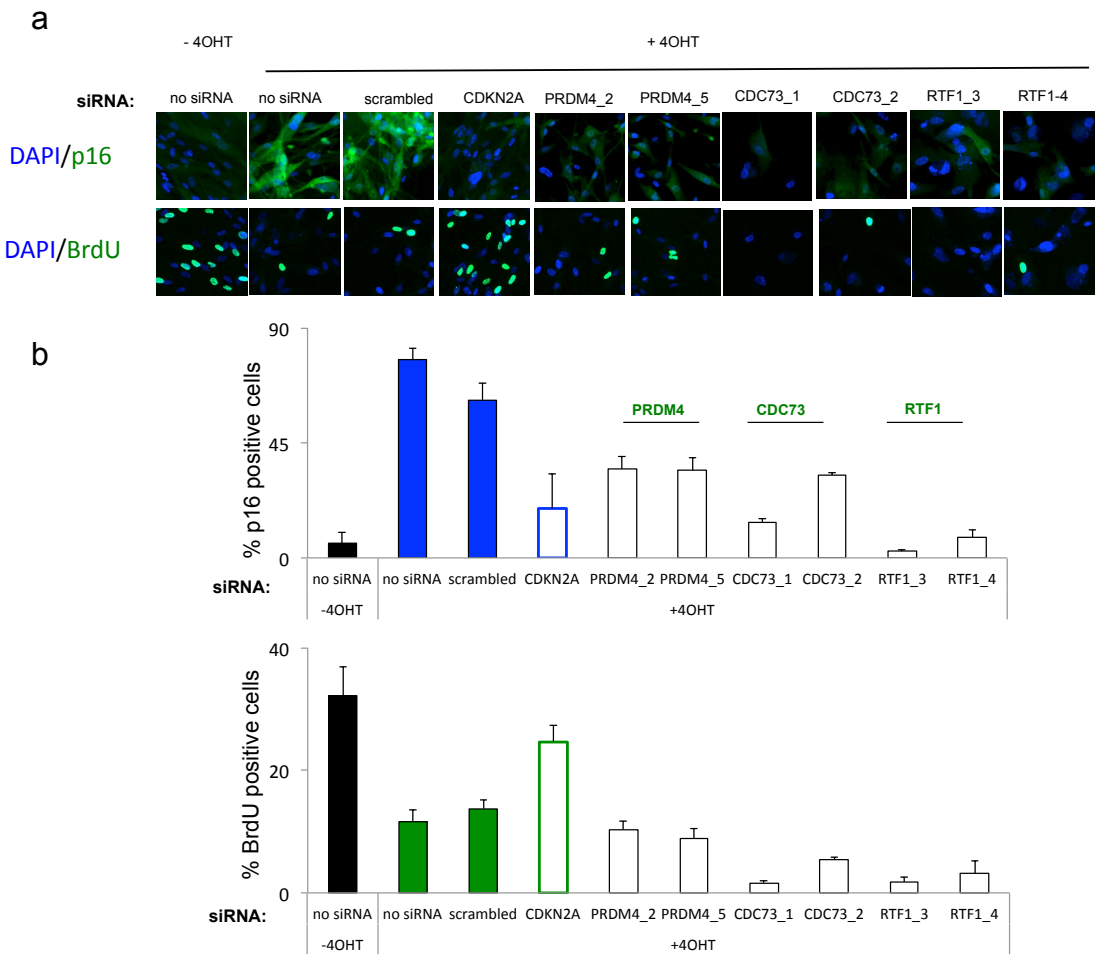


Figure 49. Example of hits preventing p16 induction without rescuing proliferation. siPRDM4, siCDC73 and siRTF1 are shown as examples of hits leading to a reduced p16 expression without affecting the cell cycle arrest. a) Immunofluorescence images acquired with the IN Cell Analyzer 2000. b) Quantification of the images collected by high throughput microscopy. Knockdown of the PAF1C complex units, RTF1 and CDC73, leads to additional reduction of the percentage of proliferating cells.

Additionally, knockdown of 5 genes led to decreased p16 levels while not affecting or reinforcing the proliferation arrest. This group consisted of the genes CDC73, PKN1, PRDM4, RTF1 and SUPT5H, involved in histone methylation, acetylation and phosphorylation and pre-mRNA processing and transcription elongation (**Figure 49**) (Chittka et al., 2012; Harrison et al., 2010; Krogan et al., 2003; Lindstrom et al., 2003). While PRDM4 is a PR-Domain containing protein, RTF1 and CDC73 are two related genes belonging to the PAF1C complex with functions in RNA polymerase II mediated transcription and in the transcriptional activity of KMT2A/MLL1 (Chittka et al., 2012; Krogan et al., 2003). Interestingly, knockdown of both genes led to a marked decrease in p16 expression suggesting a potential role of PAF1C complex in the regulation of CDKN2A expression (**Figure 49**). The downregulation of p16 expression was accompanied with a further reinforcement of the proliferation arrest for both genes, suggesting that these genes might control additional pathways regulating cell cycle progression. A role for PAF1C complex in stem cell identity has been reported, hence, understanding how the balance between these two processes affects senescence would be of interest (Ding et al., 2009).

To seek for potential negative regulators of p16 expression, we performed the secondary screen on normal proliferating IMR90 cells. The knockdown of EP300, AOF2, FOXA3, JMJD1C, ATXN7, NCOR1 and PRDM10 and PRDM11 induced p16 expression in IMR90 fibroblasts (data not shown). Indeed, siATXN7 and siFOXA3 induced an increase in p16 expression similar to that observed upon CBX7 knockdown, a known negative regulator of p16 (**Figure 50**). ATXN7 is a member of the STAGA and TFTC chromatin remodeling complexes and FOXA3 a DNA binding factor that helps opening chromatin for further remodeling (Motallebipour et al., 2009; Zhao et al., 2008)

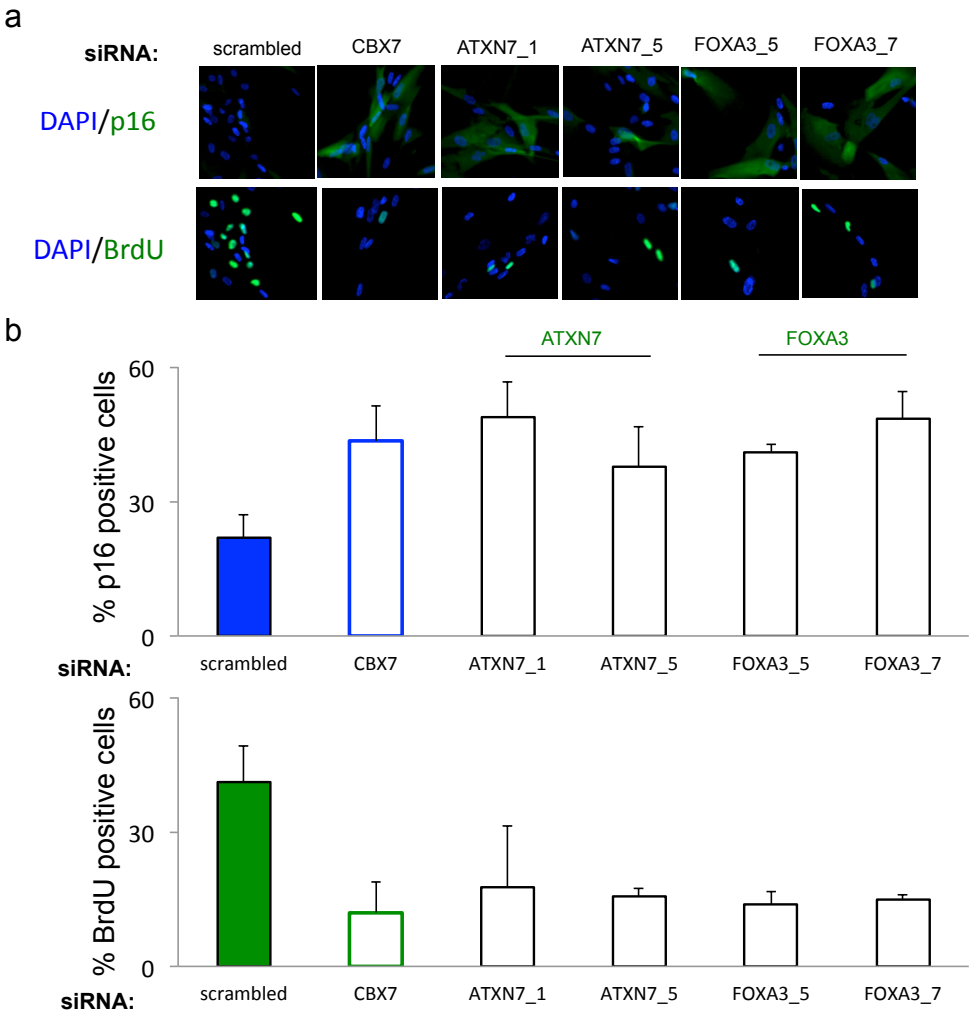


Figure 50. Example of genes inducing p16 expression. Knockdown of ATXN7 and FOXA3 increases p16 expression in proliferating IMR90 cells. a) In Cell generated images of immunofluorescence of readout markers, p16 and BrdU. b) Percentage of p16 and BrdU positive cells upon knockdown of ATXN7 and FOXA3. An induction of p16 similar to that induced by CBX7 knockdown can been seen upon silencing of the indicated genes.

5.4 Validating the bypass of the cell cycle arrest with shRNAs

Senescence is first and foremost characterized by a stable cell cycle arrest. Consequently we focused on genes whose knockdown led to a bypass of the cell cycle arrest (**Figure 46**). To confirm the bypass of the proliferation arrest observed with transient transfection, a total of 12 of the indentified candidates were selected and knocked down using lentiviral vectors expressing shRNAs (**Figure 46b**).

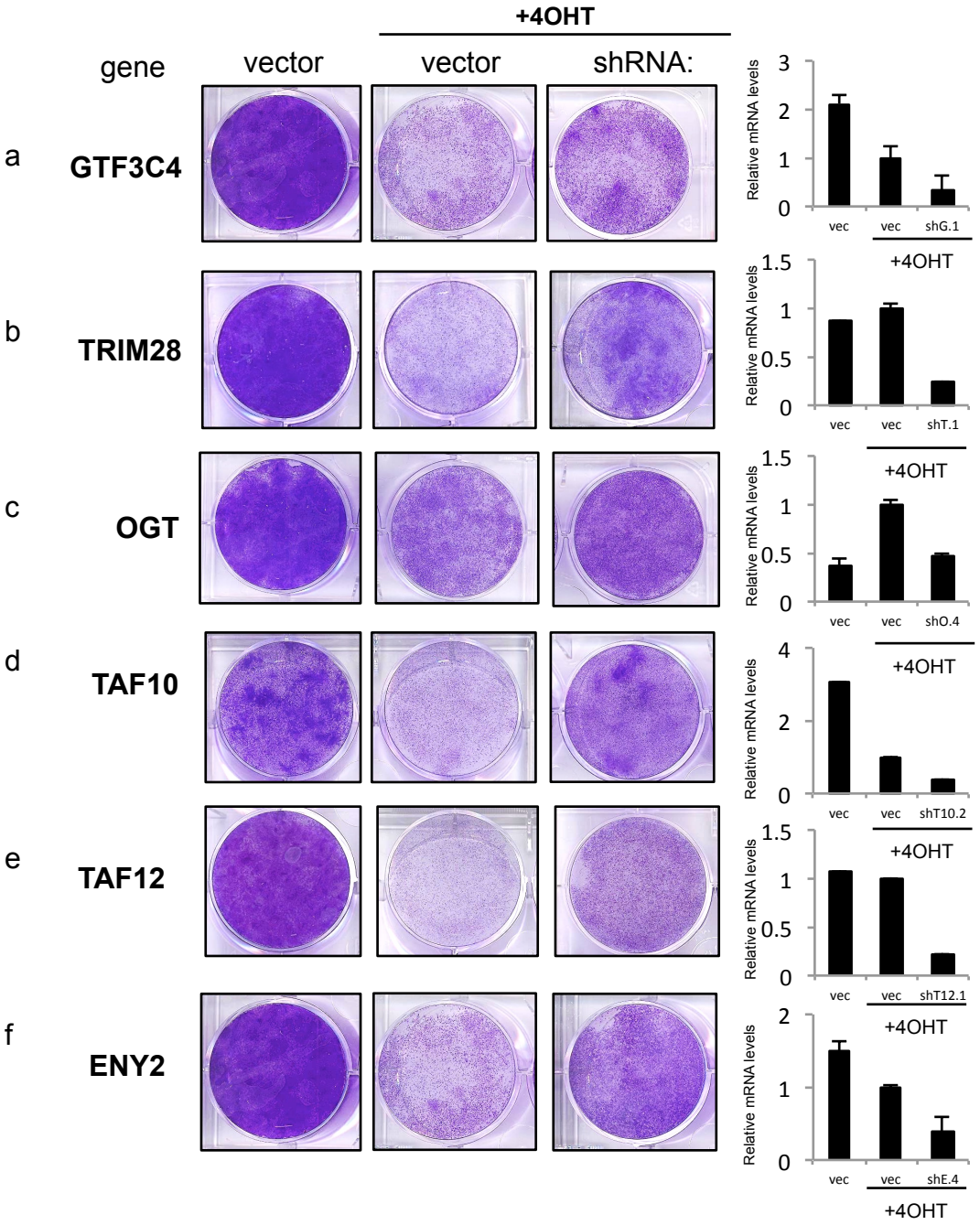


Figure 51. Stable knockdown of candidate genes bypasses growth arrest during OIS. Cadidate genes were independently targeted with shRNAs. Genes for which shRNA-mediated knockdown led to an increased colony formation are shown. Hairpin infected IMR90 ER:RAS cells were plated for low density assay and treated with 4-OHT to activate RAS and induce OIS. (a-f) Knockdown of GTF3C4, TRIM28, OGT, TAF10, TAF12 and ENY2 bypassed the cell cycle arrest as assessed by crystal violet staining (left panel). Knockdown efficiency of each gene was determined by RT-qPCR (right panel).

IMR90 ER:RAS cells were infected with lentiviral particles containing a single shRNA sequence against the target genes and infected cells were selected with puromycin.

To assess the ability of the shRNAs to bypass OIS, we plated the shRNA infected IMR90 ER:RAS fibroblasts at a low density, induced cells to undergo OIS with 4-OHT and monitored cell growth. Out of the 12 analyzed genes, the stable knockdown of 5 genes led to a bypass of the cell cycle arrest as measured by increased crystal violet staining of low-density plates. In parallel, the knockdown of TRIM28, a gene selected directly in the primary screen, resulted in an increased number of colonies of cells upon treatment with 4-OHT. Once again, cells depleted of GTF3C4 bypassed the cell cycle arrest imposed by overexpressing RAS in IMR90 cells, confirming our earlier observations (**Figure 39, Figure 51**). In this experiment we report that also shRNAs targeting TRIM28, OGT, TAF10, TAF12 and ENY2 prevented the senescence-associated growth arrest (**Figure 51**). This group of genes detained exciting characteristics: 1) - they occupy distinct functional niches, some new to senescence, from roles in chromatin remodeling complexes and protein glycosylation, to functions as co-repressors and general transcription factors; 2) - the different magnitude of p16 knockdown upon depletion of each of the aforementioned genes suggests that some might act via alternative routes to control the cell cycle arrest. Hence, we set out to investigate and characterize in more detail, how these genes affect the prototypical senescence-associated prototypical pathways and, consequently, regulate OIS.

5.5 Discussion and conclusions

5.5.1. siRNA-based screens as a tool for identifying novel regulators of OIS

Compound- or RNAi-based screens have been widely used to identify genes with functional relevance in the most diverse biological settings, routinely in *C. elegans and Drosophila* cells but also in mammalian cells (Mohr and Perrimon, 2012). Loss- or gain- of function screens have also been used to discover novel genes in senescence and associated pathways, by assessing acquisition of different senescence features (Acosta et al., 2008; Berns et al., 2004; Bishop et al., 2010; Ewald et al., 2009; Lahtela et al., 2013; Rovillain et al., 2011). For instance, Berns et al. and Rovilain et al. set out to identify novel genes involved in p53 or pRb-mediated cell cycle arrest and senescence upon shRNA-mediated knockdown of target genes (Berns et al., 2004; Rovillain et al., 2011). Verschure's and Jarrard's labs identified genes whose knockdown induced senescence, by looking at several features such as proliferation and SA-β-gal activity (Ewald et al., 2009; Lahtela et al., 2013). Also Bishop et al., by means of an siRNA genome-wide screen identified Hedgehog signaling as a mediator of p16 expression (Bishop et al., 2010).

RNAi is indeed a very attractive technology for the silencing of target mRNAs and potentially useful for cancer therapy (Guo et al., 2013). Since the discovery of RNAi in 1993, by Fire and collaborators, we have an increasing understanding of its mechanics, what has improved the technology, reducing off-target effects and increasing silencing efficiency (Fire et al., 1998; Jackson and Linsley, 2010; Rao et al., 2009b). The RNAi technology comes in two flavors, siRNA-based and vector-based methods (shRNA), each presenting advantages and disadvantages (Rao et al., 2009b). For ease of usage and to perform large-scale approaches, several libraries are available for both technologies (Echeverri and Perrimon, 2006; Falschlehner et al., 2010; Silva et al., 2005).

Due to the relevance of chromatin regulation in senescence, we planned to perform a loss-of-function screen to indentify epigenetic regulators of

oncogene-induced senescence. For that purpose an siRNA-based approach was chosen. To avoid off-target effects several methods are systematically applied (Dahlgren et al., 2008; Falschlehner et al., 2010; Jackson et al., 2006a). A very common strategy to circumvent the unwanted effect of off-targets is pooling several siRNAs together, as different siRNAs have different spectrums of off-target mRNAs (Kittler et al., 2007). Alternativey, a much simpler way to detect off targets, in the case of small libraries to medium libraries, is to use several siRNAs (different sequences) individually, as the recurrent observation of a specific phenotype will suggest an on-target result (Cullen, 2006; Echeverri et al., 2006).

In this work, an siRNA library targeting 456 genes involved in epigenetic regulation was purchased from QIAGEN. The library came in a 96 well plate format and contained 2 different and individual siRNA sequences per gene, as to perform a loss-of-function *systematic screening* (individual siRNAs as opposed to pools). Libraries as such represent a very convenient way of assessing the effect of several siRNAs, however assaying in multi-well plates frequently leads to some experimental artifacts, the most common being “edge effect” due to evaporation of the outermost rows and columns (Falschlehner et al., 2010; Jean-Philippe et al., 2012). To avoid this we did not include any target siRNA in those wells.

Until recent years screens were often performed using colorimeters or fluorescence plate readers that assessed expression of luminescence reporters, providing limited insight into cell morphology and localization. This, however, came to change with the development of high-content microscopy that allows for multi-parametric readouts. A large number of automated microscopy platforms are now available permitting an automated acquisition of both bright field and multi-channel fluorescence microscopy (Echeverri and Perrimon, 2006). The great amount of data generated ushered the need of developing image-analysis tools to annotate expression or morphological data from both fixed and live cells, in a quantitative unbiased way (Pepperkok and Ellenberg, 2006; Zanella et al., 2010).

In this study the high-throughput microscope IN Cell Analyzer 2000 and the In Cell Investigator Software (GE Healthcare) were used, allowing the collection

of thousands of fluorescence microscopy images of p16 and BrdU expression, used as readouts of the screen (**Figure 42**, **Figure 43**). These markers were selected due to their robustness as well as for providing a relatively wide dynamic range between the analyzed conditions. Despite the strong proliferation arrest and p16 induction, both features could be efficiently prevented upon transfection with control siRNAs (sip16 and sip53) hence providing a feasible readout system for the bypass of OIS (**Figure 43**).

The screen was performed in triplicate and once data was acquired, we normalized it to allow for inter-plate comparisons. Several screen normalization methods exist (Birmingham et al., 2009; Malo et al., 2006). A comparative study of normalization procedures for an RNAi screen, showed that no particular approach surpassed the others (Wiles et al., 2008). Notwithstanding, several parameters should be taken into account, such as inter-plate variation in the screen and whether samples in a plate are distributed in an unbiased way. If these premises are true then the data can be normalized using B-scores (Birmingham et al., 2009). Indeed, c*ellHTS2*-mediated B-score normalization, collected for both BrdU incorporation and p16 expression of model cells transduced with the epigenetics library, allowed for a good-enough separation of positive controls (sip16) from both negative controls (scrambled), and background data points allowing the analysis of all screen plates together (**Figure 44**) (Pelz et al., 2010).

Hit selection can be an arbitrary process although there are logical ways to approach it (Birmingham et al., 2009). One of such methods consists of setting a standard deviation threshold. Because most internal controls in the present experiment differed by at least 2 deviations from the mean (B-score 2 or -2), this value was selected to set the threshold for hit identification. Any gene for which at least 3 of the 6 independent siRNAs (2 siRNAs x 3 replicates) passed this threshold was classified as a hit. This rendered 69 potential hits, meaning a 15 % hit discovery rate. Since the threshold is rather permissive it could result in false positives. This could be circumvented by including more siRNAs per gene, therefore making the selection process lie on how many different siRNAs scored. To overcome this limitation, a second screen was performed with 4 siRNAs per gene. Because here the low-hit and

unbiased-population assumption could not be met, data were not normalized and hits were selected with basis on how many different siRNAs diverged from the negative controls (scrambled) by at least 20% for either p16 or BrdU. This approach reduced the candidates to 25 genes, suggesting that indeed the primary screen resulted in a significant number of false positives.
In conclusion, an unbiased siRNA screen targeting epigenetic factors in a model of OIS allowed the identification of several novel potential regulators of proliferation and p16 expression, two of the main features of senescence.

5.5.2 Loss-of-function screen identifies a group of genes involved in diverse aspects of epigenetic regulation

The loss-of-function screen allowed the identification of genes regulating senescence, with basis on the effect on proliferation and p16 expression. Reinforcing this premise is the fact that siRNAs against known regulators of senescence such as BRD7 and PBRM1 impaired p16 induction and led to an increase of proliferation while siEP300 had the opposite effect (**Figure 45**) (Burrows et al., 2010, Yan et al., 2013). A group of siRNAs was found to impair the cell cycle arrest, another to downregulate p16 expression, while some siRNAs had an opposite phenotype by increasing p16 expression (**Figure 46**). The latter was an interesting finding, as indeed, an important step towards senescence is the induction of the *INK4/ARF* locus. The group of genes which repressed p16 in normal fibroblasts included for instance ATXN7, a member of the hSAGA and TFTC chromatin remodeling complexes, and FOXA3, a DNA binding factor that helps opening chromatin (**Figure 50**) (Motallebipour et al., 2009; Zhao et al., 2008). This could be further assessed by investigating whether knockdown of these genes could accelerate the induction of p16 upon OIS induction.
The main goal of the screen was, however, the identification of siRNAs bypassing OIS and therefore of genes positively regulating senescence.
Amongst this group we identified the transcription factors TAF10, TAF12 and GTF3C4, which form part of complexes involved in histone and nucleosome acetylation (Grant et al., 1998; Kundu et al., 1999; Vassilev et al., 1998).

Additionally, genes involved in histone glycosylation and DNA damage, represented by OGT and PAXIP1, seemed to regulate the proliferation arrest during OIS (Callen et al., 2012; Deplus et al., 2013; Miura et al., 2012; Wu et al., 2009). This goes at odds with previous reports where knockdown of these genes impaired proliferation (Cho et al., 2003; Itkonen et al., 2013; Kamigaito et al., 2013).

The most enriched functional group identified associated with H3K9 and H3K4 methylation. The proteins PRDM4, PRDM8 and PRDM10 and PRDM11 belong to the PR domain containing protein family, a domain related to the SET methyltransferase domain (Hohenauer and Moore, 2012; Wu et al., 2009). PRDM4 and PRDM8 have been related with histone arginine methylation and establishment of H3K9me, respectively, and siRNA mediated knockdown led to a decreased p16 expression (Chittka et al., 2012; Eom et al., 2009). Conversely, PRDM10 and PRDM11 do not regulate methylation and their knockdown in normal fibroblasts upregulated p16 (data not shown). Members of PR domain containing protein family can act both as oncogenes and tumour suppressors (Hohenauer and Moore, 201). Thus, it is feasible that different members could also have opposite roles in senescence. Of note, PRDM4 has been associated with the maintenance of the “stem-like” cellular state of neural stem cells (Chittka et al., 2012). Histone methyltransferases ASH2L, EHMT1 (also known as GLP) and the already mentioned PAXIP1, involved in H3K4 and H3K9 methylation, were identified as positive regulators of OIS (Callen et al., 2012; Cho et al., 2007; Steward et al., 2006; Tachibana et al., 2005). Conversely, H3K4 and H3K9 histone demethylases, JMJD1C and AOF2, appear to negatively regulate p16 expression (data not shown) (Kim et al., 2010; Klose and Zhang, 2007). Finally, knockdown of the PAF1C complex genes RTF1 and CDC73, involved in RNA polymerase II mediated transcription and in the transcriptional activity of the H3K4 histone methyltransferase complex KMT2A/MLL1, downregulated p16 expression (**Figure 49**) (Krogan et al., 2003). In addition to preventing p16 expression, knockdown of RTF1 and CDC73 reduced the percentage of proliferating cells suggesting these genes might regulate parallel pathways to those regulating p16 expression. Indeed, a role for this complex in regulating stem cell identity

has been reported, suggesting it can positively regulate proliferation (Ding et al., 2009).

Histone methylation plays a major role in senescence (Narita et al., 2006; Chandra et al., 2012). The establishment of the repressive marks H3K27me3 and H3K9me3 is key for regulating the expression of p16 and for the formation of the SAHF (Chandra et al., 2015; Gil and Peters., 2006; Narita et al., 2006). Also, while a study showed that loss of H3K4me2 and H3K4me3 (marks of active chromatin) via a pRb and JARID1a/b mediated mechanism allows repression of cell cycle genes, another demonstrated that the maintenance of large-scale domains of H3K4me3-enriched chromatin is important for senescence (Chicas et al., 2012; Shah et al., 2013). Here novel factors positively and negatively regulating both H3K9 and H3K4 methylation were identified as potential inducers and repressors of senescence, respectively, suggesting the importance of the maintenance of these marks during senescence. A role for some of these factors has been described in senescence and in the regulation of associated pathways. For instance, both EHMT1 and AOF2 can negatively regulate p53 (Chen et al., 2010; Huang et al., 2007). Also DNA damage-dependent degradation of EHMT1 in senescence has been reported, which goes at odds with the results herein presented (Takahashi et al., 2012). Thus a more extensive analysis of these factors is needed to understand how they could regulate senescence, if their role involves the methylation of H3K4 and H3K27 and what downstream genes they affect.

As a second validation step of the hits indentified with the siRNA screen, we used shRNAs to target genes for which the knockdown bypassed the growth arrest during OIS (**Figure 51**). This not only provided a way to confirm the results obtained with the transient experiment, where knockdown levels were not assessed, as allowed the establishment of a stable-knockdown model to further evaluate the role of target genes in several types of senescence. Knockdown of TRIM28, OGT, GTF3C4, ENY2, TAF10 and TAF12 led to decreased levels of target genes and bypassed the proliferation arrest during OIS (**Figure 51**). The reason behind the lack of a loss-of-function phenotype upon knockdown of the additional tested genes was not assessed, but could

arise either from the fact that target genes had scored as false positive on the siRNA screen or false negatives during experiments with shRNAs, due to infection-derived toxicity.

In summary, we identified novel chromatin factors with a potential role in OIS by performing an siRNA screen, using p16 expression and BrdU incorporation as readouts. A secondary screen reduced the number of candidates to 25 genes encompassing a group positive regulators of p16 expression and/or of the proliferation arrest, and another group negatively regulating p16 expression. From those genes whose siRNAs bypassed the proliferation arrest, shRNA-mediated knockdown of 6 resulted in increased proliferation and cell number during OIS, thus, validating TRIM28, ENY2, OGT, GTF3C4, TAF10 and TAF12 as novel regulators of oncogene-induced senescence.

Chapter 6. Identification of novel chromatin factors regulating OIS

The loss of function screen yielded 5 novel genes with a potential role in regulating senescence. Indeed, siRNAs against TRIM28, OGT, ENY2, TAF10 and TAF12 bypassed the senescence-associated cell cycle arrest, later validated making full use of a stable knockdown system (shRNAs). Here we to provide an initial characterization of how these genes may control OIS.

6.1 A role for TRIM28/KAP1 in regulating senescence

We identified siRNAs targeting TRIM28 in our primary screen for their effect in preventing p16 induction **(Figure 45)**. Specifically, knockdown of TRIM28 downregulated p16 levels and conferred a proliferation advantage (**Figure 52**). This prompted us to investigate the role of this gene in senescence.

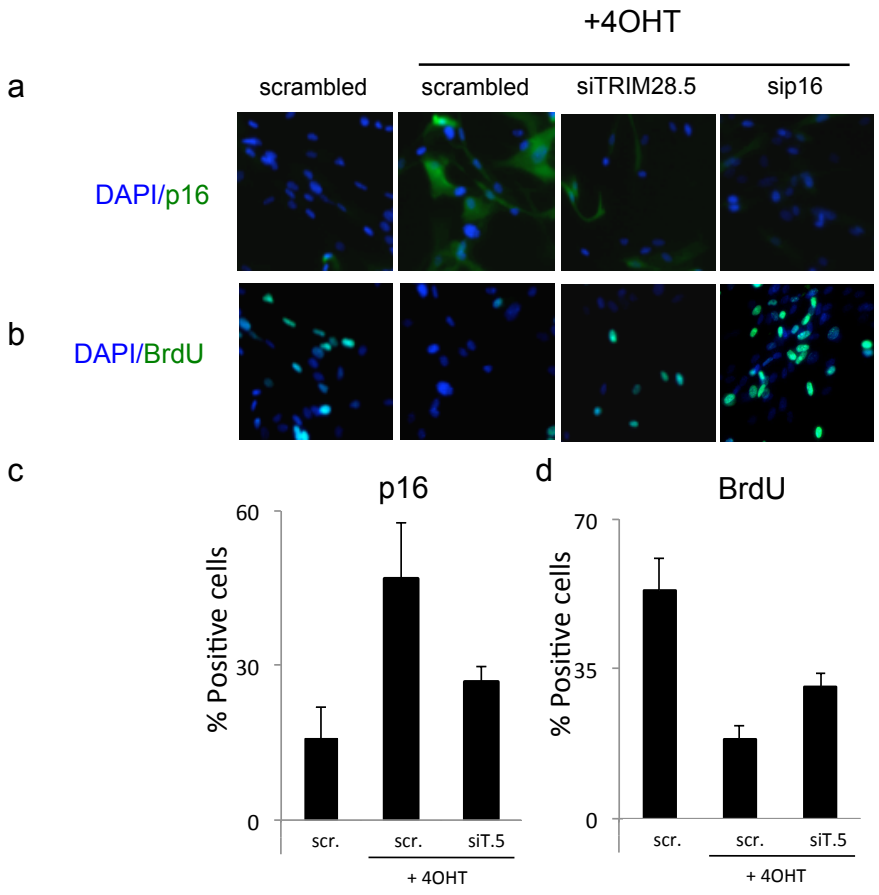


Figure 52. siRNA-mediated knockdown of TRIM28 prevents p16 induction and cell cycle arrest during OIS a-b) Immunofluorescence images collected with the IN Cell Analyzer 2000 for p16 (top) and BrdU (bottom), respectively, for IMR90 ER:RAS fibroblasts infected with either siTRIM28 or scrambled siRNAs, upon 4 days of treatment with 4-OHT. **c)** Quantification of the percentage of p16 positive cells upon siRNA-mediated silencing of TRIM28. **d)** siTRIM28 leads to decreased percentage of BrdU incorporating cells when compared with the IMR90 ER:RAS cells transfected with the scrambled siRNA.

6.1.1 TRIM28 is phosphorylated on Serine 824 during OIS

The main function of TRIM28 is to act as a co-repressor for the KRAB zinc finger proteins and is post-translationally regulated via phosphorylation on serines (Ser 473 and 824) and tyrosines (Tyr 499 , 458 and 517) as well as by sumoylation (Bolderson et al., 2012; Kubota et al., 2013; Li et al., 2007). To start dissecting the role of TRIM28 in senescence, we analyzed the expression of TRIM28 and its phosphorylation at serine S824, upon RAS activation and induction of senescence in IMR90 ER:RAS cells. This phosphorylation is known to induce the relaxation of the DNA by interfering with the silencing machinery, allowing the assembly of the repair machinery onto the double-strand breaks sites (White et al., 2012).

We observed a slight increase in the levels of TRIM28, upon induction of RAS with 4-OHT (**Figure 53a**). Interestingly, RAS activation induced the phosphorylation of TRIM28 at serine 824 (**Figure 53b**). It has been shown that TRIM28 is phosphorylated upon DNA damage by either ATM/CHK2 or ATR/CHK1 complexes (Bolderson et al., 2012 ; Ziv et al., 2006). Since the activation of a persistent DNA damage response (DDR) is one of the hallmarks of senescence, it is logical to observe the phosphorylation of TRIM28.

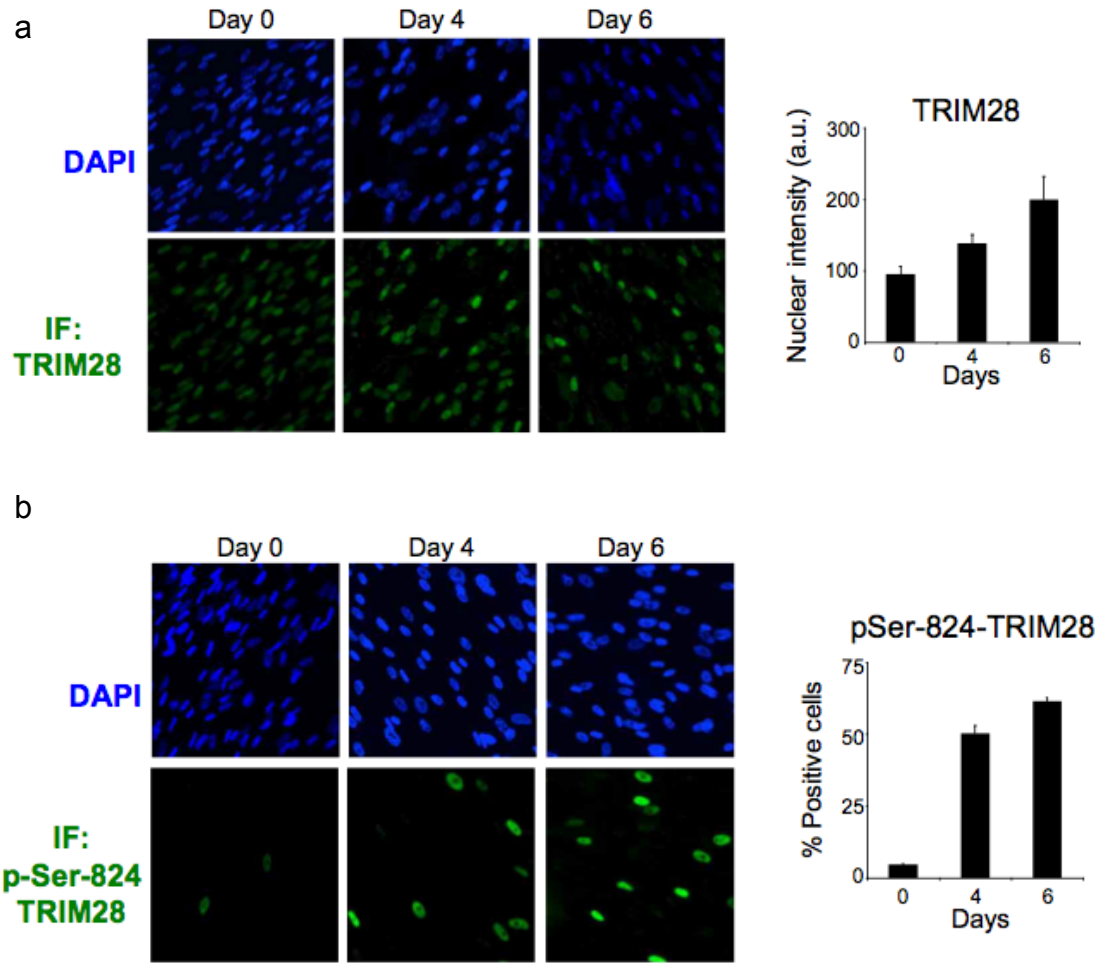


Figure 53. TRIM28 is expressed and phosphorylated on serine 824 during OIS. a) IMR90 ER:RAS cells were treated with 4-OHT and fixed at the indicated times for IF. Staining with a specific anti-KAP1 antibody revealed TRIM28 is increasingly expressed in senescence until day 6 of 4-OHT. **b)** Immunofluorescence against pKAP1 (ser824) shows TRIM28 is *de novo* phosphorylated during oncogene-induced senescence.

6.1.2 Stable knockdown of TRIM28 leads to a bypass of the cell cycle arrest

To investigate the role of TRIM28 in OIS, we tested several shRNAs against TRIM28. shTRIM28.1 and shTRIM28.2 were selected as they were the most efficient in knocking down TRIM28 levels, both mRNA and protein, as assessed by RT-qPCR and immunofluorescence and western blot analysis (**Figure 54a-c**).

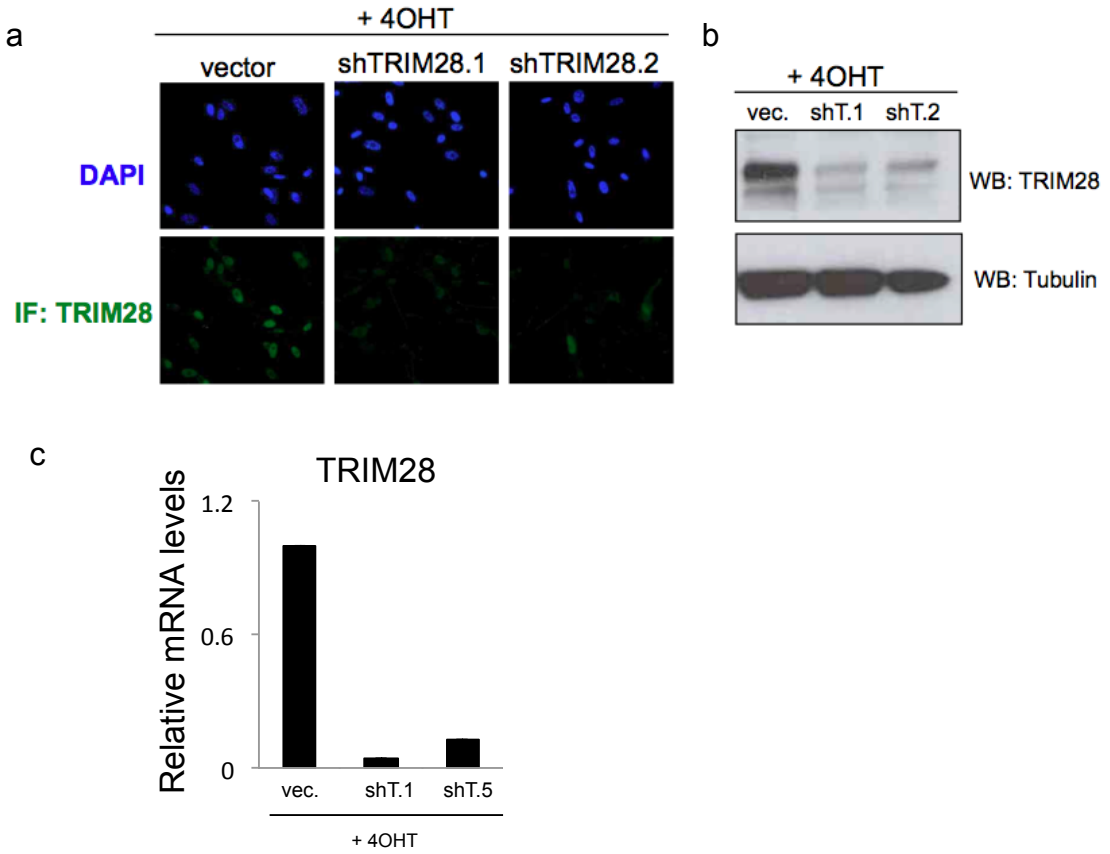


Figure 54. Two independent shRNAs efficiently knockdown TRIM28. a) Immunofluorescence of IMR90 ER:RAS infected with shTRIM28.1 and shTRIM28.2, showed decreased levels of TRIM28 as compared with the same cells infected with the vector alone. **b)** Validation of the TRIM28.1 and TRIM28.2 mediated knockdown of TRIM28 in 4-OHT treated IMR90 ER:RAS by western blot. **c)** RT-qPCR for TRIM28 shows both hairpins efficiently knockdown TRIM28 mRNA.

We then analyzed what effect knocking down TRIM28 had in OIS. IMR90 ER:RAS cells were infected with lentiviruses expressing the aforementioned shRNAs and the effects on the cell arrest were first evaluated by culturing cells at low density and staining with crystal violet. We observed that TRIM28 depletion resulted in increased cell growth upon OIS induction, similar to that observed upon p53 knockdown (**Figure 55a**). A BrdU incorporation assay showed that, 6 days after treating the cells with 4-OHT, a higher percentage of IMR90 ER:RAS with depleted TRIM28 incorporated BrdU, suggesting that depletion of TRIM28 partially prevented OIS (**Figure 55b,c**).

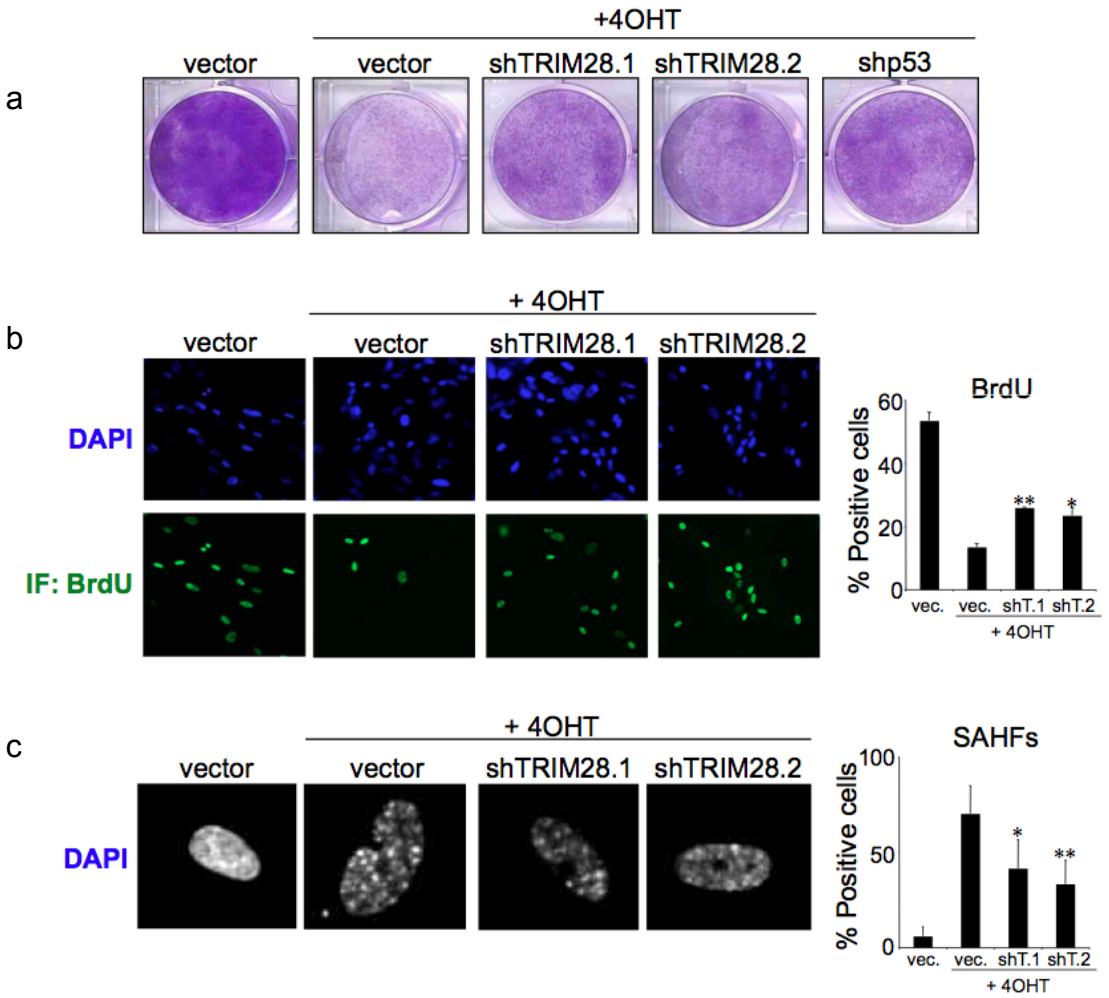


Figure 55. Depletion of TRIM28 bypasses the cell cycle arrest and leads to decreased SAHF formation. a) IMR90 ER:RAS cells were plated at a density of 20k cells per well in a 6-well plate, induced with 4-OHT and fixed for crystal violet staining. TRIM28-depleted cells displayed an increased ability to form colonies. b) IF of BrdU at day 6 showed increased BrdU incorporation upon TRIM28 knockdown. **c)** DAPI staining of nuclei where a decreased percentage of TRIM28-depleted cells dispaying senescence-associated heterochromatin foci could be seen.

Knockdown of TRIM28 also led to a decreased percentage of cells presenting senescence-associated heterochromatin foci (SAHF,**Figure 55c**). Overall, knocking down TRIM28 partially prevented OIS,

6.1.3 TRIM28 affects p16 induction

In order to investigate how the knockdown of TRIM28 affected senescence, we looked into the expression of the key tumour suppressors involved in the

implementation of senescence, p53, p21 and p16. To this end, IMR90 ER:RAS cells were infected with shRNAs targeting TRIM28 and OIS induced upon treatment with 4-OHT.

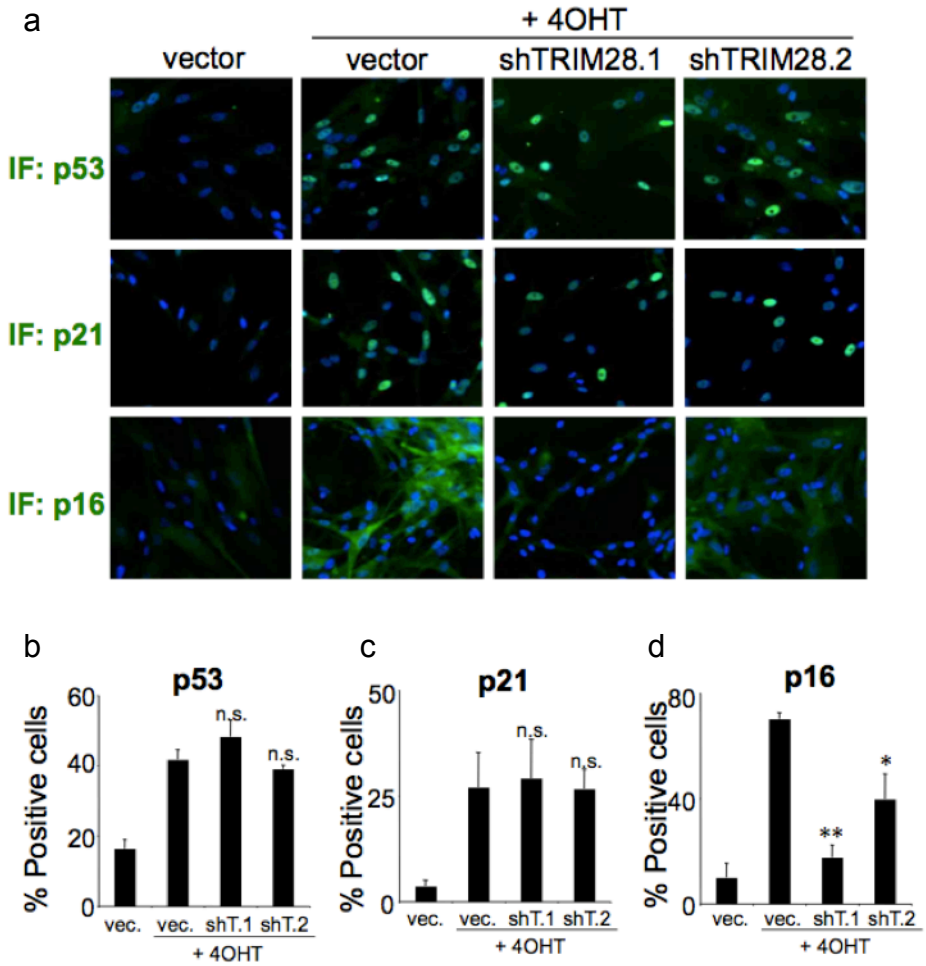


Figure 56. TRIM28 knockdown leads to decreased p16 expression in IMR90 ER:RAS cells. a) Expression of tumour suppressor genes p53, p21 and p16 was assessed by immunofluorescence upon treatment of IMR90 ER:RAS with 4-OHT. **b-c)** Expression of p21 and p53 was unaffected by low levels of TRIM28. **d)** shTRIM28.1 and shTRIM28.2 led to decreased p16 protein levels in IMR90 ER:RAS cells induced to undergo OIS.

Induction of p53 and p21 during OIS was not affected by TRIM28 knockdown (**Figure 56a-c**). However, p16 levels were lower upon knock down of TRIM28 (**Figure 56a,d**), suggesting that TRIM28 could regulate, directly or indirectly, p16 expression to control senescence.

6.1.4 TRIM28 knockdown prevents full induction of the SASP

Finally we decided to analyze the senescence-associated secretory phenotype (SASP) upon depletion of TRIM28 in OIS.

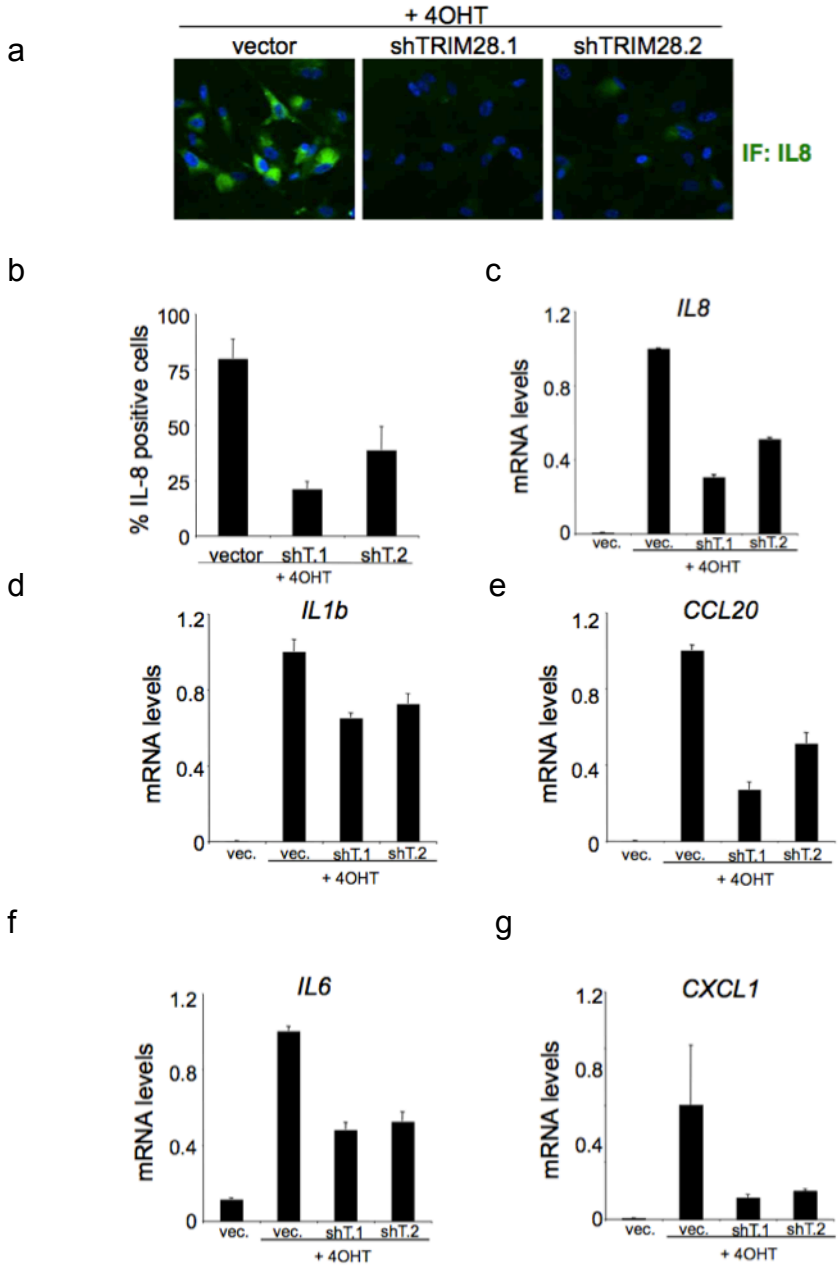


Figure 57. Knockdown of TRIM28 affects the SASP. a) IMR90 ER:RAS cells were treated with 4-OHT for 8 days to induce the secretory phenotype and stained for the prototypical SASP member IL8. TRIM28 knockdown abrogated Il8 expression in RAS overexpressing cells. **b-g)** RT-qPCRs of IL8, Il1b,CCL20,IL6 and CXCL1 reveal an overall reduction in the SASP upon TRIM28 depletion in 4-OHT treated IMR90 ER:RAS fibroblasts.

IMR90 ER:RAS fibroblasts infected with shRNAs targeting TRIM28, were induced to express RAS and sampled for immunofluorescence 8 days upon induction in order to look at the SASP. Interestingly, depletion of TRIM28 strongly suppressed IL8 induction during OIS as assessed by IF (**Figure 57a,b**).

This prompted us to analyze the expression of IL8 and additional SASP components by qRT-PCR in IMR90 ER:RAS cells in which TRIM28 expression had been depleted using shRNAS. In addition to IL8, we analyzed the expression of IL1β, CCL20, IL6 and CXCL1, factors that we and others have previously shown to be part of the SASP and play an important role in SASP functions (Acosta et al., 2013, Kuilman et al., 2008) We observed that TRIM28 expression partially suppressed the induction of these factors during OIS (**Figure 57c-g**).

6.2 OGT has a role in oncogene-induced senescence

OGT (O-linked-N-acetylglucosamine transferase) is an enzyme involved in the modification of proteins (including histones) by the addition of an O-linked-acetylglucosamine residue (Vocadlo, 2012).

siRNAs against OGT were indentified in the secondary screen as top hits for the bypass of the cell cycle arrest, suggesting OGT could play a role in regulating the senescence-associated proliferation arrest upon oncogene signaling (**Figure 58a-c**). We therefore set out to investigate OGT in OIS and its possible implications for the main senescence-associated molecular pathways.

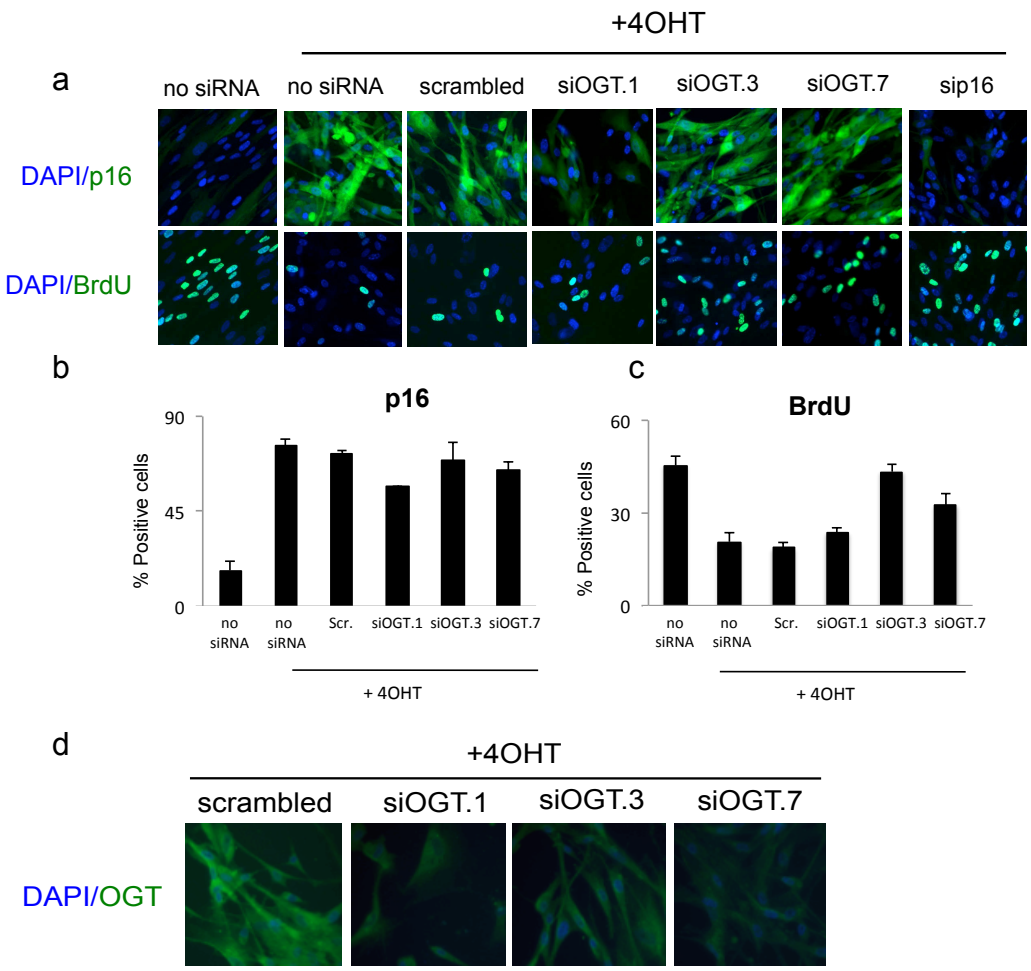


Figure 58 siRNA-mediated depletion of OGT prevents the cell cycle arrest but not p16 induction in OIS. a) Immunofluorescence against BrdU (bottom) and p16 (top) collected with the IN Cell Analyzer during the secondary siRNA screen show that depletion of OGT does not affect expression of p16 but leads to increased proliferation of IMR90 ER:RAS cells. **b-c)** Quantification of the IN Cell Analyzer images shown in a) by HCA, using the In Cell Investigator software. **d)** Immunofluorescence against OGT in 4-OHT-treated IMR90 ER:RAS fibroblasts shows decreased expression of OGT upon transfection with siOGT1.3 and 7, respectively. Immunofluorescence has been performed 5 days after 4-OHT induction.

6.2.1 Expression of OGT during OIS

In order to check OGT expression and levels of O-GlcNAc during OIS, we performed immunofluorescence of IMR90 ER:RAS cells treated with 4-OHT for 5 days. Interestingly, OGT was expressed in a very similar fashion by senescent and control cells, presenting an intense cytoplasmic expression localizing as well to the nucleus in both cell types (**Figure 59a**). Indeed, the

overall levels of OGT were similar across senescent, quiescent and proliferating cells (**Figure 59c**). Since the only known function of OGT is protein O-GlcNAcylation, we looked at O-GlcNAc levels. Surprisingly, we found an increase in the intensity of O-GlcNAc during senescence, mainly in what resembled the nuclear periphery (**Figure 59b**). This suggested that OGT could modify proteins that are structural components or associate with the nuclear envelope.

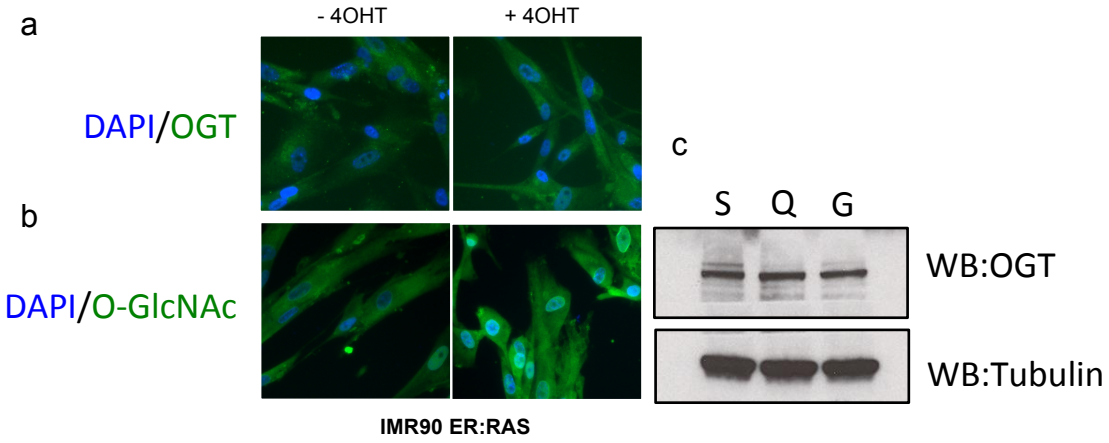


Figure 59 Specific antibodies against OGT and its modification, O-GlcNAc, show both are expressed during OIS. a) OGT is equally expressed in normal and cells undergoing OIS both in the cytoplasm and nucleus, as assessed by IF. **b)** Immunofluorescence targeting O-GlcNAc shows widespread expression of OGT-modified proteins during OIS. **c)** Western blot analysis of total extracts of senescent (Sen), quiescent (Qui) and growing (Gro) fibroblasts reveals a stable expression of OGT across cell types.

6.2.2 Stable knockdown of OGT prevents the senescence-associated cell cycle arrest

For a more detailed investigation of the impact OGT depletion has on proliferation, we infected IMR90 ER:RAS cells with several hairpins targeting OGT (**Figure 60c**). IMR90 ER:RAS cells infected with hairpins against OGT and the corresponding control were plated at low density, treated with 4-OHT and cultured for approximately 1.5 weeks.

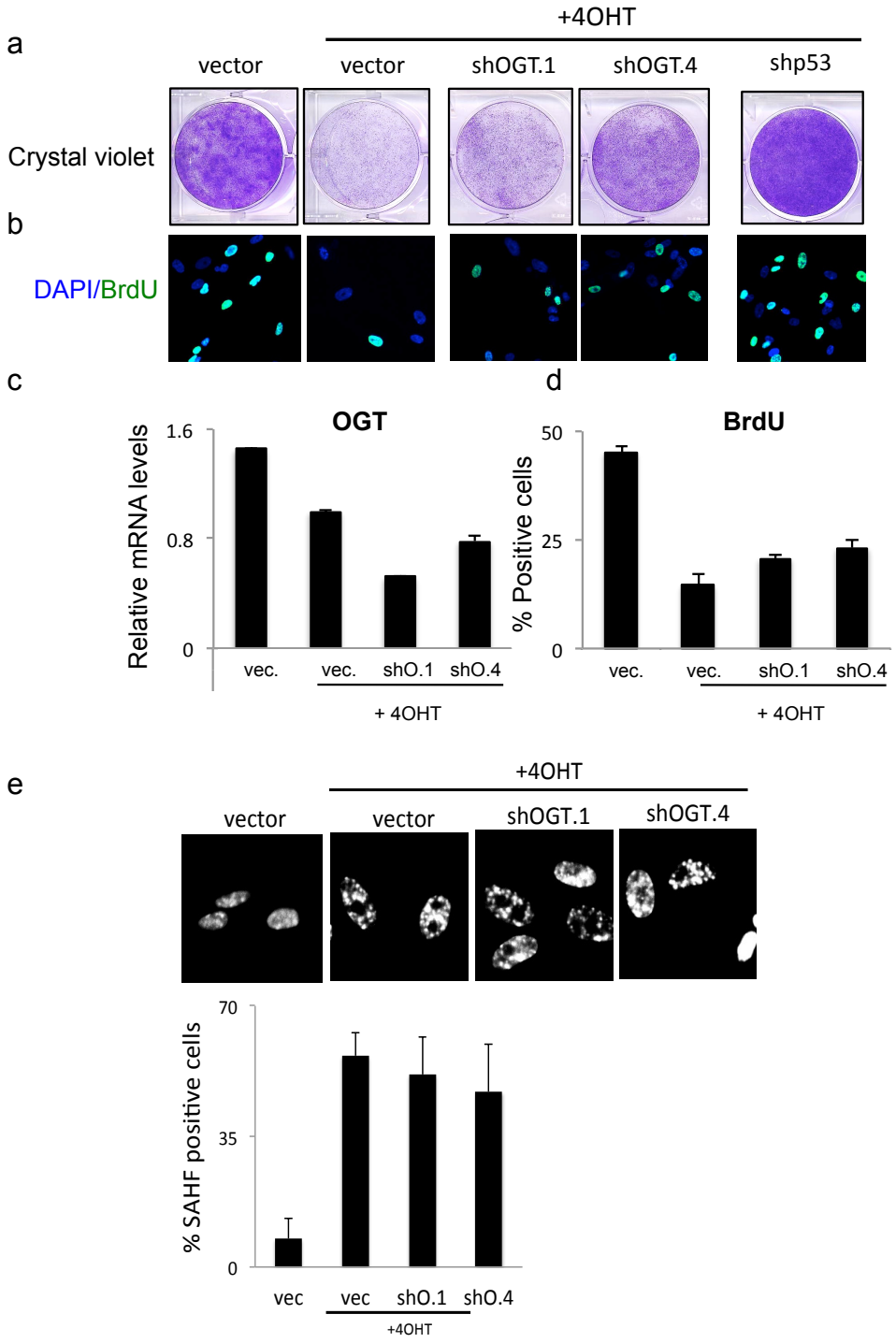


Figure 60 Stable knockdown of OGT with 2 independent short-hairpin RNAs bypasses the cell cycle arrest. a) Crystal violet staining of low density plates of shOGT expressing IMR90 ER:RAS cells reveals that OGT depletion delays the cell cycle arrest during OIS. **b)** OGT knockdown leads to increased percentage of BrdU incorporating cells. **c)** RT-qPCR of shOGT.1 and shOGT.4 expressing cells showing decreased levels of OGT mRNA levels. **d)** In Cell Investigator generated quantification of b), shows increased percentage of BrdU positive cells upon OGT knockdown. **e)** DAPI stained nuclei depicting foci of heterochromatin (SAHF). OGT knockdown did not interfere with the formation of SAHF.

To assess the ability to form colonies, cells were fixed and stained with crystal violet. OGT knockdown conferred a proliferation advantage upon activation of RAS (**Figure 60a**).

Additionally, the same cells were plated for BrdU incoporation assay. In accordance with the previous results, there was a significant increase in the proliferation rate of OGT depleted cells when compared to empty vector cells, both treated with 4-OHT (**Figure 60b, d**). Additionally, OGT knockdown did not affect the formation of the SAHF, as foci of heterochromatin could be seen in the nuclei of cells carrying an shRNA against OGT. These results suggest that OGT plays a role in senescence, by at least regulating the cell cycle arrest.

6.2.3 OGT knockdown impacts on the SASP

To better understand how OGT depletion affects oncogene-induced senescence we proceeded with investigating the main tumour suppressor pathways. We could see only a small decrease in the levels of p16 upon knockdown of OGT, especially with shOGT.4 (**Figure 61a,c**). Also the p53/p21 pathway was not affected, as the protein levels of p53 as well as the mRNA levels of p21 remained unaltered upon OGT knockdown (**Figure 61b, d, e**).

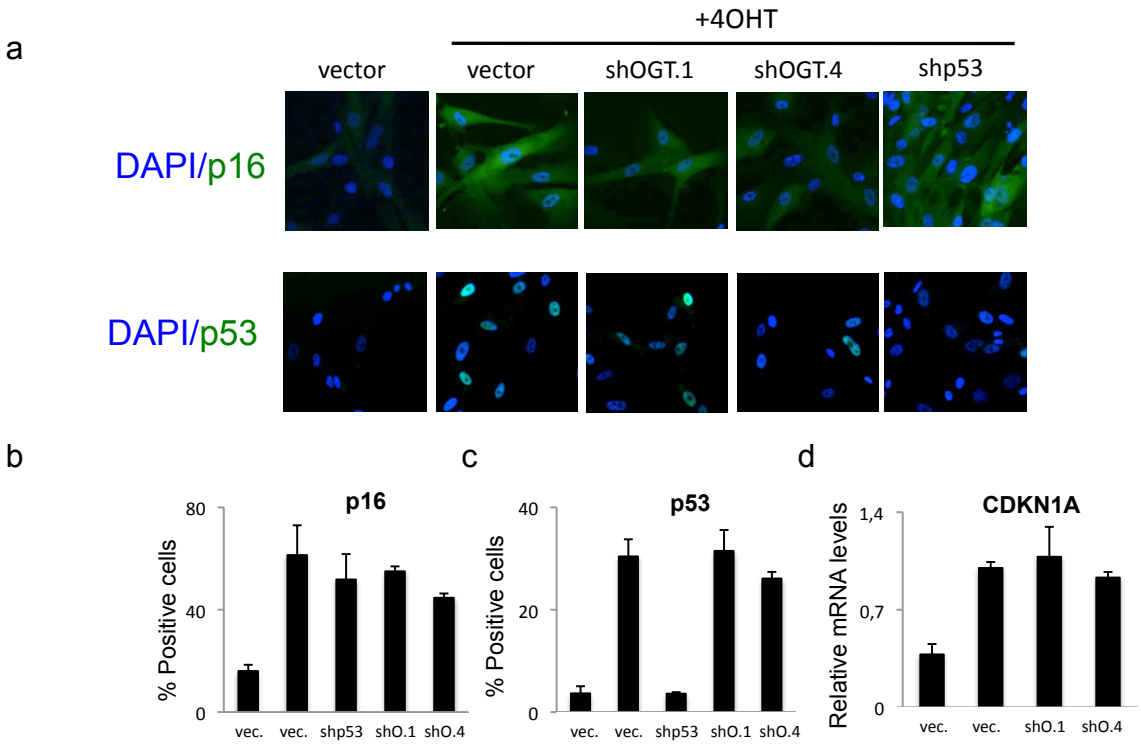


Figure 61. Stable knockdown of OGT does not affect p53 and p21 expression, and only slightly decreases p16 levels. a) Immunfluorescence of OGT-depleted IMR90 ER:RAS against the main tumour suppressor genes in OIS, p16 and p53. **b)** OGT depletion has a minimal impact on the percentage of p16 expressing cells. **c)** shOGT does not affect p53 expression as assessed by IF. **d)** RT-qPCR of shOGT infected IMR90 ER:RAS cells reveals p21 expression is also not affected by OGT levels during OIS.

These results suggest that, although OGT might slightly (directly or indirectly) affect p16 expression during OIS this alone is mostly unlikely to account for the bypass of the cell cycle arrest upon OGT knockdown. The p53/p21 pathway remained unaltered upon OGT knockdown, and consequently does not account for the role of OGT in senescence. We then decided to investigate additional characteristic hallmarks of senescence such as the SASP. As a preliminary approach to analyze the effect of the OGT knockdown on the SASP we performed immunofluorescence against IL8 using shOGT.4. Interestingly, we found IL8 expression to be impaired upon shRNA mediated knockdown of OGT (**Figure 62a, b**).

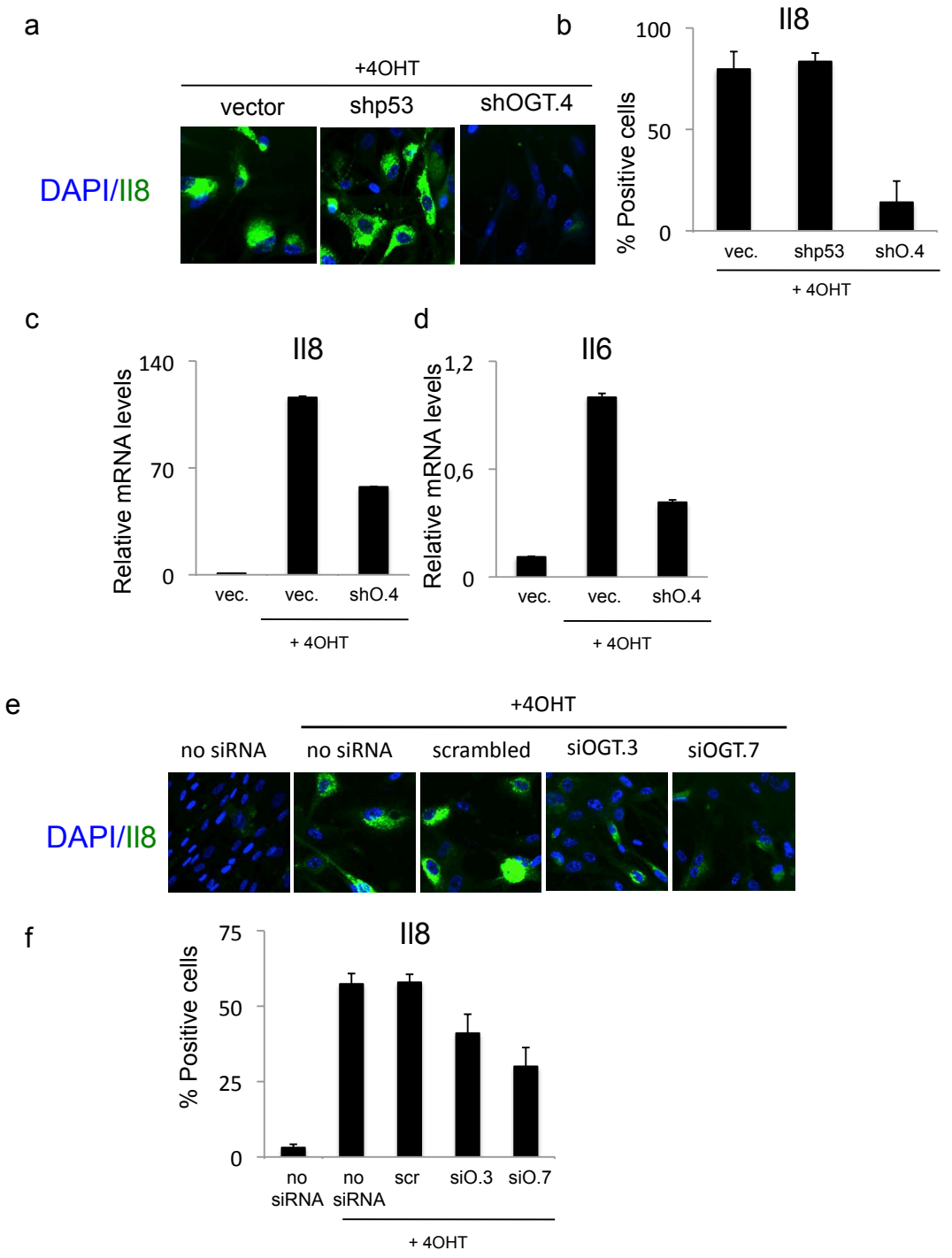


Figure 62. OGT knockdown impacts on the SASP. a) Immunofluorescence against IL8 upon stable knockdown of OGT with shOGT.4 after treating IMR0 ER:RAS cells with 4-OHT for 8 days. **b)** Quantification of the percentage of IL8 positive cells for IF data. **c-d)** RT-qPCR shows mRNA levels of both IL8 and IL6 are impaired upon depletion of OGT. **e)** Transient knockdown of OGT with siRNAs led to decreased production of IL8 as assessed by IF. **f)** Quantification of the percentage of IL8 positive cells shown in e).

Indeed, analysis of the RNA levels of both IL8 and IL6, revealed a reduced expression of the transcripts of both proinflammatory cytokines in cells depleted for OGT (**Figure 62c,d**). The same was observable upon transient knockdown of OGT, using the previously validated siRNAs (**Figure 62e, f**). These results suggest that OGT might play a role in the establishment of the senescence-associated secretory phenotype (SASP).

6.2.4 The DNA damage response is slightly affected by OGT depletion

Another hallmark of senescence is the engagement of DNA damage signaling pathways, leading to persistent foci of DNA damage that can be monitored by the accumulation of several proteins such as γH2Ax and 53BP1. In order to assess whether the DDR was intact upon OGT knockdown, we performed immunofluorescence using the high-throughput microscope IN Cell Analyzer 2000 and quantified the number of foci of 53BP1 per nuclei. Upon RAS activation, IMR90 ER:RAS cells displayed an increased number of DNA damage foci when compared to non induced cells (**Figure 63a, c**). To quantify accumulation of DNA damage we used the In Cell Investigator software to segment and count foci of 53BP1. Both the stable (**Figure 63a-b**) and transient (**Figure 63c-d**) knockdown of OGT led to a small but consistent reduction in the number of 53BP1 foci across experiments upon induction of RAS. These results are consistent with a previously reported role of OGT in the regulation of the DDR response and suggest that partial deregulation of the DNA damage signaling might account for the effects of OGT depletion in OIS (Miura et al., 2012).

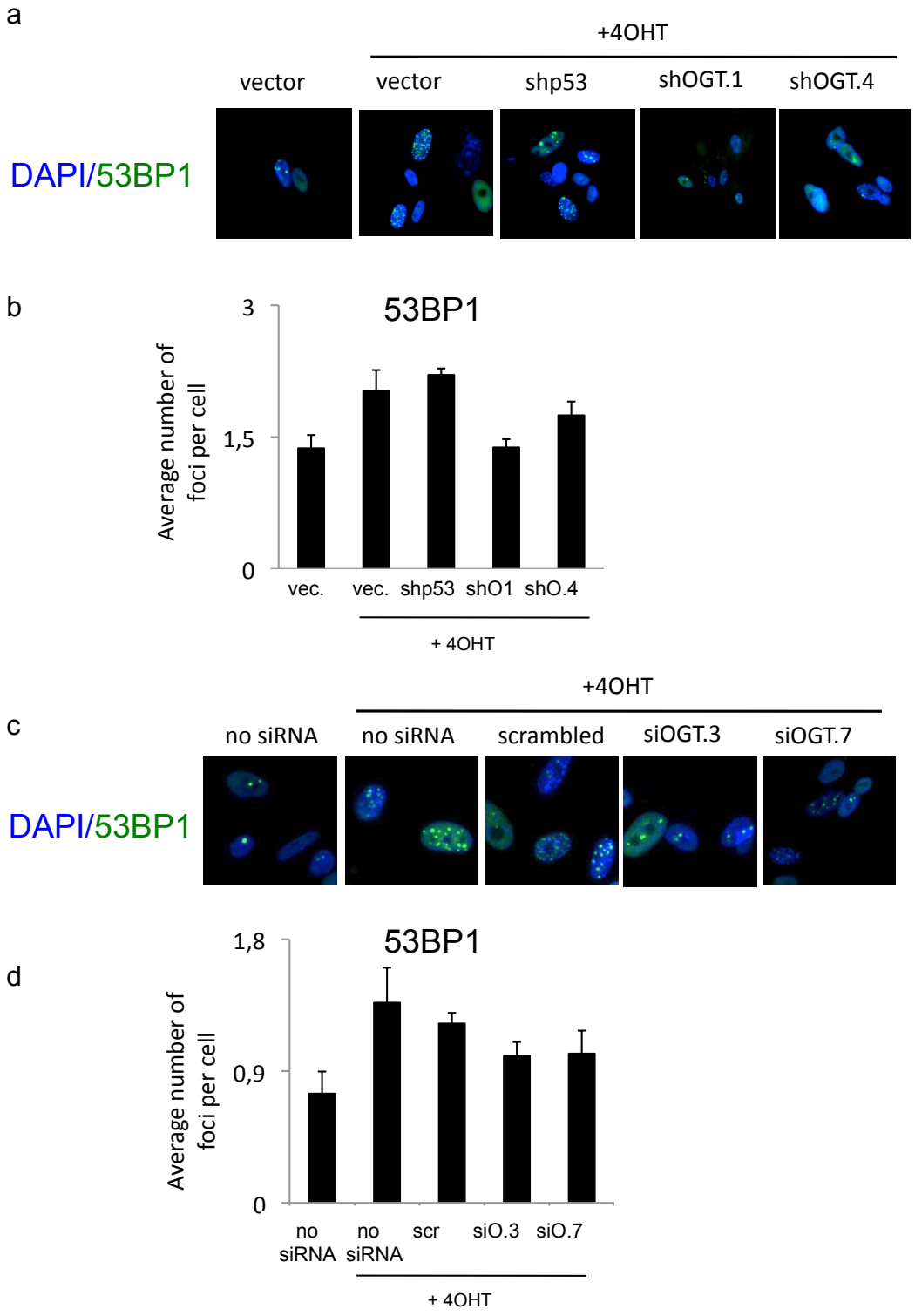


Figure 63. The accumulation of 53BP1-positive DNA damage foci is reduced upon depletion of OGT. a) Immunofluorescence against 53BP1 shows foci of DNA damage accumulate during OIS and this accumulation is slightly impaired upon OGT stable knockdown. **b)** Quantification of the average number of foci per nucleus of the images seen in a). shOGT leads to a decrease in the average number of DNA damage foci per nucleus. **c-d)** Transient knockdown of OGT with siRNAs also leads to a decreased accumulation of 53BP1 foci of IMR90 ER:RAS cells treated with 4-OHT.

6.3 SAGA complex members ENY2, TAF10 and TAF12 mediate OIS

The genes ENY2, TAF12 and TAF10, were identified in the secondary screen, and had in common the fact they were all members of the SAGA complex, a histone modifying complex involved in histone acetylation and deubiquitination (Koutelou et al.,2010). In addition to their role in the aforementioned complex, ENY2 has been reported to hold independent functions, involved for instance in mRNA export, while the TBP-associated factors (TAFs) counterparts are also part of the $TF_{II}D$ complex involved in RNA polymerase II mediated transcription (Bhaumik, 2011; Chen and Hampsey, 2002; Ogryzko et al., 1998). We then set out to investigate what function these genes had in OIS and to what extent the SAGA complex was involved.

6.3.1 Depletion of ENY2 leads to OIS bypass

Several siENY2 were selected as top candidates for siRNAs bypassing the growth arrest during OIS in the secondary screen. This suggested ENY2 could act as a mediator in one of the molecular routes for the establishment of the proliferation arrest induced by activated oncogenes. Indeed, we additionally observed attenuation of p16 expression during OIS upon ENY2 knockdown with multiple siRNAs, suggesting regulation of the *INK4a* locus could partly account for the outcome of siENY2 on the cell cycle arrest (**Figure 64**).

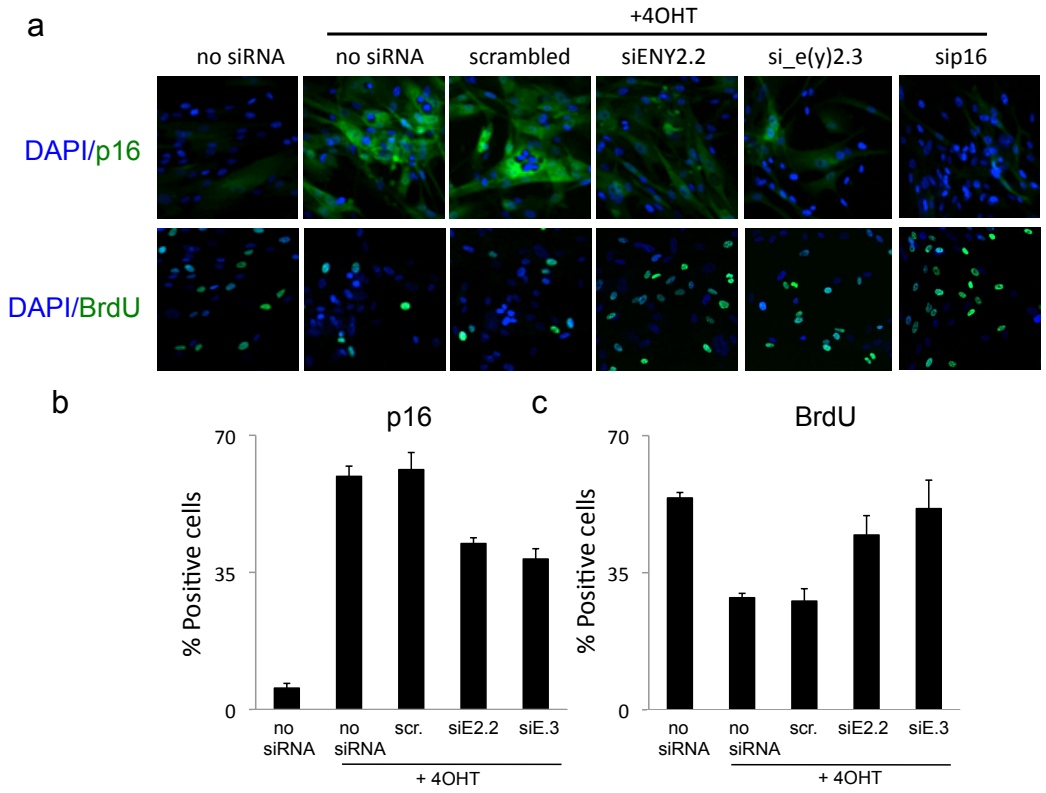


Figure 64. Knockdown of ENY2 bypasses the cell cycle arrest and downregulates p16 levels. a) Immunofluorescence against p16 and BrdU showing impairment in p16 expression and increased proliferation upon transient transfection with siRNAs targeting ENY2. **b)** Transient knockdown of ENY2 leads to decreased p16 expression when compared with untransfected cells or those transduced with the scrambled sequence. **c)** Increased percentage of cells incorporating BrdU could be seen upon ENY2 knockdown.

6.3.1.1 Bypass of the cell cycle arrest upon stable ENY2 knockdown

As a way to validate the bypass of OIS obtained with the experiments with siRNAs, we infected IMR90 ER:RAS cells with multiple hairpins against ENY2. Three shRNAs efficiently knocked down ENY2 mRNA (**Figure 65c**). Infected cells were plated for a low density assay, treated with 4-OHT to induce RAS expression and cultured until controls (non treated or shp53 + 4-OHT) were confluent. Consistently with the results obtained for the transient knockdowns, depletion of ENY2 with 3 independent shRNAs bypassed the cell cycle arrest, visualized by staining the cells with crystal violet (**Figure 65a**). Additionally, these cells incorporated more BrdU than vector cells when

treated with 4-OHT, suggesting ENY2 could function to promote the senescence-associated arrest (**Figure 65b, d**).

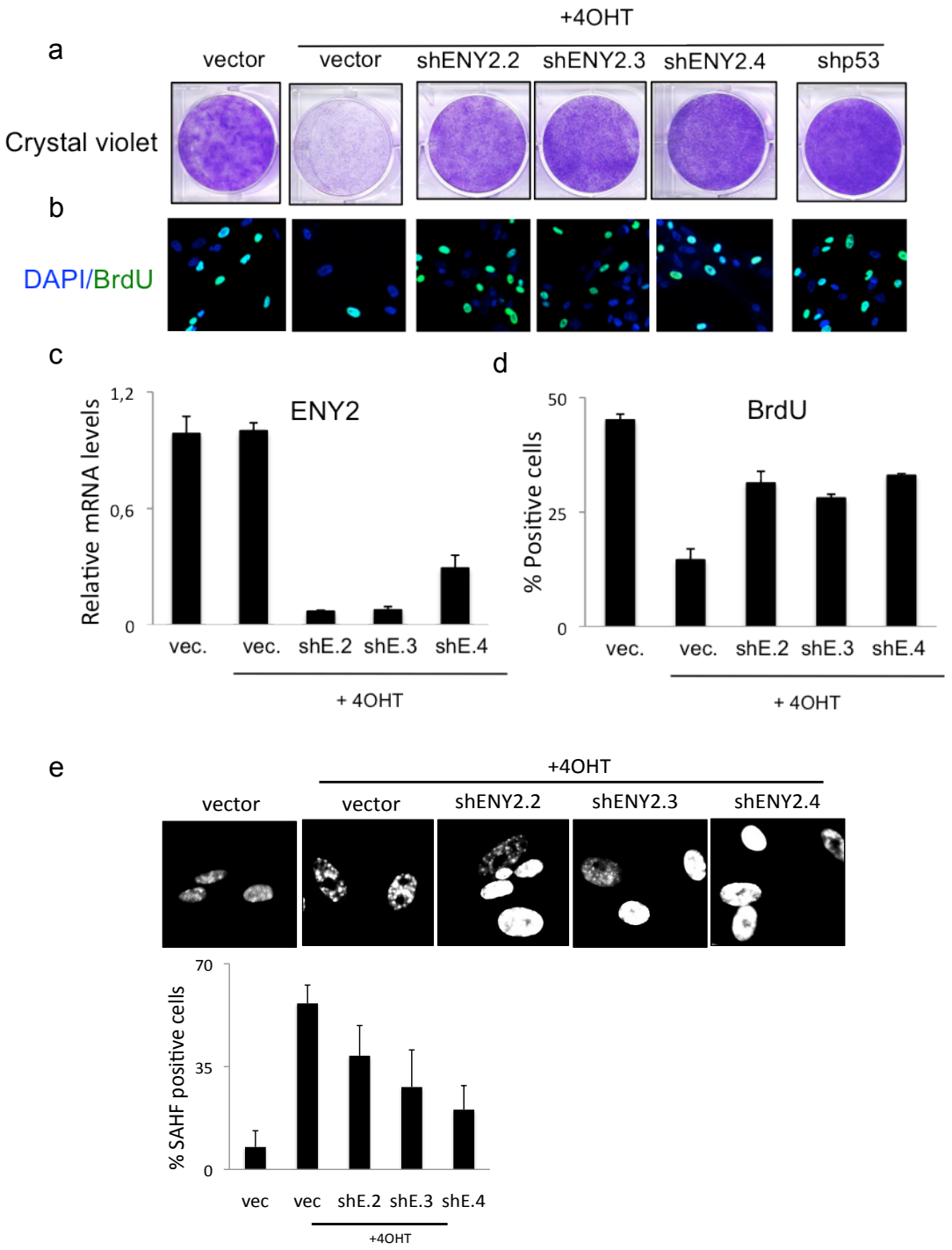


Figure 65. Stable knockdown of ENY2 prevents the senescence-associated cell cycle arrest. a) Crystal violet staining of low density plates revelaled shENY2 expressing cells severely bypassed the proliferation arrest. **b)** Immunofluorescence against BrdU shows increased incorporation of BrdU by ENY2 depleted cells. **c)** Three independent shRNAs efficiently knockdown ENY2 mRNA levels, as assessed by RT-qPCR. **d)** Quantification of the percentage of BrdU positive cells for the immunofluorescence images collected with the IN Cell Analyzer 2000 at day 4 post induction with 4-OHT. **e)** Knocking down ENY2 severely impacts on the formation of the SAHF.

Next, we looked at the SAHF in ENY2 depleted cells. Surprisingly, ENY2 knockdown had a strong impact on the formation of the senescence-associated heterochromatin foci, as assessed by immunofluorescence of nuclei stained with DAPI (**Figure 65e**).

6.3.1.2 Regulation of p16 and p21 could explain the effect of ENY2 in senescence

To address if ENY2 could operate through any of the main tumour suppressor pathways known to regulate senescence, we performed immunofluorescence against p16, p21, p53 and the DDR marker 53BP1, upon ENY2 knockdown.
Again, ENY2 depletion prevented full p16 expression in cells induced to undergo OIS (**Figure 66a**). Stable knockdown of ENY2 with shRNAs did not affect p53 expression or accumulation of 53BP1 foci during Ras-induced senescence (**Figure 66a,c,d**), however these markers appeared slightly reduced upon siRNA mediated knockdown of ENY2 (**Figure 67a,c,d**).
Interestingly, ENY2 knockdown resulted in a decreased percentage of p21 expressing IMR90 ER:RAS cells. (**Figure 67a,b**). Altogether, these results suggested that ENY2 could play a role in OIS via, direct or indirect, regulation of the CDK inhibitors, p16 and p21, possibly in a DDR and p53-independent manner.

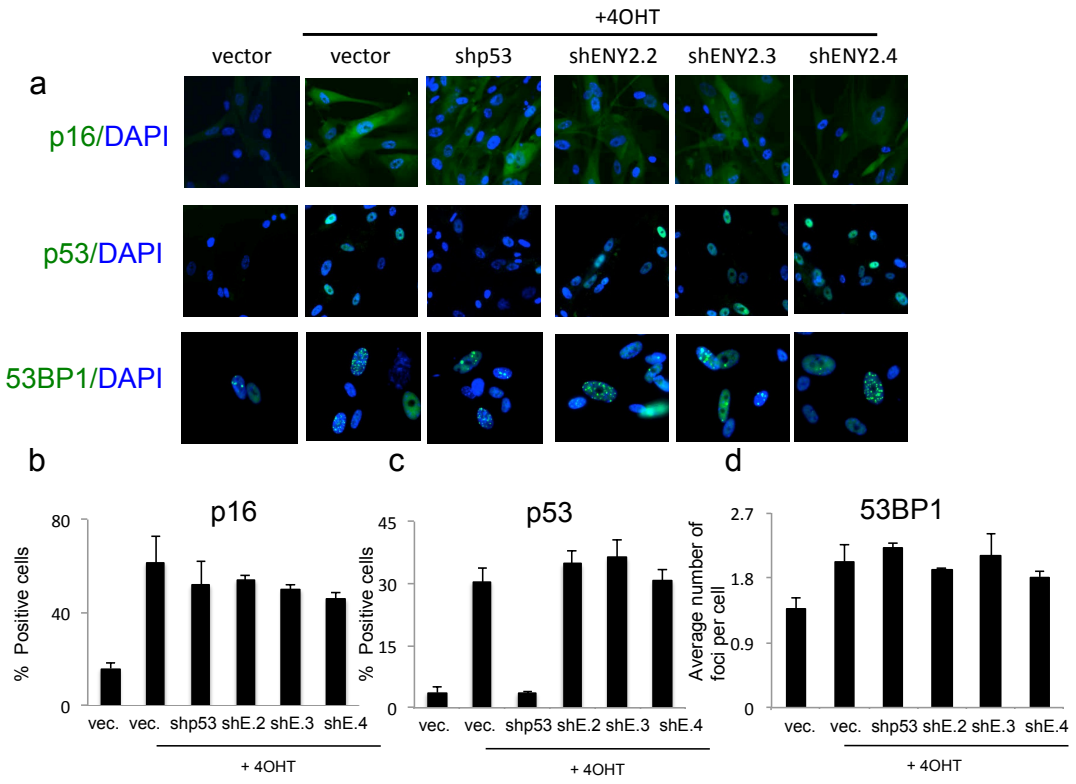


Figure 66. Stable knockdown of ENY2 has a minimal impact on p16 expression, but does not affect p53 or 53BP1 foci accumulation. a) Immunofluorescence against p16, p53 and 53BP1 collected by high-throughput microscopy, upon targeting ENY2 with 3 independent shRNAs. **b)** Stable knockdown of ENY2 led to a slight decrease in the percentage of p16 expressing cells 6 days post treating cells with 4-OHT. **c)** Analysis of the expression of p53, suggests depletion of ENY2 leads to increased expression of this tumour suppressor gene. **d)** Accumulation of DNA damage foci, as measured by IF against 53BP1 seems overall unaffected by ENY2 depletion. Only shENY2.4 led to a small decrease in the average number of 53BP1 foci per nucleus.

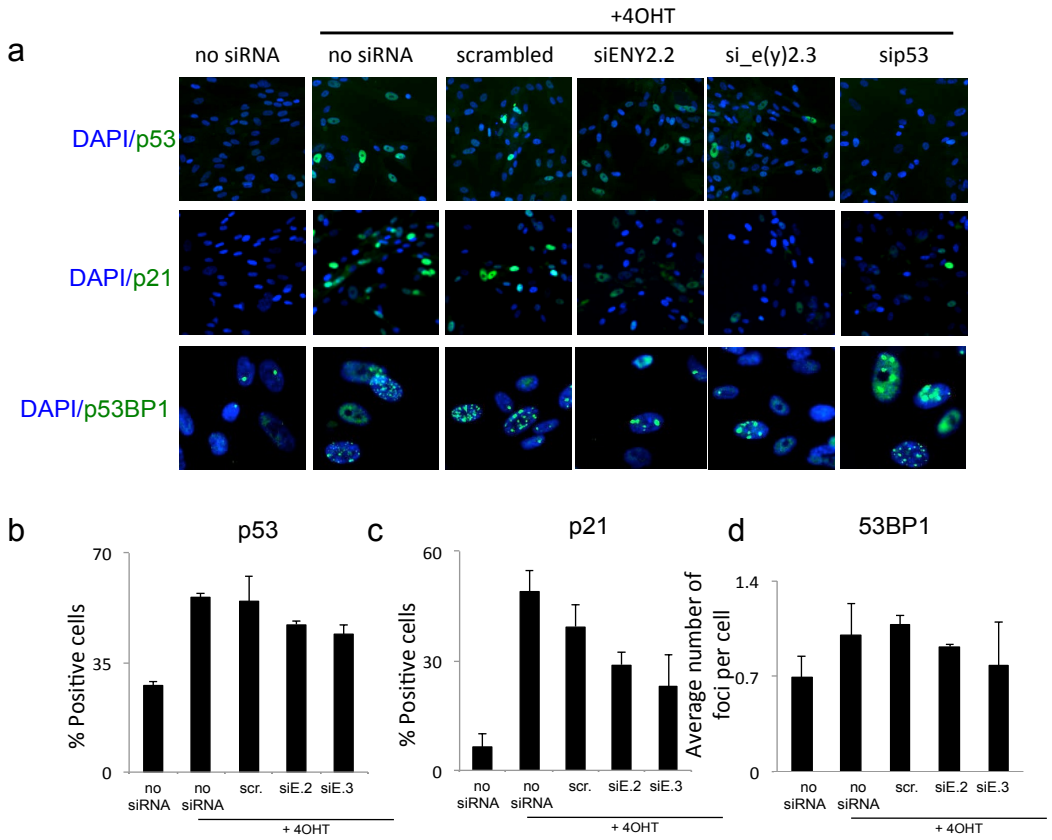


Figure 67. ENY2 trasnsient knockdown impacts on p21 expression and on 53BP1 foci accumulation but only slightly affects p53 expression. a) Immunofluorescence against p53, p21 and 53BP1 shows decreased expression of p21 and 53BP1 upon ENY2 knockdown. **b-d)** In Cell Investigator-generated quantification of the percentage of p21 and p53 expressing cells and of the average number of 53BP1 foci per cell (DNA damage).

6.3.1.3 Potential implications of ENY2 in irradiation-induced senescence

We then wondered if ENY2 could mediate senescence induced by other stresses. First we addressed whether ENY2 could mediate replicative senescence by performing serial passaging of IMR90 cells infected with shRNAs against ENY2. However, ENY2 depletion did not confer any significant lifespan extension when compared with control fibroblasts (data not shown). An additional type of premature senescence is that induced upon irradiation-derived genotoxic stress.

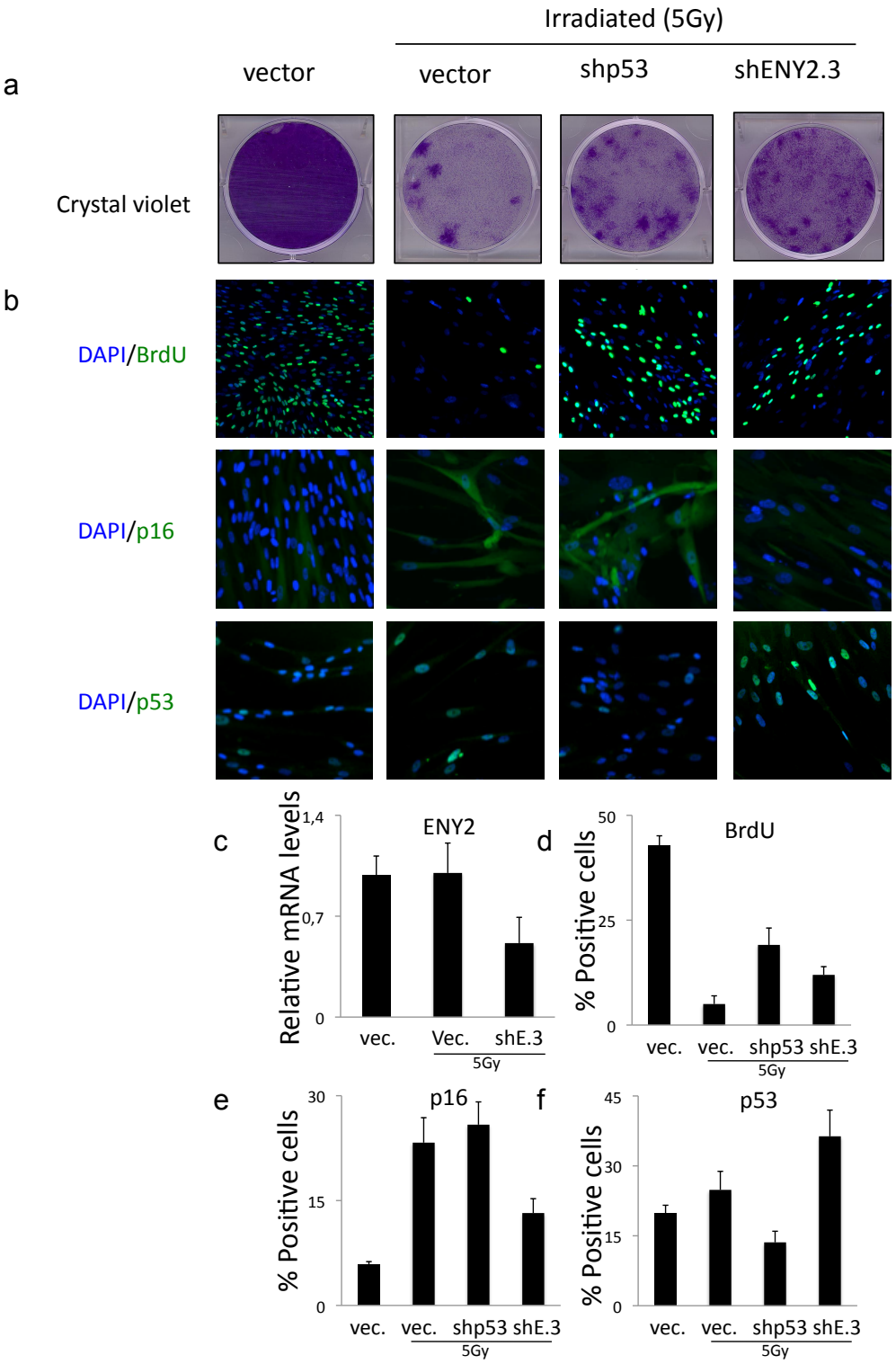


Figure 68. Irradiation-induced senescence is delayed upon ENY2 knockdown. a) Crystal violet staining of ENY2 depleted IMR90 cells submitted to gamma irradiation (5Gy) shows increased colony formation by shENY2 expressing cells when compared with control cells infected with the vector alone. **b)** Immunofluorescence against BrdU, p16 and p53. **c)** RT-qPCR shows shENY2.3 downregulates ENY2 mRNA levels in irradiated cells. **d)** ENY2 depletion leads to increased proliferation upon submitting IMR90 cells to gamma irradiation. **e)** p16 expression was analyzed by immunofluorescence and was impaired upon depletion of ENY2. **f)** Percentage of p53 expressing cells increases upon submitting ENY2-deficient cells to irradiation.

To this end IMR90 cells infected with hairpins against ENY2 were subjected to gamma irradiation (5Gy) and plated at low density to assess the ability of these cells to proliferate. Staining with crystal violet revealed a higher number of colonies in plates containing ENY2 depleted cells, suggesting a partial bypass of the cell cycle arrest (**Figure 68a, b**). Indeed, ENY2 depleted cells incorporated more BrdU than vector cells treated with the same dose of irradiation (**Figure 68c**). Interestingly, while depletion of ENY2 increased p53 levels it impaired the induction of p16 upon irradiation (**Figure 68e, f**).

6.3.2 TAF10 and TAF12 play a role in senescence

TAF10 and TAF12 are two related transcription factors selected as potential regulators of OIS in our screen for their effect on both p16 and proliferation.
The similar outcome of their knockdown in senescence, together with the fact that they are part of the same protein complexes, suggested the possibility of both TAF proteins (via the activity of one of the complexes they take part of) regulating OIS.

6.3.2.1 TAFs 10 and 12 could mediate senescence through the p16/pRb and/or p53/p21 pathways

As previously mentioned knockdown of either TAF reduced p16 expression and delayed the cell cycle arrest of cells pushed to undergo senescence in response to oncogenic RAS (**Figure 69a-d**). This was a consistent result across several experiments that suggested that TAF10 and TAF12 could mediate p16 de-repression during OIS.

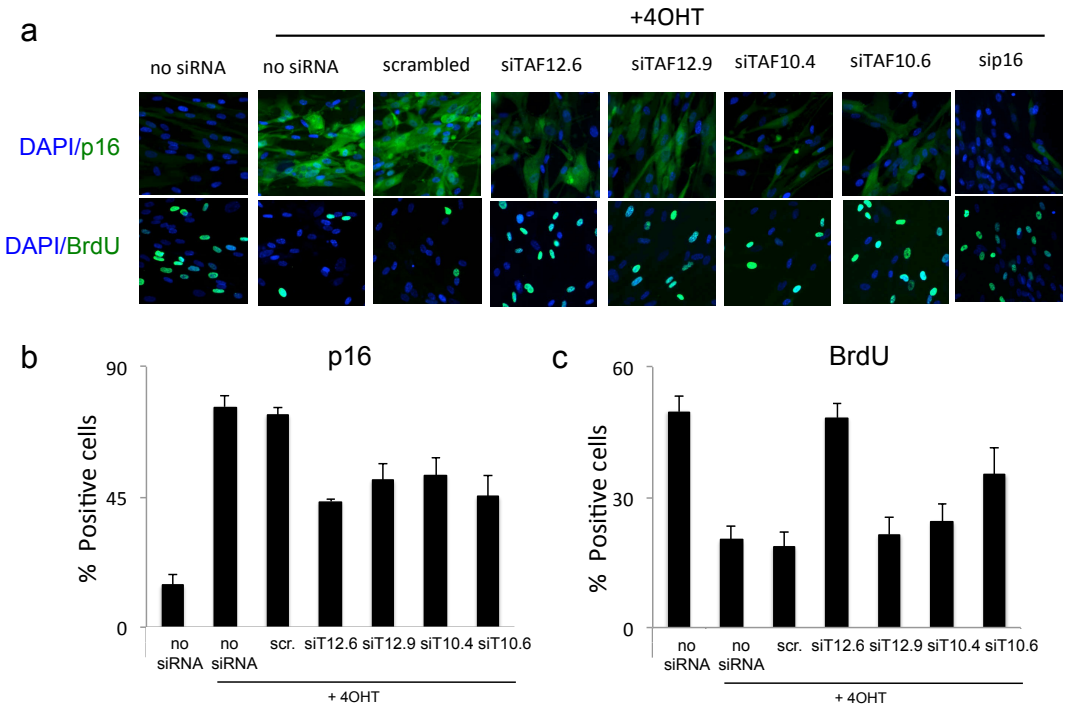


Figure 69. Transient knockdown of TAF10 and TAF12 concomitantly bypassed the proliferation arrest and prevented p16 induction during OIS. a) IF data collected with the IN Cell Analyzer 2000 showed a clear decrease in p16 expression with all siRNAs as well as an increase in the number of BrdU positive nuclei upon TAF10 and TAF12 knockdown. **b)** Knockdown of TAF10 and TAF12 led to decreased percentage of p16 expressing cells. **c)** The percentage of cells incorporating BrdU increases upon transient depletion of both TAF proteins.

We then set out to address whether TAFs depletion could impact on the expression of additional markers of senescence. To this end we performed immunofluorescence against several senescence regulators on IMR90 ER:RAS cells infected with siRNAs against TAF10 and TAF12.

Knockdown of both TAF10 and TAF12 independently resulted in decreased expression of p53 assessed 5 days post inducing RAS expression (**Figure 70a, b**). Moreover, also the levels of p21 were reduced upon knockdown of either TAF protein with independent siRNAs (**Figure 71a, b**).

This suggested, that in addition to the pronounced effect on p16 expression, TAF 10 and 12 could detain an additional role somewhere down the cascade of events regulating p53 and p21 expression during oncogene-induced senescence.

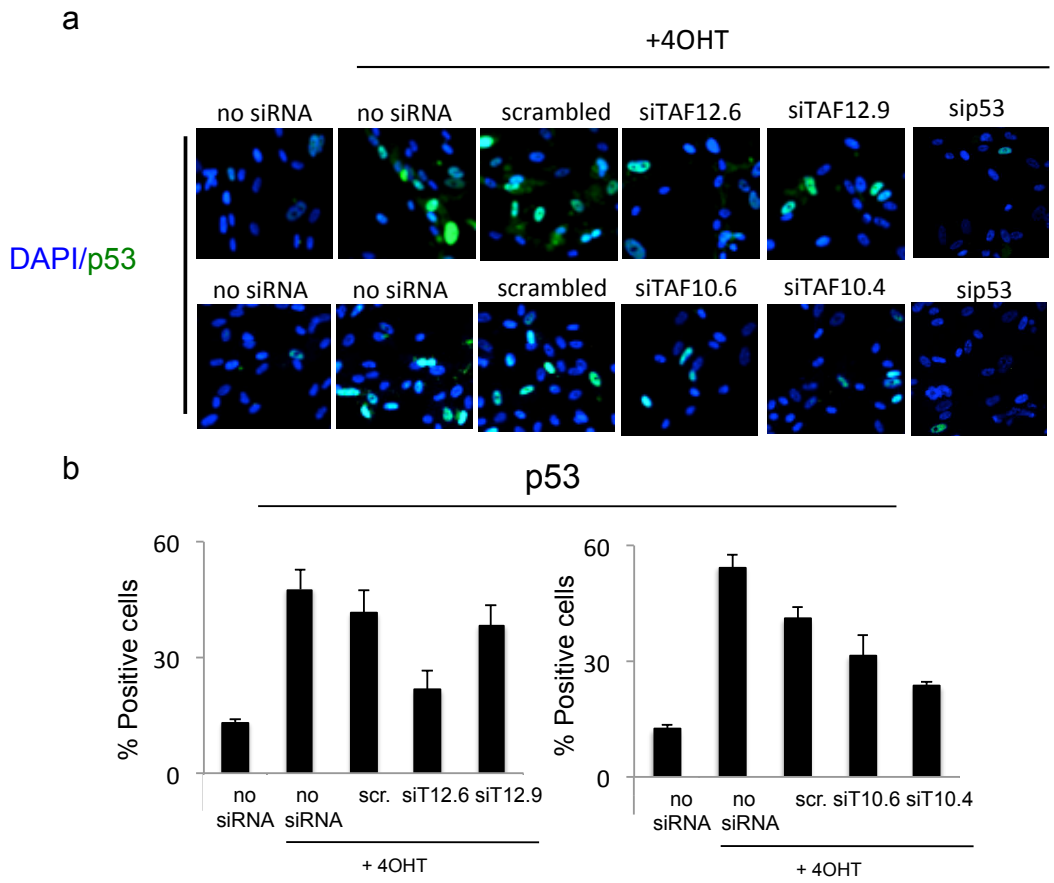


Figure 70. Expression of the tumour suppressor gene p53 is slightly impaired upon transient knockdown of the TAF proteins. a) Immunofluorescence images showing p53 expression of IMR90 ER:RAS cells transduced with siRNAs against TAF12 and TAF10. **b)** Quantification of the percentage p53 positive cells upon knockdown of TAF12 and TAF10, showing that depletion of both TAF proteins slightly reduced the pool of p53 expressing cells when compared with the untransfected or scrambled transfected cells.

Of note, the accumulation of 53BP1 and the production of the SASP did not suffer from TAF10 and TAF12 depletion during OIS (data not shown).

Altogether these results suggest that TAF10 and TAF12 regulate OIS probably by regulating the expression of the cyclin-dependent kinase inhibitors p16 and p21 and the tumour suppressor p53 to affect the cell cycle in a DDR-independent way.

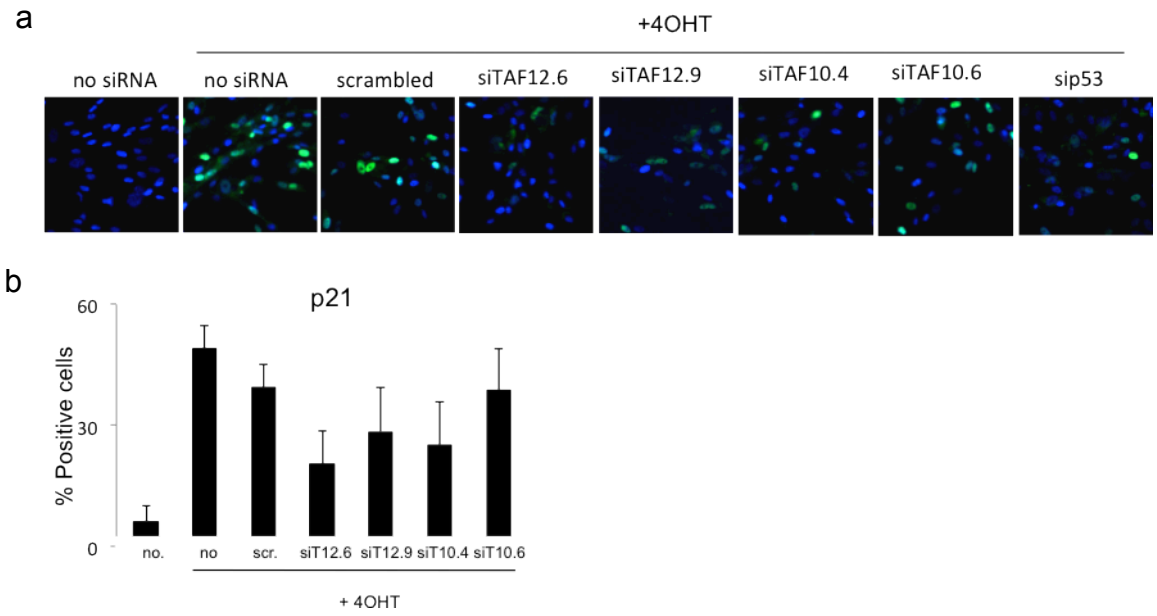


Figure 71. Transient depletion of TAF10 and TAF12 leads to decreased expression of p21. a) Immunofluorescence images for p21 upon knockdown of both TAF10 and TAF12 show a decrease in the number of p21 expressing nuclei when compared with the mock or scrambled control. **b)** Quantification of data obtained by immunofluorescence. Knockdown of both TAF proteins leads to a reduction in the percentage of p21 positive cells.

6.3.2.2 Knockdown of TAF10 and TAF12 extends the lifespan of IMR90 fibroblasts

To investigate whether TAFs function could extend to additional forms of senescence we studied the effect of TAF10 and TAF12 knockdown, with shRNAs, during γ-irradiation-induced and replicative senescence.

Depletion of TAF10 or TAF12 did not render conclusive results when cells were induced to senesce upon exposure to γ-irradiation (data not shown). However, when IMR90 fibroblasts were infected with multiple shRNAs targeting TAF10 and TAF12 and consecutively passaged, we observed an extension in the lifespan of cells depleted for either TAF, when compared with control cells. Specifically, while passage 27 fibroblasts, infected with the empty vector, underwent a strong cell cycle arrest, cells infected with shRNAs against TAF10 and TAF12 continued proliferating and forming colonies along time (**Figure 72a- d**).

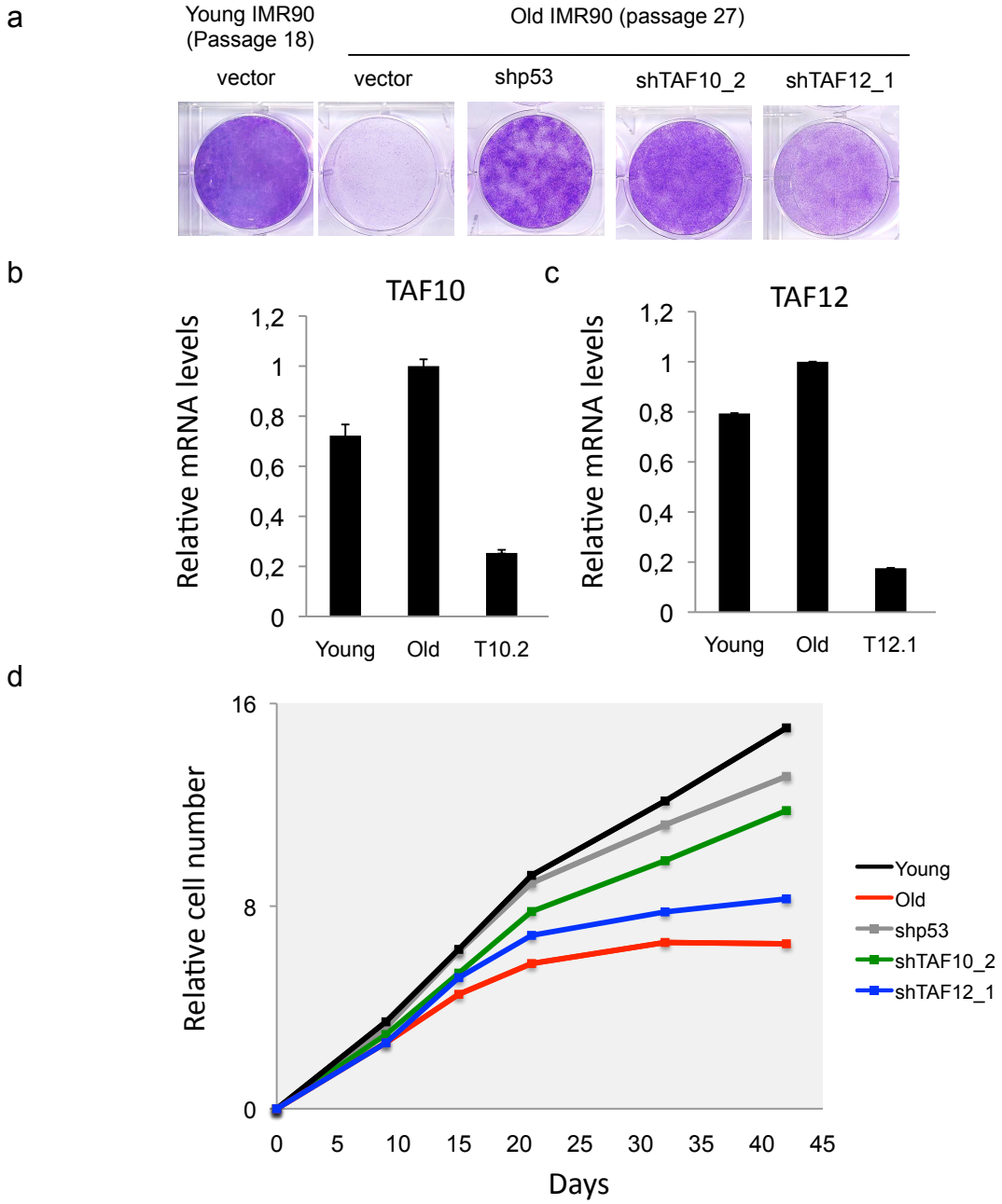


Figure 72. The lifespan of IMR90 fibroblasts is extended upon knockdown of TAF10 and TAF12. a) Cristal violet staining of passage 27 IMR90 cells infected with shRNAs against TAF10 and TAF12. Depletion of TAF10 and TAF12 delays the establishment of replicative senescence. **b-c)** RT-qPCR for TAF12 and TAF10 upon knockdown with specific hairpins, showing the genes were effectively knocked down. **d)** Growth curves for passage 27 IMR90 cells infected with hairpins targeting both TAF proteins show a growth advantage of these cells compared to cells infected with the vector alone. shp53 passage 27 cells and early-passage IMR90s (p18) were used as controls.

This suggested a possible implication for both TAF proteins in replicative senescence. Interestingly, knockdown of ENY2 under the same circumstances did not lead to a delayed entry of IMR90 cells in senescence (data not shown). These consist of preliminary experiments and therefore, a repetition should be made in order to validate results and clarify whether ENY2 affects replicative senescence to the same extent of TAF proteins.

6.3.2.3 Stable knockdown of TAF12 bypasses OIS

Finally, to confirm the results obtained with siRNAs we set out to investigate TAF10 and TAF12 knockdown in OIS using shRNAs. Unfortunately, lack of reproducibility for shRNA-mediated knockdown of TAF10 did not allow for conclusive results on this gene.

IMR90 ER:RAS were infected with the best shRNA against TAF12 and plated for colony formation assessment upon OIS induction. In line with the results previously obtained with siRNA-mediated knockdown, shTAF12-expressing cells displayed increased number of colonies when compared with control cells after RAS induction with 4-OHT (**Figure 73a**). Interestingly, however, we did not see a significant increase in the percentage of TAF12 deficient cells incorporating BrdU (**Figure 73b, c**). Furthermore, TAF12 knockdown led to a significant reduction in the percentage of cells with SAHFs (**Figure 73e**), further suggesting a role for TAF12 in senescence.

Additionally, we performed immunofluorescence against the prototypical senescence associated markers, and analyzed their expression via high-content analysis. In agreement with the results obtained for the transient knockdowns, shRNA-mediated depletion of TAF12 prevented full p16 and p21 induction and only slightly affected p53 expression (**Figure 73f-g**). Although consisting of preliminary analysis, these results reinforce the idea that TAF10 and TAF12 could regulate senescence by mediating both p16/pRb and p53/p21 pathways as well as chromatin remodeling.

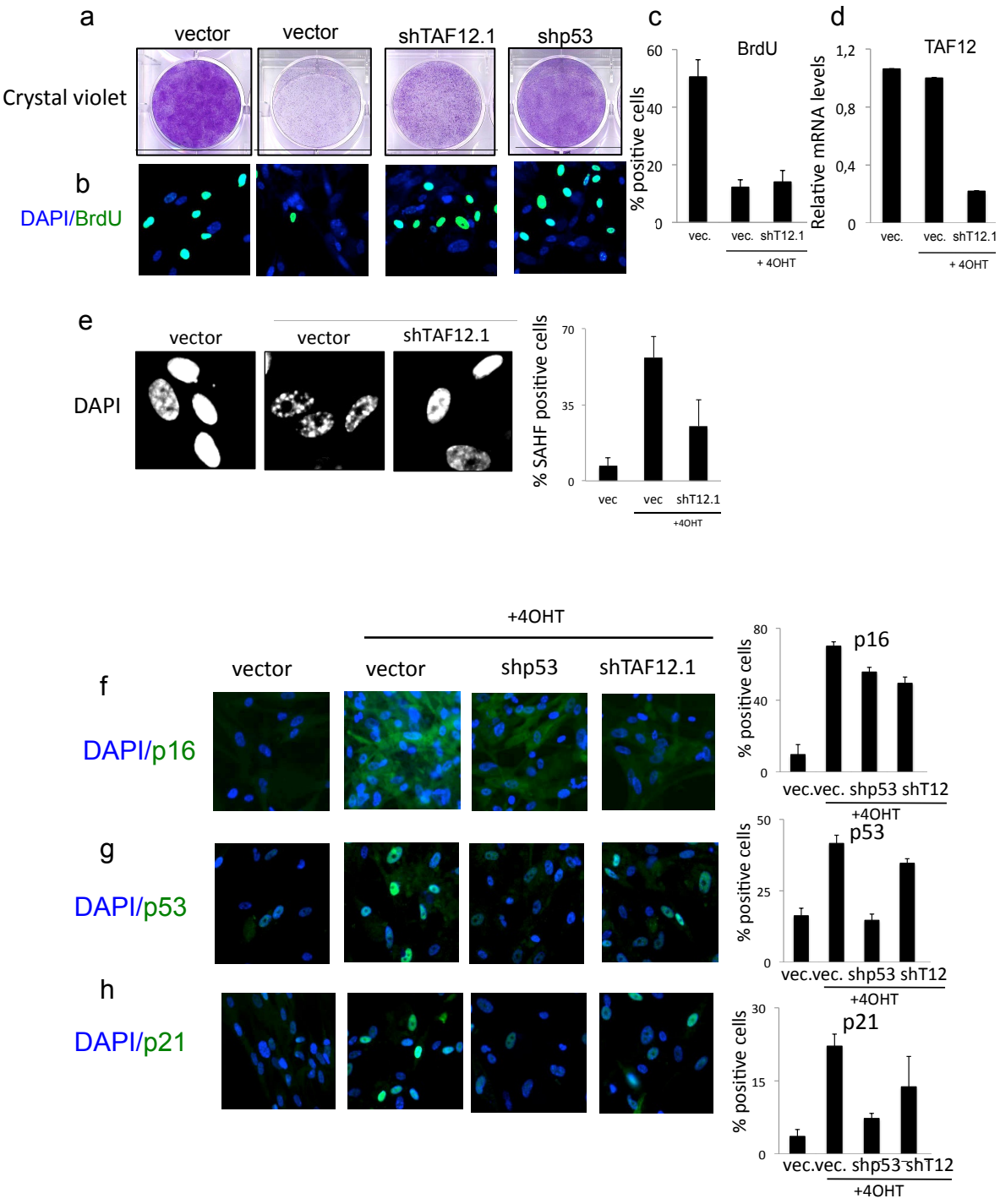


Figure 73. Stable knockdown of TAF12 bypases OIS. a) Cristal violet staining of low density plates shows increased cell number upon infection of IMR90 ER:RAS fibroblasts with shTAF12. **b)** Immunofluorescence of BrdU positive cells. **c)** RT-qPCR for TAF12 mRNA shows shTAF12 efficiently knocks TAF12 down in cells undergoing OIS. **d)** Quantification of the percentage of BrdU positive cells upon TAF12 depletion **e)** Immunofluorescence of DAPI stained nuclei shows a clear reduction in the formation of SAHFs upon TAF12 knockdown. **f-g)** IF against p16, p53 and p21 (left) and respective quantifications (right) shows TAF12 depletion with shRNA impairs both p16 and p21 expression while only slightly affecting p53.

6.4 Discussion and conclusions

6.4.1 TRIM28 is a new regulator of senescence

TRIM28 (also known as KAP1 or TIF1b) is a member of the Tripartite Motif (TRIM) family of E3 ligases characterized by the presence of a RING-finger motif, zinc-binding motifs and a coiled–coil region (Hatakeyama, 2011). The main function of TRIM28 is to act as a co-repressor for the KRAB-ZFPs transcriptional regulators by interacting with HP1 proteins and additional complexes involved in transcriptional repression such as SETDB1, NurD and HDACs leading to the formation of heterochromatin (Nielsen et al., 1999; Schultz et al., 2002; Schultz et al., 2001).

The activity of TRIM28 is regulated post-translationally via phosphorylation on serine (Ser 473 and 824) and tyrosine (Tyr 499, 458 and 517) residues. The most widely studied TRIM28 modification is its phosphorylation on serine 824 by ATM upon DNA damage (Li et al., 2007). Specifically, phosphorylation of TRIM28 leads to the release of the repressive machinery from the chromatin and therefore the relaxation of the DNA, allowing the assembly of the repair machinery onto the damaged sites (Goodarzi et al., 2011). A similar scenario is observed upon CHK2 mediated phosphorylation of TRIM28 at serine 473, which alleviates repression of cell cycle genes and induces cell proliferation (Bolderson et al., 2012; Chang et al., 2008). In addition to being phosphorylated, TRIM28 can also also be modified by sumoylation, what facilitates its interaction with SETDB1 contributing to gene repression (Ivanov et al., 2007; Iyengar and Farnham, 2011; Zeng et al., 2008)

TRIM28 can regulate multiple cellular processes, such as cell growth and differentiation, stem cell self-renewal and plutipotency, proliferation, apoptosis, DNA repair and oncogenic transformation (Iyengar and Farnham, 2011).

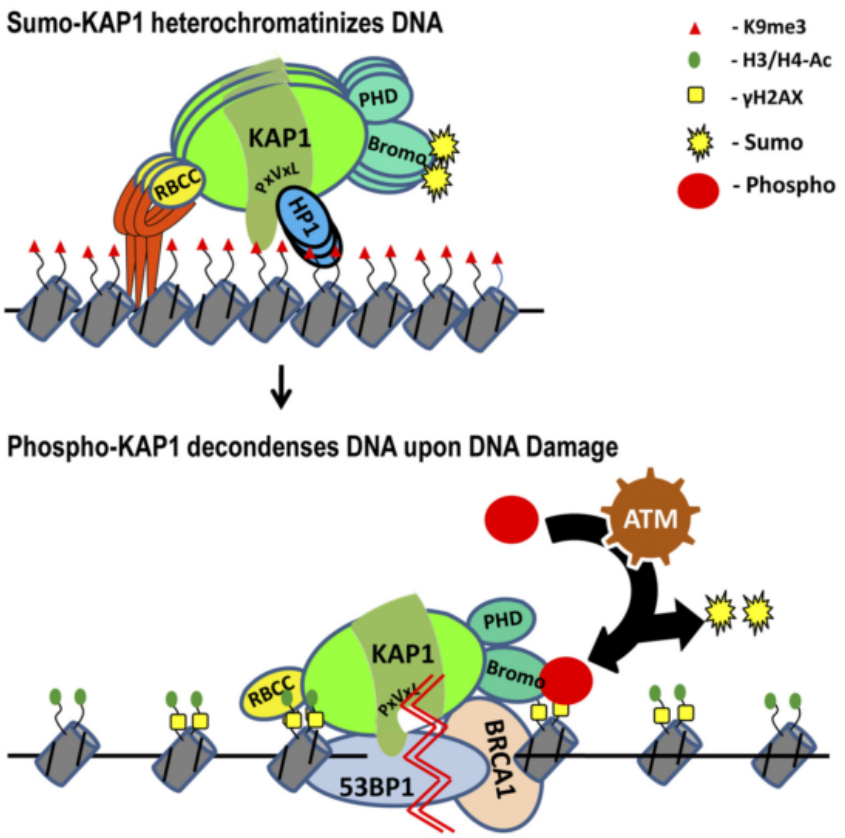


Figure 74. Regulation of gene expression by TRIM28. In unstressed cells TRIM28 (KAP1) is sumoylated and recruited to the DNA through interaction with KRAB-ZNFs. Sumoylated TRIM28 recruits repressive machinery leading to the establishment of H3K9me3. Upon DNA damage ATM phosphorylates TRIM28 (at residues S824 and S473) leading to a local dissolution of the heterochromatic structure, allowing the assembly of the DNA repair machinery (Adapted from Iyengar and Farnham, 2011).

However, the role in tumorigenesis is rather complex. In one hand, TRIM28 behaves as an oncogene and it is frequently overexpressed in tumors (Hatakeyama, 2011). Indeed, TRIM28 is a negative regulator of p53 and p21 (Iyengar and Farnham, 2011). On the other hand, knocking down TRIM28 in breast and lung cancer cell lines results in increased proliferation, due to de-repression of E2F3 and E2F4 mediated transcription. As higher expression of TRIM28 in early-stage lung tumors also increases overall survival of patients, TRIM28 has been categorized as a tumour suppressor in some genetic contexts (Lee et al., 2007; Li et al., 2007; Okamoto et al., 2006; Wang et al., 2005). Potentially contributing to this role as a tumour suppressor, a recent study suggested that the knock down of TRIM28 abrogated senescence in a mouse model of progeria (Chen et al., 2012).

Zmpste24$^{-/-}$ mice undergo accelerated ageing linked to p53 signaling activation (Varela et al., 2005), and mouse embryonic fibroblasts (MEFs) derived from *Zmpste24*$^{-/-}$ mice undergo premature senescence. Interestingly, this senescence response can be rescued by knocking down TRIM28 (Liu et al., 2013), but how exactly TRIM28 functions and what are the implications remains unexplored.

In the present study, using a model for OIS, we continued these observations showing that TRIM28 regulates OIS. Specifically, TRIM28 knockdown partially prevented OIS, and depletion of TRIM28 resulted in reduced p16 levels, without affecting the activation of the p53/p21 pathway (**Figure 55**, **Figure 56**). Whether the effect of TRIM28 on p16 induction is direct, indirect or just a consequence of TRIM28 inhibiting senescence is not clear yet but will be worth investigating. Since the main function of TRIM28 is acting as a transcriptional repressor, a possible explanation could be that TRIM28 represses a negative regulator of p16. Indeed, the expression of p16, and by extension the *INK4/ARF* locus, is subjected to strict transcriptional and epigenetic control (Liu et al., 2013).

Interestingly, we observed that TRIM28 is de novo phosphorylated on serine 824 during OIS (**Figure 53**). This phosphorylation is known to occur in response to the activation of the DNA damage response (Gil and Peters, 2006). Ser 824 phosphorylation has been shown to interfere with TRIM28 repressive abilities. It is not clear how to reconcile this observation with the effects that TRIM28 has on p16 expression during senescence. As TRIM28 activity is regulated by multiple phosphorylation and SUMOylation events further work would be needed to understand the precise mechanism(s) by which TRIM28 controls senescence.

TRIM28 connects the DNA damage response with heterochromatin organization. A DNA damage response is one of the key triggers initiating OIS (Li et al., 2007). A profound chromatin reorganization, that includes heterochromatin redistribution in SAHFs also occurs during senescence (Narita et al., 2003). Interestingly, TRIM28 knockdown reduced the percentage of cells with SAHFs, suggesting that TRIM28 could influence chromatin organization in senescence (**Figure 55**). Since it has been

proposed that the chromatin reorganization observed during senescence contributes to limit the DNA damage response, then TRIM28 could be a key factor coordinating these responses during OIS (Di Micco et al., 2006).

Our results also showed that TRIM28 depletion suppresses the SASP (**Figure 57**). Given that depletion of TRIM28 partially prevents senescence, the easiest explanation is that decreased senescence results in decreased SASP. However, it would be interesting to investigate whether TRIM28 directly regulates the SASP. Interestingly, besides its role in controlling chromatin formation, TRIM28 directly binds, and inhibits proteins of the IRF family involved in inflammatory signaling (Eames et al., 2012; Liang et al., 2011). TRIM28 has also been shown to negatively regulate IL6 expression by interfering with NF-κβ acetylation and recruitment to promoters (Kamitani et al., 2011; Tsuruma et al., 2008). Although it is not clear how that could be reconciled with our observations, the relation between TRIM28 and IRFs opens additional venues to explain how TRIM28 could control the SASP.

Interestingly, during ES cells differentiation sumoylated TRIM28 relocalizes from the pericentric heterochromatin to nucleoplasmic bodies, which the authors denominated KAKA foci, co-localizing with HP1 proteins and KRAB-ZFPs (Briers et al., 2009). The relevance of these foci is not clear but they are adjacent to PML bodies, which are important for senescence (Vernier et al., 2011). Whether the role of TRIM28 in senescence could advent from this association with PML needs further investigation.

The role of TRIM28 in cancer is complex, and probably context dependent (Hatakeyama, 2011). Our data would suggest that, by mediating senescence, TRIM28 could have tumour suppressive functions. Overall, here we have described a role for TRIM28 in regulating senescence that joins the list of other cellular processes regulated by TRIM28. Given the unique position of TRIM28 in the coordination of the DNA damage response and heterochromatin formation, understanding fully the role of TRIM28 in senescence will be important to further evaluate the possibility of targeting TRIM28 in disease.

6.4.2 The O-GlcNAc transferase (OGT) is a novel regulator of OIS

The O-linkage of N-acetylaglucosamine residues to proteins was first shown in 1984 by Torres and Hart while working with plasma membranes of lymphocytes (Torres and Hart, 1984). O-GlcNAc has since shown to be a widespread modification occurring in both nuclear and cytoplasmatic proteins (Haltiwanger et al., 1990; Haltiwanger Rs, 1992; Holts and Hart, 1986). UDP-GlcNAc is produced through the hexosamine biosynthetic pathway (HBP) and added to proteins by the O-linked N-acetylaglucosamine transferase (OGT) (Fardini et al., 2013). OGT (or O-GlcNAc transferase) is the only known enzyme able to catalyze the addition of O-GlcNac to the hydroxyl groups of serine or threonine residues, action that is counteracted by the β-N-acetylglucosaminidase (or O-GlcNAcase –OGA) (Dong and Hart, 1994; Holts and Hart, 1986; Kreppel et al., 1997; Torres and Hart, 1984; Vocadlo, 2012) (**Figure 75**).

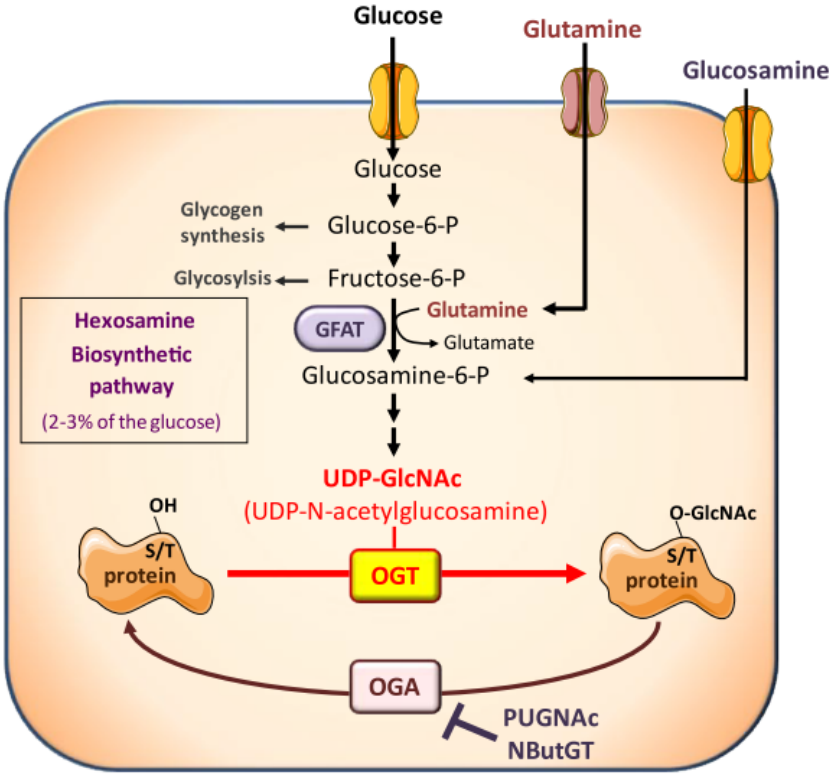


Figure 75. Protein O-GlcNAcylation results from concerted action of the enzymes OGT and OGA. The Hexosamine biosynthetic pathway feeds the cell with uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). The O-GlcNAc-transferase (OGT) catalyses the O-linkage of N-acetylaglucosamine residues to proteins (including histones). On its turn O-GlcNAcase (OGA) catalyses the removal of O-GclNAc. Therefore, the levels of O-GlcNAcylated proteins are maintained by concerted action of OGA and OGT (Adapted from Fardini et al., 2013)

The O-GlcNAcylation of proteins can regulate their function at several levels. O-GlcNAc can affect protein-protein binding, DNA-transcription factor binding, stability and nucle-cytoplasmatic shuttling of several proteins (Ozcan et al., 2010). Transcription initiation and repression has also been shown to happen via direct and indirect regulation of Pol II, respectively, by OGT (Ranuncolo et al., 2012; Yang et al., 2002). A direct role for epigenetic regulation by OGT has also been reported. OGT can modify core histones, through addition of O-GlcNAc residues as well as by indirectly promoting histone methylation (Chen et al., 2013; Deplus et al., 2013; Fong et al., 2012; Fujiki et al., 2011; Sakabe et al., 2010; Zhang et al., 2011b). H2B modification by OGT is dependent on association with TET2 (Chen et al., 2013; Fujiki et al., 2011). Association of OGT with TET1, TET2 and TET3 leads to increased levels of O-GlcNAcylated proteins on the CpG islands and TSS of TET proteins- regulated promoters, but generally not to an increase in 5-hydroxymethylation (5hmC) (Deplus et al., 2013; Vella et al., 2013). Other study, however, showed that O-GlcNAcylation of TET1 by OGT positively regulates 5hmC deposition (Shi 2013). Additionally, Deplus and coworkers showed that the association of OGT with TET2 and TET3 led to O-GlcNAcylation of HCF1, a member of the SET1/Compass H3K4 methyltransferase complex, thus providing an additional layer of epigenetic regulation by OGT (Deplus et al., 2013).

The extensive spectrum of OGT-regulated proteins suggests that O-GlcNAc can be an important post-translational modification for several cellular processes ranging from nutrient sensing to gene transcription. Altered levels of O-GlcNAc are responsible for diseases such as type II diabetes, neurodegeneration and cancer (Bond and Hanover, 2013).

The current work shows that OGT is also involved in regulating senescence. Knockdown of OGT in IMR90 ER:RAS cells bypassed the senescence-associated arrest (**Figure 58**,**Figure 60**). This result contradicts what has been observed in cancer cell lines. For instance, previous work has shown that OGT is upregulated is prostate cancer cells and tumours and contributes to cellular proliferation in a c-Myc mediated way. OGT is also associated with poor prognosis (Itkonen et al., 2013; Kamigaito et al., 2013). Additionally, knockdown of OGT in breast cancer cell lines reduces proliferation, leading to

upregulation of p27 (Caldwell et al., 2010). Indeed, increased O-GlcNAcylation and OGT expression is observed in several cancer types leading to the postulation of this mark as a cancer hallmark (Fardini et al., 2013). OGT can also regulate stem cell pluripotency and inhibit differentiation (Kim et al., 2009; Myers et al., 2011; Shi et al., 2013). Nevertheless, other reports describe decreased O-GlcNAcylation in breast and thyroid cancer, although no link with OGT expression was made (Krzeslak et al., 2010; Slawson et al., 2001). OGT can also O-GlcNAcylate the tumour suppressor p53 on Ser149, leading to its stabilization and reduced viability of cells upon doxyrubicin induced DNA damage, suggesting p53 modification by OGT could control its role in apoptosis and cell cycle arrest (Yang et al., 2006). However, in our results, OGT knockdown did not affect levels of p53 (**Figure 61**).

The function of OGT in controlling the cell cycle is intricate, as levels of O-GlcNAc oscillate during the cell cycle (Fardini et al., 2013; Zhang et al., 2011b). Senescent cells undergo G1 cell cycle arrest, although some cells also arrest in G2 (Dulic et al., 1993; Mao et al., 2012). In one experiment with human mammary gland cells, Drougat and collaborators showed that O-GlcNAcylation increased as cells progressed though G1, and rapidly decreased when cells progressed through S phase, an event that was attributed to increased OGA levels during S phase (Drougat et al., 2012). Additionally, they identified several differentially O-GlcNAcylated proteins during the G1/ S transition, including the lamina associated proteins, lamin A/C and lamin B. Therefore O-GlcNAcylation could play a role in the G1/S cell cycle checkpoint and entrance in senescence, for instance by counteracting phosphorylation events of key proteins. OGT can affect the phosphorylation of important proteins during mitosis (Wang et al., 2010). Indeed, O-GlcNAcylation of H3 Ser10 and H3 Thr32 reduces the mitosis-associated phosphorylation of Ser10, Ser28 and Thr32, and incomplete removal of the O-GlcNAc groups hinders mitosis (Fong et al., 2012; Zhang et al., 2011a). Of note, lowering O-GlcNac levels before the G1 phase leads to a quiescence-like cell cycle arrest (Slawson et al., 2005).

In the present work, levels of OGT and its modification were assessed by immunofluorescence and western blot (**Figure 59**,data not shown). OGT and

O-GlcNAc modified proteins were ubiquitously expressed in the nucleus and cytoplasm (Haltiwanger et al., 1990; Holts and Hart, 1986; Kreppel et al., 1997). Although no increase in OGT was detected, there was an increase in the expression of O-GlcNAcylated proteins, around the nucleus (**Figure 59**). It is worthy noting that glycosylation of the nuclear envelope has been previously reported however the relevance of that has not been addressed (Holts and Hart, 1986).

Consistently with the lack of effect on p53, also p21 levels seemed unaffected by OGT knockdown, however we observed a small decrease in p16 expression (**Figure 61**). This result goes at odds with the previously reported function of OGT in positively regulating Polycomb (Chu et al., 2014; Gambetta et al., 2009). Specifically, OGT has been shown to O-GlcNAcylate EZH2 at Ser75 leading to its stabilization and increase in H3K27me3 formation, a chromatin mark known to inhibit p16 expression (Chu et al., 2014). Additionally PRC2 is needed for OGT stability and O-GlcNAcylation of proteins in stem cells (Myers et al., 2011). Considering that the *INK4b-ARF-INK4a* locus is target of Polycomb-mediated repression, it is conceivable that it could be modified by OGT. In light of this, the O-GlcNAcylation status of the *INK4b-ARF-INK4a* locus should be investigated.

O-GlcNAcylation increases upon induction of DNA damage (Miura et al., 2012). Previous work has shown that OGT directly O-GlcNAcylates ATM, facilitating its phoshorylation and activity, while decreased O-GlcNAc levels would delay the DNA damage cascade (Miura et al., 2012). In the current work a small decrease of 53BP1 was seen 5 days after treating cells with 4-OHT **(Figure 63)**. It would be relevant to perform a time course study to evaluate the accumulation of this or other DNA damage associated markers at earlier and later time points to verify whether OGT knockdown delayed the DNA damage response triggered upon oncogene signaling as well.

Finally the most striking result that we have observed upon OGT knockdown was a significant decrease of the SASP markers IL8 and IL6 **(Figure 62)**. Indirect IL8 regulation by OGT has been reported (Allison et al., 2012). Specifically, a study with human and murine cells revealed that OGT modifies Rel-A/p65 allowing its acetylation by p300 and together localize to NF-κβ

regulated promoters. Since p65 is a regulator of the SASP, and considering that also IL6 mRNA levels were impaired after OGT depletion then it is feasible that OGT could be more broadly involved in regulating the secretory phenotype. Therefore would be interesting to investigate whether and how OGT regulates the SASP.

Interestingly, the shRNA providing the best bypass of the cell cycle arrest was the one that conferred the lowest knockdown of OGT (**Figure 60**). This suggests that perhaps an accentuated reduction of OGT levels would not be compatible with viability. Indeed, previous work suggested that depleting OGT in fibroblasts led to a brief cell cycle arrest followed by cell death (Donnell et al., 2004; Kazemi et al., 2010).

While the present work provided novel insights into OGT function in OIS, several questions remain to be answered. Further experiments should address how OGT activity is regulated and modulates OIS. For instance, OGT positively regulates the PI3K signaling pathway, as well as Erk1/2 activity, thus it is likely that OGT could specifically modulate Ras signaling (Fardini et al., 2013; Tallent et al., 2009). However OGT could additionally O-GlcNAcylate proteins with a more general role in senescence. Thus, cataloguing the pattern of O-GlcNAcylated proteins across different types of senescence and identifying the processes they regulate would be key to understand the role of OGT in senescence. Nevertheless, while protein O-GlcNAcylation is the only known function of OGT additional functions might exist, for what understanding whether its role in senescence depends on its enzymatic activity would also be relevant.

The fact that O-GlcNAcylated proteins accumulate during aging in rats suggests OGT could play a role in senescence-associated processes (Fulop et al., 2008). Thus, untangling its role in senescence could allow a better understanding of several pathologies and open venues for novel therapeutic approaches.

6.4.3 ENY2, TAF10 and TAF12 regulate OIS: SAGA or independent stories?

ENY2 is a small nuclear protein whose structure is evolutionary conserved. ENY2 can be found within the multisubunit SAGA (Spt–Ada–Gcn5 Acetyltransferase) and TREX-2 (Transcription and Export complex 2) complexes, which regulate histone deubiquitination and acetylation and mRNA export, respectively (Garcia-Oliver et al., 2012; Kohler et al., 2006; Koutelou et al., 2010; Rodriguez-Navarro et al., 2004; Samara and Wolberger, 2011; Zhao et al., 2008).

In the present work we observed that ENY2 knockdown strongly bypassed the senescence-associated arrest (**Figure 65**). Additionally, in the context of OIS, ENY2 knockdown prevented p16 and p21 induction and decreased the percentage of cells with SAHFs (**Figure 66**,**Figure 67**)

Interestingly, our screen also indentified siRNAs against two genes functionally related to ENY2. The TAF (TBP-associated factors) proteins, TAF10 and TAF12, are integral components of the previously mentioned SAGA complex, the histone acetylase complex PCAF and together with TBP are part of the TFIID complex, necessary for RNA Pol II mediated transcription (Bhaumik, 2011; Chen and Hampsey, 2002; Ogryzko et al., 1998). The knockdown of either TAF10 or TAF12 led to a marked bypass of the proliferation arrest and concomitant downregulation of p16, p21 and p53 without affecting accumulation of 53BP1 (**Figure 69**, **Figure 70**, **Figure 71**). Moreover, shTAF12 affected SAHF formation during OIS and depletion of either TAF extended the replicative life span of normal IMR90s (**Figure 72**).

TAF10, TAF12 and ENY2 are all components of the SAGA complex. This, together with the similar phenotype obtained upon individual knockdown of each gene in OIS, raises the idea that SAGA complex could regulate senescence.

The SAGA complex is organized into 4 subcomplexes with distinct functions: the deubiquitinating module (DUB, centered on USP22), the histone acetyltransferase module (HAT, centered on GCN5), the SPT module involved in pre-initiation complex assembly and the TAF module that plays

structural functions (**Figure 76**) (Samara and Wolberger, 2011; Weake and Workman, 2012). The enzymatic activity of the complex is present on the HAT and the DUB modules. These are involved in H3 acetylation and H2A and H2B deubiquitination, respectively, and are important for transcriptional activation and elongation by RNA Pol II (Govind et al., 2007; Lang et al., 2011; Wyce et al., 2007; Zhao et al., 2008).

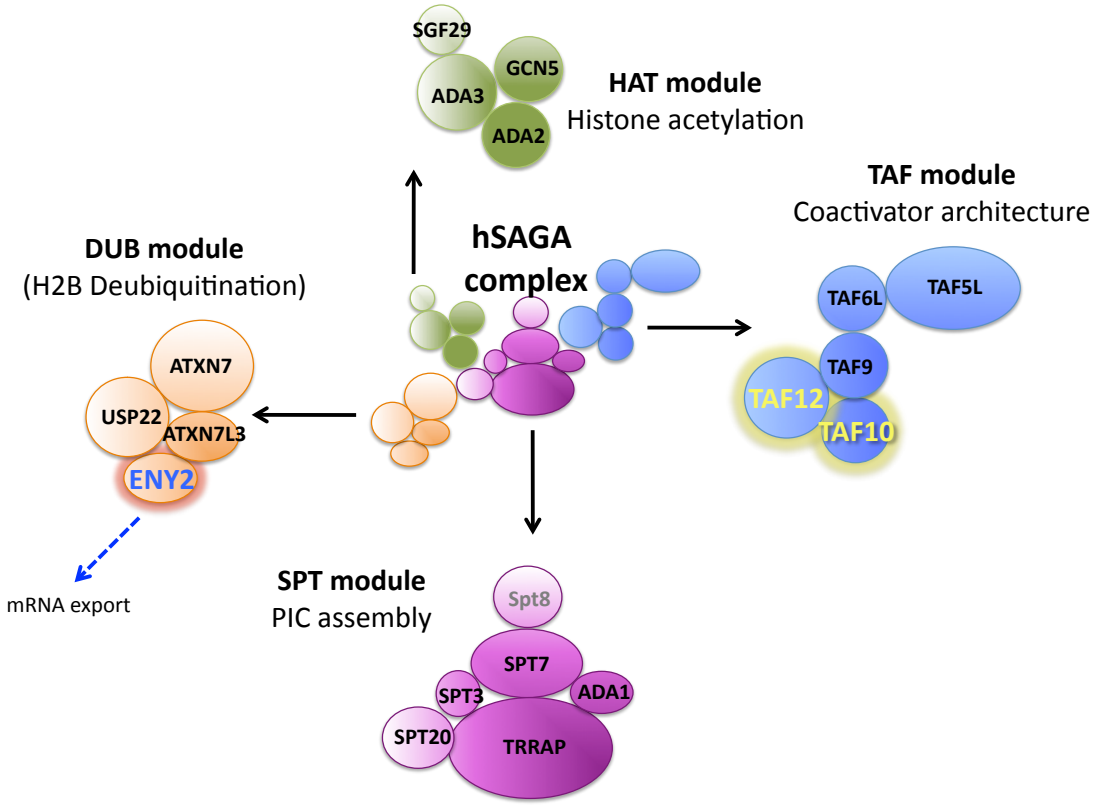


Figure 76. The human SAGA complex. The SAGA is composed by 4 multiprotein modules: the HAT module involved in histone acetylation, the DUB module with histone deubiquitinating activity, the TAF module that has structural and co-activator functions and the SPT module involved in Pre-initiation complex assembly. ENY2 belongs to the DUB module and has additional functions in mRNA export (in the TREX2 complex). TAF10 and TAF12 belong to the TAF module. The names shown correspond to the human homologs with exception of the yeast Spt8 (grey) which has not yet been described in humans. (Adapted from Samara and Wolberger, 2011)

While ENY2 belongs to the deubiquitination module, TAF10 and TAF12 are thought to contribute to the stability of the complex, similarly to the TFIID complex. However, TAF12 might additionally function to direct SAGA complex to promoters (Fishburn et al., 2005; Kohler et al., 2006; Reeves and Hahn, 2005; Wu et al., 2004; Zhao et al., 2008). The interdependence of the different modules or their ability to be recruited independently is still a matter of debate,

however it is possible that the HAT and DUB can coordinate their activities (Atanassov et al., 2009; Kohler et al., 2006; Weake and Workman, 2012).

The SAGA complex can regulate multiple processes (Galan and Rodriguez-Navarro, 2012; Koutelou et al., 2010). For instance, binding of SAGA and its acetylating activity are important for expression of p53 target genes such as p21 and Gadd45a, what would support a role for this complex in senescence (Gamper and Roeder, 2008). Additionally, deletion of members of the DUB module has recently shown to extend the replicative life span of yeast by negatively regulating SIRT2-mediated telomere-proximal gene silencing (McCormick et al., 2014). Although the HAT module alone did not interfere with the replicative life span, DUB mutants had increased replicative lifespan upon GCN5 deletion. However, SUS1 (yeast homologue of ENY2) did not seem to play a role in life span extension (McCormick et al., 2014).

Despite the tempting idea of TAF10, TAF12 and ENY2 regulating senescence through the SAGA complex, most studies suggest a role for the SAGA complex in mediating proliferation and tumourigenesis. Indeed, SAGA can cooperate with E2F and c-Myc to mediate transcriptional activation and cellular proliferation (Grant et al., 1998; Liu et al., 2003; Liu et al., 2008; McMahon et al., 1998). Specifically c-Myc has been shown to recruit USP22 (the deubiquitinase subunit of the DUB module of the SAGA complex), and consequently the SAGA complex, to mediate proliferation and transformation (Zhang et al., 2008). Moreover, depletion of USP22 leads to an accumulation of G1 arrested fibroblasts (Zhang et al., 2008). Additionally, the USP22 deubiquitinase activity is important for maintenance of TRF1 stability and telomere integrity, in a GCN5 dependent way but independently of its acetylating function (Atanassov et al., 2009). Finally, ENY2 can also regulate embryonic stem cells self-renewal (Hu et al., 2009).

The aforementioned examples go at odds with a role for the SAGA complex in senescence. Since ENY2, TAF10 and TAF12 have functions besides those related with the SAGA complex, it is possible that the role of these genes in senescence could be SAGA-independent. For instance, ENY2 is part of the TREX2 complex, which is involved in nuclear mRNA export (Jani et al., 2012; Kopytova et al., 2010; Rodriguez-Navarro et al., 2004). Previous work, mainly

in yeast has suggested that ENY2 could work as a bridge between the SAGA and TREX-2 complexes promoting contact between target genes and the nuclear pore complex (NPC), leading to transcription-coupled mRNA export (gene gating) (Cabal et al., 2006; Galan and Rodriguez-Navarro, 2012; Kohler et al., 2006; Kurshakova et al., 2007; Rodriguez-Navarro et al., 2004). However, a recent report suggested that in humans and Drosophila those complexes did not interact (Kopytova et al., 2010; Umlauf et al., 2013). Irrespective of whether or not ENY2-mediated gene gating happens in humans, it would be interesting to test whether the function of ENY2 in senescence depends on its role in mediating mRNA nuclear export. That could be addressed, for instance, by analyzing the cytoplasm-associated transcriptome of senescent cells lacking ENY2 or the TREX2 complex, to identify transcripts whose nuclear export depends on their activity.

Also TAF12 has been shown to have SAGA and TAF10-independent roles, for example, in RNA Pol I mediated transcription (Denissov et al., 2007). Also, TAF12 interacts with and recruits the Growth Arrest and DNA-damage-inducible protein (Gadd45a) to maintain promoters in a hypomethylated state (Schmitz et al., 2009). Interestingly Gadd45a is a p53 target, which contributes to p53 stability establishing a positive feed back loop in controlling cell cycle progression (Jin et al., 2003; Zhan, 2005; Zhan et al., 1999). Gadd45 has been linked with senescence via regulation of the p38/p53 signaling cascade (Bulavin et al., 2003; Passos et al., 2010). Since TAF12 can bind Gadd45a it would be interesting to investigate whether this would interfere with its ability to regulate p53 or p38. Of note, TAF12 is upregulated upon RAS signaling and important for its oncogenic properties (Voulgari et al., 2008). Additionally, TAF proteins take part of the PCAF and TFIID complexes that regulate the expression of senescence regulators. Indeed, the PCAF complex can regulate p53 acetylation in response to DNA damage (Jin et al., 2002; Love et al., 2012; Ogryzko et al., 1998) and the TFIID complex mediates recruitment of SWI/SNF members to target promoters (Sharma et al., 2003).

In summary our work identified the related factors TAF10, TAF12 and ENY2 as novel regulators of OIS. While the concerted action of these genes through

the SAGA complex is an attractive hypothesis, SAGA-independent functions exist for all of these factors. Thus, further experiments should try to address this by unveiling the gene spectrum regulated by each one of these factors and investigate whether or not they overlap. Additionally the relevance of both SAGA enzymatic activities in senescence (by specific knockdown of GCN5 or USP22) should be addressed, as it will reveal whether the role of TAF10, TAF12 and ENY2 in senescence arises from facilitating histone acetylation or deubiquitination. Role of additional relevant complexes, such as PCAF or TFIID should also be addressed in OIS. Indeed, the latter has been shown to accumulate during senescence in a previous work by Dimri and Campisi, what could explain the effects associated with TAF10 and TAF12 (Dimri and Campisi, 1994).

Overall the results herein present suggest that ENY2, TAF10 and TAF12 are novel potential regulators of senescence. Since TAF10 and TAF12 are frequently downregulated in cancer (*Tumorscape*- http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf), these results could have important biological implications. Hence, a detailed study of how these factors regulate senescence could improve our understanding of tumorigenesis and other senescence-associated diseases.

Chapter 7. Final conclusions and future work

7.1 Large-scale approaches identified novel regulators of OIS

Large-scale approaches have provided us with a detailed and integrative view of the gene networks regulating several processes. Approaches like high-content screenings, have allowed a functional characterization of these genes.

To unravel novel genes regulating OIS we performed spatial proteomics, allowing the analysis of subcellular proteomes. Several of the identified proteins were differentially expressed in senescence, mostly in the chromatin fraction. siRNA screens confirmed the functional relevance of the differentially expressed chromatin proteins, and identified GTF3C4, TRIM28, ENY2, TAF12, TAF10 and OGT as novel regulators of OIS.

The present proteomics approach compared only between proliferating and senescent cells. It will be now relevant to distinguish changes specific of OIS from those that result from a general state of cell cycle arrest or oncogene activation. For that, a wider spatial proteomics analysis is being conducted to compare between proliferating, OIS, OIS bypass and quiescent cells. Moreover, a characterization of the candidate genes should be carried out including transcriptome analysis upon gene knockdown or overexpression. The potential of these factors to induce senescence and their synergism with relevant senescence-associated pathways should be further tested upon overexpression. As the knockdown of the some candidate genes (*i.e.* TAF12 and ENY2) attenuated SAHF formation, it is likely they could play a role in OIS by regulating epigenetic mechanisms. Nevertheless, identified genes encompass alternative functions that range from glycosylation to mRNA export, for what will be key to first investigate where in the cell these genes are likely to exert their function in OIS and what proteins (chromatin - associated or not) are modified as part of their senescence program. Finally, their tumour suppressor potential should be addressed using cancer cell lines, human samples and animal models. Thorough characterization of these

genes in senescence and tumorigenesis would open novel venues for cancer therapy, potentially via chromatin-mediated modulation of gene expression.

7.2 Relevance of senescence and epigenetics for cancer therapy

Deregulation of senescence contributes to diseases ranging from liver fibrosis to cancer (review in Munoz-Espin and Serrano, 2014). OIS has long been regarded as a barrier to tumorigenesis *in vivo* (Bennecke et al., 2010; Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Lazzerini Denchi et al., 2005; Michaloglou et al., 2005).

The potential of senescence as an anticancer therapy was suggested upon observing that mainstream chemotherapeutical drugs such as doxorubicin and cyclophosphamide could trigger senescence (Chang et al., 1999a; Chang et al., 1999b; Schmitt et al., 2002). The concept of therapy-induced senescence (TIS) relies on the idea that one could modulate the expression of specific senescence-regulators during cancer, to divert cells into senescence, block proliferation and eventually clear off the tumour by action of the immune system (Baker et al., 2011; Munoz-Espin and Serrano, 2014; Sun et al., 2012; Xue et al., 2007). For instance, while using a Eμ-myc transgenic mouse lymphoma model, Dohr et al. showed that TIS leads to a highly metabolic and energy consuming senescent phenotype due to a need to counteract proteotoxic stress as a consequence of the SASP (Dorr et al., 2013). Due to their high demand in glucose, these senescent cells are sensitive to drugs eliciting the blockage of glucose intake or autophagy, ultimately undergoing apoptosis.

Several lines of TIS have been investigated and extensively reviewed, encompassing the use of p53 restoration approaches, PTEN and telomerase inactivation and CDK inhibitors (Acosta and Gil, 2012; Nardella et al., 2011). Indeed, the CDK4 inhibitor palbociclib (PD-0332991, Pfizer) has been already used and shown great results in phase II of clinical trials in the treatment of cell lymphoma, breast cancer and liposarcomas (Dickson et al., 2013; Guha, 2013; Leonard et al., 2012). Thus, discovering novel regulators of

senescence-associated pathways might provide novel targets for TIS and cancer elimination.

The identification of epigenetic regulators with a possible role in cancer (for what the study of processes like senescence might contribute) offers another layer of anti-cancer therapy. Aging and cancer have long been appreciated as epigenetic diseases (D'Aquila et al., 2013; Rodriguez-Paredes and Esteller, 2011a). The relevance of “cancer epigenetics” has been rising as not only several tumour suppressors are silenced by epigenetic mechanisms in cancer, but epigenetic regulators are also mutated in virtually all malignant tumors (Azad et al., 2013; Shen and Laird, 2013). For instance, members of the SWI/SNF complex (*i.e.* ARID1A and PBRM1) are largely mutated in cancer and members of the Polycomb proteins (*i.e.* EZH2), are overexpressed in cancer (Bracken et al., 2003; Jones et al., 2010; Wiegand et al., 2010; Kleer et al., 2003; Morin et al., 2010; Pawlowski et al., 2013; Varambally et al., 2002; Varela et al., 2011). As previously mentioned, both Polycomb and SWI/SNF regulate senescence (Agherbi et al., 2009; Bracken et al., 2007; Bracken et al., 2003; Kia et al., 2008). Thus the identification of epigenetic modulators in senescence might hint to possible genes or pathways altered in cancer.

The concept of cancer epigenetic therapy lays on two main premises: genes affected by abnormal epigenetic modulation are normally wild type, for what functional restoration is possible, and genetic abnormalities due to mutations in one chromatin regulator affect several downstream genes. Thus, epigenetic therapy, targeting a specific chromatin remodeler, will lead to a simultaneous reversal of the altered signaling pathways affected by it, restoring the function of the underlying genes (Azad et al., 2013). Although promising, and despite the large variety of epigenetic regulators described so far, only 4 drugs (as of 2014) have been approved for the epigenetic treatment of blood cancers: two HDACs inhibitors and 2 DNMTs inhibitors (Arrowsmith et al., 2012; Byrd et al., 2005; Garcia et al., 2010; Mummaneni and Shord, 2014; O'Connor et al., 2006; Piekarz et al., 2009; Rodriguez-Paredes and Esteller, 2011b). Hence, an exhaustive characterization of the senescence (such as the one herein

presented) and cancer epigenome should be undertaken in order to expand the arsenal of known epigenetic modulators of tumorigenesis and open novel venues for anti-cancer therapy.

7.3 Concluding remark

The emerging need of a more efficient and specialized anti-cancer medicine implies the study of all biological processes that could detain anti- or pro-tumorigenic effects. Since senescent cells harbor features of cancer cells, particularly regarding the signaling pathways and the epigenetic mechanisms they activate, the search for novel regulators of senescence and their thorough functional characterization will hopefully amplify our understanding of cancer and other aging-associated diseases.

Here, using a combined approach of spatial proteomics and siRNA screens, 6 novel chromatin factors regulating OIS were identified: TRIM28, OGT, ENY2, TAF12, TAF10 and GTF3C4. Efforts should now center on better understanding the biological and epigenetic value of these genes in senescence and in cancer.

References

Aan, G.J., Hairi, H.A., Makpol, S., Rahman, M.A., and Karsani, S.A. (2013). Differences in protein changes between stress-induced premature senescence and replicative senescence states. Electrophoresis *34*, 2209-2217.

Abbas, T., and Dutta, A. (2009). p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer *9*, 400-414.

Abdelmohsen, K., Panda, A., Kang, M.J., Xu, J., Selimyan, R., Yoon, J.H., Martindale, J.L., De, S., Wood, W.H., Becker, K.G.*, et al.* (2013). Senescence-associated lncRNAs: Senescence-associated long noncoding RNAs. Aging Cell *12*, 890-900.

Acosta, J.C., and Gil, J. (2012). Senescence: a new weapon for cancer therapy. Trends Cell Biol *22*, 211-219.

Acosta, J.C., Banito, A., Wuestefeld, T., Georgilis, A., Janich, P., Morton, J.P., Athineos, D., Kang, T.-W., Lasitschka, F., Andrulis, M.*, et al.* (2013a). A complex secretory program orchestrated by the inflammasome controls paracrine senescence. Nature cell biology *15*, 978-990.

Acosta, J.C., O'Loghlen, A., Banito, A., Guijarro, M.V., Augert, A., Raguz, S., Fumagalli, M., Da Costa, M., Brown, C., Popov, N.*, et al.* (2008). Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell *133*, 1006-1018.

Acosta, J.C., Snijders, A.P., and Gil, J. (2013b). Unbiased characterization of the senescence-associated secretome using SILAC-based quantitative proteomics. Methods Mol Biol *965*, 175-184.

Adams, P.D. (2007). Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. Gene *397*, 84-93.

Agger, K., Cloos, P.a.C., Rudkjaer, L., Williams, K., Andersen, G., Christensen, J., and Helin, K. (2009). The H3K27me3 demethylase JMJD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence. Genes & development *23*, 1171-1176.

Agherbi, H., Gaussmann-Wenger, A., Verthuy, C., Chasson, L., Serrano, M., and Djabali, M. (2009). Polycomb mediated epigenetic silencing and replication timing at the INK4a/ARF locus during senescence. PloS one *4*, e5622-e5622.

Alani, R.M., Young, a.Z., and Shifflett, C.B. (2001). Id1 regulation of cellular senescence through transcriptional repression of p16/Ink4a. Proceedings of the National Academy of Sciences of the United States of America *98*, 7812-7816.

Alcorta, D.a., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J.C. (1996). Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proceedings of the National Academy of Sciences of the United States of America *93*, 13742-13747.

Alessio, N., Squillaro, T., Cipollaro, M., Bagella, L., Giordano, a., and Galderisi, U. (2010). The BRG1 ATPase of chromatin remodeling complexes is involved in modulation of mesenchymal stem cell senescence through RB-P53 pathways. Oncogene *29*, 5452-5463.

Alimonti, A., Nardella, C., Chen, Z., Clohessy, J.G., Carracedo, A., Trotman, L.C., Cheng, K., Varmeh, S., Kozma, S.C., Thomas, G.*, et al.* (2010). A novel type of cellular senescence that can be enhanced in mouse models and human tumor xenografts to suppress prostate tumorigenesis. Journal of Clinical Investigation *120*, 681-693.

Aliouat-Denis, C.M. (2005). p53-Independent Regulation of p21Waf1/Cip1 Expression and Senescence by Chk2. Molecular Cancer Research *3*, 627-634.

Allison, D.F., Wamsley, J.J., Kumar, M., Li, D., Gray, L.G., Hart, G.W., Jones, D.R., and Mayo, M.W. (2012). Modification of RelA by O - linked N - acetylglucosamine links glucose metabolism to NF- ŒÍ B acetylation and transcription. Proceedings of the National Academy of Sciences of the United States of America *109*, 16888-16893.

Althubiti, M., Lezina, L., Carrera, S., Jukes-Jones, R., Giblett, S.M., Antonov, a., Barlev, N., Saldanha, G.S., Pritchard, C.a., Cain, K.*, et al.* (2014). Characterization of novel markers of senescence and their prognostic potential in cancer. Cell Death and Disease *5*, e1528-e1528.

Ancrile, B., Lim, K.H., and Counter, C.M. (2007). Oncogenic Ras-induced secretion of IL6 is required for tumorigenesis. Genes and Development *21*, 1714-1719.

Arrowsmith, C.H., Bountra, C., Fish, P.V., Lee, K., and Schapira, M. (2012). Epigenetic protein families: a new frontier for drug discovery. Nat Rev Drug Discov *11*, 384-400.

Aslanian, A., Yates, J.R., 3rd, and Hunter, T. (2014). Mass spectrometry-based quantification of the cellular response to methyl methanesulfonate treatment in human cells. DNA Repair (Amst) *15*, 29-38.

Atanassov, B.S., Evrard, Y.a., Multani, A.S., Zhang, Z., Tora, L.s., Devys, D., Chang, S., and Dent, S.Y.R. (2009). Gcn5 and SAGA regulate shelterin protein turnover and telomere maintenance. Molecular cell *35*, 352-364.

Avantaggiati, M.L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A.S., and Kelly, K. (1997). Recruitment of p300/CBP in p53-dependent signal pathways. Cell *89*, 1175-1184.

Azad, N., Zahnow, C.a., Rudin, C.M., and Baylin, S.B. (2013). The future of epigenetic therapy in solid tumours--lessons from the past. Nature reviews Clinical oncology *10*, 256-266.

Baillat, D., Hakimi, M.A., Naar, A.M., Shilatifard, A., Cooch, N., and Shiekhattar, R. (2005). Integrator, a multiprotein mediator of small nuclear RNA processing, associates with the C-terminal repeat of RNA polymerase II. Cell *123*, 265-276.

Baker, D.J., Wijshake, T., Tchkonia, T., LeBrasseur, N.K., Childs, B.G., van de Sluis, B., Kirkland, J.L., and van Deursen, J.M. (2011). Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature *479*, 232-236.

Banito, A., Rashid, S.T., Acosta, J.C., Li, S., Pereira, C.F., Geti, I., Pinho, S., Silva, J.C., Azuara, V., Walsh, M.*, et al.* (2009). Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev *23*, 2134-2139.

Barascu, A., Le Chalony, C., Pennarun, G., Genet, D., Imam, N., Lopez, B., and Bertrand, P. (2012). Oxidative stress induces an ATM-independent senescence pathway through p38 MAPK-mediated lamin B1 accumulation. EMBO J *31*, 1080-1094.

Barradas, M., Anderton, E., Acosta, J.C., Li, S., Banito, A., Rodriguez-Niedenfuhr, M., Maertens, G., Banck, M., Zhou, M.M., Walsh, M.J.*, et al.* (2009). Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS. Genes Dev *23*, 1177-1182.

Bartel, D.P., Lee, R., and Feinbaum, R. (2004). MicroRNAs : Genomics , Biogenesis , Mechanism , and Function Genomics : The miRNA Genes. *116*, 281-297.

Barth, T.K., and Imhof, A. (2010). Fast signals and slow marks: the dynamics of histone modifications. Trends in biochemical sciences *35*, 618-626.

Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L.-V.F., Kolettas, E., Niforou, K., Zoumpourlis, V.C.*, et al.* (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*, 633-637.

Beer, D.G., Kardia, S.L.R., Huang, C.-C., Giordano, T.J., Levin, A.M., Misek, D.E., Lin, L., Chen, G., Gharib, T.G., Thomas, D.G.*, et al.* (2002). Gene-expression profiles predict survival of patients with lung adenocarcinoma. Nature medicine *8*, 816-824.

Behrens, P., Brinkmann, U., and Wellmann, A. (2003). CSE1L/CAS: its role in proliferation and apoptosis. Apoptosis *8*, 39-44.

Bennecke, M., Kriegl, L., Bajbouj, M., Retzlaff, K., Robine, S., Jung, A., Arkan, M.C., Kirchner, T., and Greten, F.R. (2010). Ink4a/Arf and oncogene-induced senescence prevent tumor progression during alternative colorectal tumorigenesis. Cancer cell *18*, 135-146.

Benvenuti, S., Cramer, R., Bruce, J., Waterfield, M.D., and Jat, P.S. (2002). Identification of novel candidates for replicative senescence by functional proteomics. Oncogene *21*, 4403-4413.

Berns, K., Hijmans, E.M., Mullenders, J., Brummelkamp, T.R., Velds, A., Heimerikx, M., Kerkhoven, R.M., Madiredjo, M., Nijkamp, W., Weigelt, B.*, et al.* (2004). A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature *428*, 431-437.

Beronja, S., Janki, P., Heller, E., Lien, W.-H., Keyes, B.E., Oshimori, N., and Fuchs, E. (2013). RNAi screens in mice identify physiological regulators of oncogenic growth. Nature *501*, 185-190.

Bhargava, P., Reese, J.C., Gjidoda, A., and Henry, R.W. (2013). RNA polymerase III repression by the retinoblastoma tumor suppressor protein. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms *1829*, 385-392.

Bhaumik, S.R. (2011). Distinct regulatory mechanisms of eukaryotic transcriptional activation by SAGA and TFIID. Biochim Biophys Acta *1809*, 97-108.

Bianchi-Smiraglia, A., and Nikiforov, M.a. (2012). Controversial aspects of oncogene-induced senescence. Cell Cycle *11*, 4147-4151.

Bieging, K.T., Mello, S.S., and Attardi, L.D. (2014). Unravelling mechanisms of p53-mediated tumour suppression. Nature reviews Cancer *14*, 359-370.

Birmingham, A., Selfors, L.M., Forster, T., Wrobel, D., Kennedy, C.J., Shanks, E., Santoyo-Lopez, J., Dunican, D.J., Long, A., Kelleher, D.*, et al.* (2009). Statistical methods for analysis of high-throughput RNA interference screens. Nature methods *6*, 569-575.

Bischof, O., Kirsh, O., Pearson, M., Itahana, K., Pelicci, P.G., and Dejean, A. (2002). Deconstructing PML-induced premature senescence. EMBO Journal *21*, 3358-3369.

Bishop, C.L., Bergin, A.-M.H., Fessart, D., Borgdorff, V., Hatzimasoura, E., Garbe, J.C., Stampfer, M.R., Koh, J., and Beach, D.H. (2010). Primary cilium-dependent and -independent Hedgehog signaling inhibits p16(INK4A). Molecular cell *40*, 533-547.

Bodnar, a.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., and Wright, W.E. (1998). Extension of life-span by introduction of telomerase into normal human cells. Science (New York, NY) *279*, 349-352.

Boisvert, F.M., Lam, Y.W., Lamont, D., and Lamond, A.I. (2010). A quantitative proteomics analysis of subcellular proteome localization and changes induced by DNA damage. Mol Cell Proteomics *9*, 457-470.

Boisvert, F.M., and Lamond, A.I. (2010). p53-Dependent subcellular proteome localization following DNA damage. Proteomics *10*, 4087-4097.

Bolderson, E., Savage, K.I., Mahen, R., Pisupati, V., Graham, M.E., Richard, D.J., Robinson, P.J., Venkitaraman, A.R., and Khanna, K.K. (2012). Kruppel-associated Box (KRAB)-associated co-repressor (KAP-1) Ser-473 phosphorylation regulates heterochromatin protein 1beta (HP1-beta) mobilization and DNA repair in heterochromatin. J Biol Chem *287*, 28122-28131.

Bond, M.R., and Hanover, J.a. (2013). O- GlcNAc Cycling: A Link Between Metabolism and Chronic Disease. Annual Review of Nutrition *33*, 205-229.

Boultwood, J., and Wainscoat, J.S. (2007). Gene silencing by DNA methylation in haematological malignancies. British Journal of Haematology *138*, 3-11.

Bracken, A.P., Ciro, M., Cocito, A., and Helin, K. (2004). E2F target genes: unraveling the biology. Trends in biochemical sciences *29*, 409-417.

Bracken, A.P., and Helin, K. (2009). Polycomb group proteins: navigators of lineage pathways led astray in cancer. Nature reviews Cancer *9*, 773-784.

Bracken, A.P., Kleine-Kohlbrecher, D., Dietrich, N., Pasini, D., Gargiulo, G., Beekman, C., Theilgaard-Monch, K., Minucci, S., Porse, B.T., Marine, J.-C.*, et al.* (2007). The Polycomb group proteins bind throughout the INK4A-ARF

locus and are disassociated in senescent cells. Genes & development *21*, 525-530.

Bracken, A.P., Pasini, D., Capra, M., Prosperini, E., Colli, E., and Helin, K. (2003). EZH2 is downstream of the pRB-E2F pathway , essential for proliferation and amplified in cancer. *22*, 5323-5335.

Brady, C.a., Jiang, D., Mello, S.S., Johnson, T.M., Jarvis, L.a., Kozak, M.M., Kenzelmann Broz, D., Basak, S., Park, E.J., McLaughlin, M.E.*, et al.* (2011). Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. Cell *145*, 571-583.

Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A.H.F.M., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C.A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. Nature *436*, 660-665.

Brideau, C., Gunter, B., Pikounis, B., and Liaw, A. (2003). Improved statistical methods for hit selection in high-throughput screening. Journal of biomolecular screening : the official journal of the Society for Biomolecular Screening *8*, 634-647.

Briers, S., Crawford, C., Bickmore, W.a., and Sutherland, H.G. (2009). KRAB zinc-finger proteins localise to novel KAP1-containing foci that are adjacent to PML nuclear bodies. Journal of cell science *122*, 937-946.

Brookes, S., Rowe, J., Gutierrez Del Arroyo, A., Bond, J., and Peters, G. (2004). Contribution of p16INK4a to replicative senescence of human fibroblasts. Experimental Cell Research *298*, 549-559.

Brookes, S., Rowe, J., Ruas, M., Llanos, S., Clark, P.a., Lomax, M., James, M.C., Vatcheva, R., Bates, S., Vousden, K.H.*, et al.* (2002). INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. The EMBO journal *21*, 2936-2945.

Brown, J.P., Wei, W., and Sedivy, J.M. (1997). Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. Science (New York, NY) *277*, 831-834.

Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T., and Hannon, G.J. (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature *377*, 552-557.

Brummelkamp, T.R. (2002). A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. *296*, 550-553.

Bulavin, D.V., Kovalsky, O., Hollander, M.C., and Jr, A.J.F. (2003). Loss of Oncogenic H-ras-Induced Cell Cycle Arrest and p38 Mitogen-Activated Protein Kinase Activation by Disruption of Loss of Oncogenic H-ras-Induced Cell Cycle Arrest and p38 Mitogen-Activated Protein Kinase Activation by Disruption of Gadd45a. *23*, 3859-3871.

Burd, C.E., Sorrentino, J.a., Clark, K.S., Darr, D.B., Krishnamurthy, J., Deal, A.M., Bardeesy, N., Castrillon, D.H., Beach, D.H., and Sharpless, N.E. (2013). Monitoring tumorigenesis and senescence in vivo with a p16 INK4a-luciferase model. Cell *152*, 340-351.

Burkhart, D.L., and Sage, J. (2008). Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nature reviews Cancer *8*, 671-682.

Burrows, A.E., Smogorzewska, A., and Elledge, S.J. (2010). Polybromo-associated BRG1-associated factor components BRD7 and BAF180 are critical regulators of p53 required for induction of replicative senescence. Proceedings of the National Academy of Sciences of the United States of America *107*, 14280-14285.

Byrd, J.C., Marcucci, G., Parthun, M.R., Xiao, J.J., Klisovic, R.B., Moran, M., Lin, T.S., Liu, S., Sklenar, A.R., Davis, M.E.*, et al.* (2005). A phase 1 and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia. Oncology *105*, 959-967.

Cabal, G.G., Genovesio, A., Rodriguez-Navarro, S., Zimmer, C., Gadal, O., Lesne, A., Buc, H., Feuerbach-Fournier, F., Olivo-Marin, J.-C., Hurt, E.C.*, et al.* (2006). SAGA interacting factors confine sub-diffusion of transcribed genes to the nuclear envelope. Nature *441*, 770-773.

Cairns, C.a., and White, R.J. (1998). p53 is a general repressor of RNA polymerase III transcription. EMBO Journal *17*, 3112-3123.

Caldwell, S.A., Jackson, S.R., Shahriari, K.S., Lynch, T.P., Sethi, G., Walker, S., Vosseller, K., and Reginato, M.J. (2010). Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1. Oncogene *29*, 2831-2842.

Callen, E., Faryabi, R.B., Luckey, M., Hao, B., Daniel, J.a., Yang, W., Sun, H.W., Dressler, G., Peng, W., Chi, H.*, et al.* (2012). The DNA Damage- and Transcription-Associated Protein Paxip1 Controls Thymocyte Development and Emigration. Immunity *37*, 971-985.

Campisi, J., and d'Adda di Fagagna, F. (2007). Cellular senescence: when bad things happen to good cells. Nature reviews Molecular cell biology *8*, 729-740.

Capparelli, C., Chiavarina, B., Whitaker-Menezes, D., Pestell, T.G., Pestell, R.G., Hulit, J., And√≤, S., Howell, A., Martinez-Outschoorn, U.E., Sotgia, F.*, et al.* (2012). CDK inhibitors (p16/p19/p21) induce senescence and autophagy in cancer-associated fibroblasts, "fueling" tumor growth via paracrine interactions, without an increase in neo-angiogenesis. Cell Cycle *11*, 3599-3610.

Carthew, R.W., and Sontheimer, E.J. (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell *136*, 642-655.

Catez, F.d.r., Ueda, T., and Bustin, M. (2006). Determinants of histone H1 mobility and chromatin binding in living cells. Nature structural & molecular biology *13*, 305-310.

Chandra, T., Ewels, P.A., Fraser, P., Reik, W., Schoenfelder, S., Furlan-magaril, M., and Wingett, S.W. (2015). Global Reorganization of the Nuclear Landscape in Article Global Reorganization of the Nuclear Landscape in Senescent Cells. Celrep *10*, 471-483.

Chandra, T., Kirschner, K., Thuret, J.Y., Pope, B.D., Ryba, T., Newman, S., Ahmed, K., Samarajiwa, S.A., Salama, R., Carroll, T.*, et al.* (2012). Independence of Repressive Histone Marks and Chromatin Compaction during Senescent Heterochromatic Layer Formation. Molecular Cell *47*, 203-214.

Chandra, T., and Narita, M. (2013). High-order chromatin structure and the epigenome in SAHFs. Nucleus (Austin, Tex) *4*, 23-28.

Chandris, P., Giannouli, C.C., Panayotou, G., and Kletsas, D. (2010). Compromise in mRNA processing machinery in senescent human fibroblasts: Implications for a novel potential role of Phospho-ATR (ser428). Biogerontology *11*, 421-436.

Chang, B.D., Broude, E.V., Dokmanovic, M., Zhu, H., Ruth, A., Xuan, Y., Kandel, E.S., Lausch, E., Christov, K., and Roninson, I.B. (1999a). A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. Cancer Research *59*, 3761-3767.

Chang, B.D., Xuan, Y., Broude, E.V., Zhu, H., Schott, B., Fang, J., and Roninson, I.B. (1999b). Role of p53 and p21waf1/cip1 in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs. Oncogene *18*, 4808-4818.

Chang, C.-J., Yang, J.-Y., Xia, W., Chen, C.-T., Xie, X., Chao, C.-H., Woodward, W.a., Hsu, J.-M., Hortobagyi, G.N., and Hung, M.-C. (2011). EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-β-catenin signaling. Cancer cell *19*, 86-100.

Chang, C.-W., Chou, H.-Y., Lin, Y.-S., Huang, K.-H., Chang, C.-J., Hsu, T.-C., and Lee, S.-C. (2008). Phosphorylation at Ser473 regulates heterochromatin protein 1 binding and corepressor function of TIF1beta/KAP1. BMC molecular biology *9*, 61-61

Charmpilas, N., Daskalaki, I., Papandreou, M.E., and Tavernarakis, N. (2014). Protein synthesis as an integral quality control mechanism during ageing. Ageing Research Reviews, 1-15.

Chen, B.S., and Hampsey, M. (2002). Transcription activation: Unveiling the essential nature of TFIID. Current Biology *12*, 620-622.

Chen, L., Chen, D.-T., Kurtyka, C., Rawal, B., Fulp, W.J., Haura, E.B., and Cress, W.D. (2012). Tripartite motif containing 28 (Trim28) can regulate cell proliferation by bridging HDAC1/E2F interactions. The Journal of biological chemistry *287*, 40106-40118.

Chen, Q., Chen, Y., Bian, C., Fujiki, R., and Yu, X. (2013). TET2 promotes histone O-GlcNAcylation during gene transcription. Nature *493*, 561-564.

Chen, J., and Wagner, E.J. (2010). snRNA 3' end formation: the dawn of the Integrator complex. Biochemical Society transactions *38*, 1082-1087.

Chen, L., Li, Z., Zwolinska, A.K., Smith, M.a., Cross, B., Koomen, J., Yuan, Z.-M., Jenuwein, T., Marine, J.-C., Wright, K.L*., et al.* (2010). MDM2 recruitment of lysine methyltransferases regulates p53 transcriptional output. The EMBO journal *29*, 2538-2552.

Chen, Q., Fischer, a., Reagan, J.D., Yan, L.J., and Ames, B.N. (1995). Oxidative DNA damage and senescence of human diploid fibroblast cells. Proceedings of the National Academy of Sciences of the United States of America *92*, 4337-4341.

Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.-K., Dotan, Z.a., Niki, M., Koutcher, J.a., Scher, H.I., Ludwig, T., Gerald, W*., et al.* (2005). Crucial role of

p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature *436*, 725-730.

Chesnokov, I., Chu, W.M., Botchan, M.R., and Schmid, C.W. (1996). p53 inhibits RNA polymerase III-directed transcription in a promoter-dependent manner. Molecular and cellular biology *16*, 7084-7088.

Chicas, a., Kapoor, a., Wang, X., Aksoy, O., Evertts, a.G., Zhang, M.Q., Garcia, B.a., Bernstein, E., and Lowe, S.W. (2012). H3K4 demethylation by Jarid1a and Jarid1b contributes to retinoblastoma-mediated gene silencing during cellular senescence. Proceedings of the National Academy of Sciences *109*, 8971-8976.

Chicas, A., Wang, X., Zhang, C., McCurrach, M., Zhao, Z., Mert, O., Dickins, R.A., Narita, M., Zhang, M., and Lowe, S.W. (2010). Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. Cancer Cell *17*, 376-387.

Chien, Y., Scuoppo, C., Wang, X., Fang, X., Balgley, B., Bolden, J.E., Premsrirut, P., Luo, W., Chicas, A., Lee, C.S.*, et al.* (2011). Control of the senescence-associated secretory phenotype by NF-κβ promotes senescence and enhances chemosensitivity. Genes and Development *25*, 2125-2136.

Childs, B.G., Baker, D.J., Kirkland, J.L., Campisi, J., and Deursen, J.M.V. (2014). Senescence and apoptosis : dueling or complementary cell fates ? *15*, 1139-1154.

Chittka, A., Nitarska, J., Grazini, U., and Richardson, W.D. (2012). Transcription factor positive regulatory domain 4 (PRDM4) recruits protein arginine methyltransferase 5 (PRMT5) to mediate histone arginine methylation and control neural stem cell proliferation and differentiation. Journal of Biological Chemistry *287*, 42995-43006.

Cho, E.A., Prindle, M.J., and Dressler, G.R. (2003). BRCT domain-containing protein PTIP is essential for progression through mitosis. Molecular and cellular biology *23*, 1666-1673.

Cho, Y.W., Hong, T., Hong, S., Guo, H., Yu, H., Kim, D., Guszczynski, T., Dressler, G.R., Copeland, T.D., Kalkum, M.*, et al.* (2007). PTIP associates with MLL3- and MLL4-containing histone H3 lysine 4 methyltransferase complex. J Biol Chem *282*, 20395-20406.

Christiansen, M.N., Chik, J., Lee, L., Anugraham, M., Abrahams, J.L., and Packer, N.H. (2014). Cell surface protein glycosylation in cancer. Proteomics *14*, 525-546.

Chu, C.-S., Lo, P.-W., Yeh, Y.-H., Hsu, P.-H., Peng, S.-H., Teng, Y.-C., Kang, M.-L., Wong, C.-H., and Juan, L.-J. (2014). O-GlcNAcylation regulates EZH2 protein stability and function. Proceedings of the National Academy of Sciences of the United States of America *111*, 1355-1360.

Chu, I.M., Hengst, L., and Slingerland, J.M. (2008). The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. Nature reviews Cancer *8*, 253-267.

Clair, S.S., and Manfredi, J.J. (2006). The Dual Specificity Phosphatase Cdc25C is a Direct Target for Perspective ND SC. Cell Cycle, 709-713.

Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benguria, A., Zaballos, A., Flores, J.M., Barbacid, M.*, et al.* (2005). Tumour biology: senescence in premalignant tumours. Nature *436*, 642.

Coppe, J.P., Desprez, P.Y., Krtolica, A., and Campisi, J. (2010). The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol *5*, 99-118.

Coppe, J.P., Patil, C.K., Rodier, F., Sun, Y., Munoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.Y., and Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol *6*, 2853-2868.

Coppe, J.P., Rodier, F., Patil, C.K., Freund, A., Desprez, P.Y., and Campisi, J. (2011). Tumor suppressor and aging biomarker p16(INK4a) induces cellular senescence without the associated inflammatory secretory phenotype. J Biol Chem *286*, 36396-36403

Corpet, A., Olbrich, T., Gwerder, M., Fink, D., and Stucki, M. (2014). Dynamics of histone H3.3 deposition in proliferating and senescent cells reveals a DAXX-dependent targeting to PML-NBs important for pericentromeric heterochromatin organization. Cell Cycle *13*, 249-267.

Cotta-Ramusino, C., McDonald, E.R., 3rd, Hurov, K., Sowa, M.E., Harper, J.W., and Elledge, S.J. (2011). A DNA damage response screen identifies RHINO, a 9-1-1 and TopBP1 interacting protein required for ATR signaling. Science *332*, 1313-1317.

Courtois-Cox, S., Genther Williams, S.M., Reczek, E.E., Johnson, B.W., McGillicuddy, L.T., Johannessen, C.M., Hollstein, P.E., MacCollin, M., and Cichowski, K. (2006). A negative feedback signaling network underlies oncogene-induced senescence. Cancer Cell *10*, 459-472.

Courtois-Cox, S., Jones, S.L., and Cichowski, K. (2008). Many roads lead to oncogene-induced senescence. Oncogene *27*, 2801-2809.

Cristofalo, V.J., Lorenzini, A., Allen, R.G., Torres, C., and Tresini, M. (2004). Replicative senescence: A critical review. Mechanisms of Ageing and Development *125*, 827-848.

Cruickshanks, H.a., McBryan, T., Nelson, D.M., Vanderkraats, N.D., Shah, P.P., van Tuyn, J., Singh Rai, T., Brock, C., Donahue, G., Dunican, D.S.*, et al.* (2013). Senescent cells harbour features of the cancer epigenome. Nature cell biology *15*, 1495-1506.

Cullen, B.R. (2006). Enhancing and confirming the specificity of RNAi experiments. Nat Methods *3*, 677-681.

d'Adda di Fagagna, F. (2008). Living on a break: cellular senescence as a DNA-damage response. Nature reviews Cancer *8*, 512-522.

d'Adda di Fagagna, F., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P., and Jackson, S.P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. Nature *426*, 194-198.

Dahlgren, C., Zhang, H.Y., Du, Q., Grahn, M., Norstedt, G., Wahlestedt, C., and Liang, Z. (2008). Analysis of siRNA specificity on targets with double-nucleotide mismatches. Nucleic Acids Res *36*, e53.

D'Aquila, P., Rose, G., Bellizzi, D., and Passarino, G. (2013). Epigenetics and aging. Maturitas *74*, 130-136.

Davis-Smyth, T., Duncan, R.C., Zheng, T., Michelotti, G., and Levens, D. (1996). The far upstream element-binding proteins comprise an ancient family of single-strand DNA-binding transactivators. J Biol Chem *271*, 31679-31687.

De Cecco, M., Criscione, S.W., Peckham, E.J., Hillenmeyer, S., Hamm, E.A., Manivannan, J., Peterson, A.L., Kreiling, J.A., Neretti, N., and Sedivy, J.M. (2013). Genomes of replicatively senescent cells undergo global epigenetic changes leading to gene silencing and activation of transposable elements. Aging Cell *12*, 247-256.

de Graaf, E.L., Kaplon, J., Zhou, H., Heck, A.J.R., Peeper, D.S., and Altelaar, a.F.M. (2014). Phosphoproteome dynamics in onset and maintenance of Oncogene-Induced Senescence. Molecular & cellular proteomics : MCP, 1-35.

de Keizer, P.L.J., Packer, L.M., Szypowska, A.a., Riedl-Polderman, P.E., van den Broek, N.J.F., de Bruin, A., Dansen, T.B., Marais, R., Brenkman, A.B., and Burgering, B.M.T. (2010). Activation of forkhead box O transcription factors by oncogenic BRAF promotes p21cip1-dependent senescence. Cancer research *70*, 8526-8536.

Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell *82*, 675-684.

Denissov, S., van Driel, M., Voit, R., Hekkelman, M., Hulsen, T., Hernandez, N., Grummt, I., Wehrens, R., and Stunnenberg, H. (2007). Identification of novel functional TBP-binding sites and general factor repertoires. The EMBO journal *26*, 944-954.

Deplus, R., Delatte, B., Schwinn, M.K., Defrance, M., Mendez, J., Murphy, N., Dawson, M.A., Volkmar, M., Putmans, P., Calonne, E.*, et al.* (2013). TET2 and TET3 regulate GlcNAcylation and H3K4 methylation through OGT and SET1/COMPASS. EMBO J *32*, 645-655.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P.G., Bensimon, A.*, et al.* (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

Di Micco, R., Sulli, G., Dobreva, M., Liontos, M., Botrugno, O.A., Gargiulo, G., dal Zuffo, R., Matti, V., d'Ario, G., Montani, E.*, et al.* (2011). Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. Nat Cell Biol *13*, 292-302.

Dickins, R.A., Hemann, M.T., Zilfou, J.T., Simpson, D.R., Ibarra, I., Hannon, G.J., and Lowe, S.W. (2005). Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. Nat Genet *37*, 1289-1295.

Dickson, M.a., Tap, W.D., Keohan, M.L., D'Angelo, S.P., Gounder, M.M., Antonescu, C.R., Landa, J., Qin, L.X., Rathbone, D.D., Condy, M.M.*, et al.* (2013). Phase II trial of the CDK4 inhibitor PD0332991 in patients with advanced CDK4-amplified well-differentiated or dedifferentiated liposarcoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *31*, 2024-2028.

Dietrich, N., Bracken, A.P., Trinh, E., Schjerling, C.K., Koseki, H., Rappsilber, J., Helin, K., and Hansen, K.H. (2007). Bypass of senescence by the polycomb group protein CBX8 through direct binding to the INK4A-ARF locus. EMBO J *26*, 1637-1648.

Dimauro, T., and David, G. (2009). Chromatin modifications: the driving force of senescence and aging? Aging (Albany NY) *1*, 182-190.

Dimri, G.P., and Campisi, J. (1994). Altered profile of transcription factor-binding activities in senescent human fibroblasts. Experimental cell research *212*, 132-140.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., and Pereira-Smith, O. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proceedings of the National Academy of Sciences of the United States of America *92*, 9363-9367.

Ding, L., Paszkowski-Rogacz, M., Nitzsche, A., Slabicki, M.M., Heninger, A.K., Vries, I.d., Kittler, R., Junqueira, M., Shevchenko, A., Schulz, H.*, et al.* (2009). A Genome-Scale RNAi Screen for Oct4 Modulators Defines a Role of the Paf1 Complex for Embryonic Stem Cell Identity. Cell Stem Cell *4*, 403-415.

Diotti, R., and Loayza, D. (2011). Shelterin complex and associated factors at human telomeres. *2*, 119-135.

Donati, G., Peddigari, S., Mercer, C.a., and Thomas, G. (2013). 5S Ribosomal RNA Is an Essential Component of a Nascent Ribosomal Precursor Complex that Regulates the Hdm2-p53 Checkpoint. Cell Reports *4*, 87-98.

Donnell, N.O., Zachara, N.E., Hart, G.W., and Marth, J.D. (2004). Protein Glycosylation Is a Requisite Modification in Somatic Cell Function and Embryo Viability Ogt -Dependent X-Chromosome-Linked Protein Glycosylation Is a Requisite Modification in Somatic Cell Function and Embryo Viability. *24*, 1680-1690.

Dong, D.L., and Hart, G.W. (1994). Purification and characterization of an O-GlcNAc selective N-acetyl-beta-D-glucosaminidase from rat spleen cytosol. The Journal of biological chemistry *269*, 19321-19330.

Donze, D. (2012). Extra-transcriptional functions of RNA Polymerase III complexes: TFIIIC as a potential global chromatin bookmark. Gene *493*, 169-175.

Dorr, J.R., Yu, Y., Milanovic, M., Beuster, G., Zasada, C., Dabritz, J.H., Lisec, J., Lenze, D., Gerhardt, A., Schleicher, K.*, et al.* (2013). Synthetic lethal metabolic targeting of cellular senescence in cancer therapy. Nature *501*, 421-425.

Dreesen, O., Chojnowski, A., Ong, P.F., Zhao, T.Y., Common, J.E., Lunny, D., Lane, E.B., Lee, S.J., Vardy, L.A., Stewart, C.L.*, et al.* (2013). Lamin B1 fluctuations have differential effects on cellular proliferation and senescence. Journal of Cell Biology *200*, 605-617.

Drost, J., Mantovani, F., Tocco, F., Elkon, R., Comel, A., Holstege, H., Kerkhoven, R., Jonkers, J., Voorhoeve, P.M., Agami, R.*, et al.* (2010). BRD7 is a candidate tumour suppressor gene required for p53 function. Nature cell biology *12*, 380-389.

Drougat, L., Olivier-Van Stichelen, S.p., Mortuaire, M.n., Foulquier, F.o., Lacoste, A.-S., Michalski, J.-C., Lefebvre, T., and Vercoutter-Edouart, A.-S. (2012). Characterization of O-GlcNAc cycling and proteomic identification of differentially O-GlcNAcylated proteins during G1/S transition. Biochimica et biophysica acta *1820*, 1839-1848.

Duarte, L.F., Young, A.R., Wang, Z., Wu, H.A., Panda, T., Kou, Y., Kapoor, A., Hasson, D., Mills, N.R., Ma'ayan, A.*, et al.* (2014). Histone H3.3 and its proteolytically processed form drive a cellular senescence programme. Nat Commun *5*, 5210.

Dulic, V., Drullinger, L.F., Lees, E., Reed, S.I., and Stein, G.H. (1993). Altered regulation of G1 cyclins in senescent human diploid fibroblasts: accumulation of inactive cyclin E-Cdk2 and cyclin D1-Cdk2 complexes. Proc Natl Acad Sci U S A *90*, 11034-11038.

Dumay-Odelot, H., Marck, C., Durrieu-Gaillard, S., Lefebvre, O., Jourdain, S., Prochazkova, M., Pflieger, A., and Teichmann, M. (2007). Identification, molecular cloning, and characterization of the sixth subunit of human transcription factor TFIIIC. J Biol Chem *282*, 17179-17189.

Dumay-Odelot, H.l.n., Durrieu-Gaillard, S.p., Da Silva, D., Roeder, R.G., and Teichmann, M. (2010). Cell growth- and differentiation-dependent regulation of RNA polymerase III transcription. Cell Cycle *9*, 3687-3699.

Dunaief, J.L., Strober, B.E., Guha, S., Khavari, P.A., Alin, K., Luban, J., Begemann, M., Crabtree, G.R., and Goff, S.P. (1994). The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell *79*, 119-130.

Dutta, T.A., and Anindya (2009). p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer *9*, 400-414.

Eames, H.L., Saliba, D.G., Krausgruber, T., Lanfrancotti, a., Ryzhakov, G., and Udalova, I.a. (2012). KAP1/TRIM28: an inhibitor of IRF5 function in inflammatory macrophages. Immunobiology *217*, 1315-1324.

Echeverri, C.J., Beachy, P.A., Baum, B., Boutros, M., Buchholz, F., Chanda, S.K., Downward, J., Ellenberg, J., Fraser, A.G., Hacohen, N.*, et al.* (2006). Minimizing the risk of reporting false positives in large-scale RNAi screens. Nat Methods *3*, 777-779.

Echeverri, C.J., and Perrimon, N. (2006). High-throughput RNAi screening in cultured cells: a user's guide. Nature reviews Genetics *7*, 373-384.

Eichhorn, K., and Jackson, S.P. (2001). A Role for TAF3B2 in the Repression of Human RNA Polymerase III Transcription in Nonproliferating Cells. Journal of Biological Chemistry *276*, 21158-21165.

el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-825.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001a). Duplexes of 21 ¬± nucleotide RNAs mediate RNA interference in cultured mammalian cells. *411*, 1-5.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001b). Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J *20*, 6877-6888.

Elbers, I.J., Stoopen, G.M., Bakker, H., Stevens, L.H., Bardor, M., Molthoff, J.W., Jordi, W.J., Bosch, D., and Lommen, A. (2001). Influence of growth conditions and developmental stage on N-glycan heterogeneity of transgenic immunoglobulin G and endogenous proteins in tobacco leaves. Plant Physiol *126*, 1314-1322.

Eom, G.H., Kim, K., Kim, S.M., Kee, H.J., Kim, J.Y., Jin, H.M., Kim, J.R., Kim, J.H., Choe, N., Kim, K.B.*, et al.* (2009). Histone methyltransferase PRDM8 regulates mouse testis steroidogenesis. Biochemical and Biophysical Research Communications *388*, 131-136.

Erickson, S., Sangfelt, O., Heyman, M., Castro, J., Einhorn, S., and Grander, D. (1998). Involvement of the Ink4 proteins p16 and p15 in T-lymphocyte senescence. Oncogene *17*, 595-602.

Eriksson, M., Brown, W.T., Gordon, L.B., Glynn, M.W., Singer, J., Scott, L., Erdos, M.R., Robbins, C.M., Moses, T.Y., Berglund, P.*, et al.* (2003). Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. Nature *423*, 293-298.

Esteller, M. (2008). Epigenetics in Cancer. 1-12.

Esteller, M., Corn, P.G., Baylin, S.B., and Herman, J.G. (2001). A gene hypermethylation profile of human cancer. Cancer Res *61*, 3225-3229.

Eswaran, J., Horvath, A., Godbole, S., Reddy, S.D., Mudvari, P., Ohshiro, K., Cyanam, D., Nair, S., Fuqua, S.a.W., Polyak, K.*, et al.* (2013). RNA sequencing of cancer reveals novel splicing alterations. Scientific reports *3*, 1689-1689.

Ewald, J.a., Peters, N., Desotelle, J.a., Hoffmann, F.M., and Jarrard, D.F. (2009). A high-throughput method to identify novel senescence-inducing compounds. Journal of biomolecular screening *14*, 853-858.

Falschlehner, C., Steinbrink, S., Erdmann, G., and Boutros, M. (2010). High-throughput RNAi screening to dissect cellular pathways: a how-to guide. Biotechnology journal *5*, 368-376.

Fardini, Y., Dehennaut, V., Lefebvre, T., and Issad, T. (2013). O-GlcNAcylation: A new cancer hallmark? Frontiers in Endocrinology *4*, 1-14.

Fishburn, J., Mohibullah, N., and Hahn, S. (2005). Function of a eukaryotic Felton-Edkins, Z.a., Fairley, J.a., Graham, E.L., Johnston, I.M., White, R.J., and Scott, P.H. (2003). The mitogen-activated protein (MAP) kinase ERK induces tRNA synthesis by phosphorylating TFIIIB. EMBO Journal *22*, 2422-2432.

Feng, J., Funk, W.D., Wang, S.S., Weinrich, S.L., Avilion, a.a., Chiu, C.P., Adams, R.R., Chang, E., Allsopp, R.C., and Yu, J. (1995). The RNA component of human telomerase. Science (New York, NY) *269*, 1236-1241.

Ferbeyre, G. (2002). PML a target of translocations in APL is a regulator of cellular senescence. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK *16*, 1918-1926.

Feser, J., Truong, D., Das, C., Carson, J.J., Kieft, J., Harkness, T., and Tyler, J.K. (2010). Elevated histone expression promotes lifespan extension. Molecular Cell *39*, 724-735.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

transcription activator during the transcription cycle. Molecular Cell *18*, 369-378.

Fong, J.J., Nguyen, B.L., Bridger, R., Medrano, E.E., Wells, L., Pan, S., and Sifers, R.N. (2012). beta-N-Acetylglucosamine (O-GlcNAc) is a novel regulator of mitosis-specific phosphorylations on histone H3. J Biol Chem *287*, 12195-12203.

Franza, B.R., Jr., Maruyama, K., Garrels, J.I., and Ruley, H.E. (1986). In vitro establishment is not a sufficient prerequisite for transformation by activated ras oncogenes. Cell *44*, 409-418.

Freund, A., Laberge, R.M., Demaria, M., and Campisi, J. (2012). Lamin B1 loss is a senescence-associated biomarker. Mol Biol Cell *23*, 2066-2075.

Freund, A., Patil, C.K., and Campisi, J. (2011). p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. The EMBO journal *30*, 1536-1548.

Fujiki, R., Hashiba, W., Sekine, H., Yokoyama, A., Chikanishi, T., Ito, S., Imai, Y., Kim, J., He, H.H., Igarashi, K.*, et al.* (2011). GlcNAcylation of histone H2B facilitates its monoubiquitination. Nature *480*, 557-560.

Fulop, N., Feng, W., Xing, D., He, K., Not, L.G., Brocks, C.A., Marchase, R.B., Miller, A.P., and Chatham, J.C. (2008). Aging leads to increased levels of protein O-linked N-acetylglucosamine in heart, aorta, brain and skeletal muscle in Brown-Norway rats. Biogerontology *9*, 139-151.

Fumagalli, M., Rossiello, F., Clerici, M., Barozzi, S., Cittaro, D., Kaplunov, J.M., Bucci, G., Dobreva, M., Matti, V., Beausejour, C.M.*, et al.* (2012). Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. Nature Cell Biology *14*, 355-365.

Funakoshi, Y., and Suzuki, T. (2009). Glycobiology in the cytosol: the bitter side of a sweet world. Biochim Biophys Acta *1790*, 81-94.

Funayama, R., Saito, M., Tanobe, H., and Ishikawa, F. (2006). Loss of linker histone H1 in cellular senescence. The Journal of cell biology *175*, 869-880.

Galan, A., and Rodriguez-Navarro, S. (2012). Sus1/ENY2: a multitasking protein in eukaryotic gene expression. Crit Rev Biochem Mol Biol *47*, 556-568.

Gambetta, M.C., Oktaba, K., and M√ºller, J.r. (2009). Essential Role of the Glycosyltransferase Sxc/Ogt in Polycomb Repression. 93-97.

Gamerdinger, M., Hajieva, P., Kaya, A.M., Wolfrum, U., Hartl, F.U., and Behl, C. (2009). Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. The EMBO journal *28*, 889-901.

Gamper, A.M., and Roeder, R.G. (2008). Multivalent binding of p53 to the STAGA complex mediates coactivator recruitment after UV damage. Molecular and cellular biology *28*, 2517-2527.

Garcia, J.S., Jain, N., and Godley, L.a. (2010). An update on the safety and efficacy of decitabine in the treatment of myelodysplastic syndromes. OncoTargets and Therapy *3*, 1-13.

Garcia-Oliver, E., Garcia-Molinero, V., and Rodriguez-Navarro, S. (2012). mRNA export and gene expression: the SAGA-TREX-2 connection. Biochim Biophys Acta *1819*, 555-565.

Gerland, L.M., Peyrol, S., Lallemand, C., Branche, R., Magaud, J.P., and Ffrench, M. (2003). Association of increased autophagic inclusions labeled for beta-galactosidase with fibroblastic aging. Experimental Gerontology *38*, 887-895.

Gatto, L., Breckels, L.M., Burger, T., Nightingale, D.J., Groen, A.J., Campbell, C., Nikolovski, N., Mulvey, C.M., Christoforou, A., Ferro, M.*, et al.* (2014). A foundation for reliable spatial proteomics data analysis. Mol Cell Proteomics *13*, 1937-1952

Gewirtz, D.a. (2013). Autophagy and senescence: A partnership in search of definition. Autophagy *9*, 808-812.

Ghavidel, A., and Schultz, M.C. (2001). TATA binding protein-associated CK2 transduces DNA damage signals to the RNA polymerase III transcriptional machinery. Cell *106*, 575-584.

Gil, J., Bernard, D., Martinez, D., and Beach, D. (2004). Polycomb CBX7 has a unifying role in cellular lifespan. Nat Cell Biol *6*, 67-72.

Gil, J., and Peters, G. (2006). Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol *7*, 667-677.

Golden, D.E., Gerbasi, V.R., and Sontheimer, E.J. (2008). An inside job for siRNAs. Mol Cell *31*, 309-312.

Goldman, R.D., Shumaker, D.K., Erdos, M.R., Eriksson, M., Goldman, A.E., Gordon, L.B., Gruenbaum, Y., Khuon, S., Mendez, M., Varga, R.e.*, et al.* (2004). Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome. Proceedings of the National Academy of Sciences of the United States of America *101*, 8963-8968.

Goodfellow, S.J., and White, R.J. (2007). Regulation of RNA polymerase III transcription during mammalian cell growth. Cell Cycle *6*, 2323-2326.

Goodarzi, A.a., Kurka, T., and Jeggo, P.a. (2011). KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response. Nature structural & molecular biology *18*, 831-839.

Gorgoulis, V.G., and Halazonetis, T.D. (2010). Oncogene-induced senescence: the bright and dark side of the response. Current opinion in cell biology *22*, 816-827.

Govind, C.K., Zhang, F., Qiu, H., Hofmeyer, K., and Hinnebusch, A.G. (2007). Gcn5 promotes acetylation, eviction, and methylation of nucleosomes in transcribed coding regions. Molecular cell *25*, 31-42.

Grant, P.a., Schieltz, D., Pray-Grant, M.G., Steger, D.J., Reese, J.C., Yates, J.R., and Workman, J.L. (1998). A subset of TAF(II)s are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation. Cell *94*, 45-53.

Grimm, D., and Kay, M.A. (2007). Review series Therapeutic application of RNAi : is mRNA targeting finally ready for prime time ? *117*, 3633-3641.

Grimm, D., Streetz, K.L., Jopling, C.L., Storm, T.a., Pandey, K., Davis, C.R., Marion, P., Salazar, F., and Kay, M.a. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature *441*, 537-541.

Grossman, S.R. (2001). p300/CBP/p53 interaction and regulation of the p53 response. Eur J Biochem *268*, 2773-2778.

Guha, M. (2013). Blockbuster dreams for Pfizer's CDK inhibitor. Nature biotechnology *31*, 187-187.

Guo, G.E., Ma, L.W., Jiang, B., Yi, J., Tong, T.J., and Wang, W.G. (2010). Hydrogen peroxide induces p16(INK4a) through an AUF1-dependent manner. J Cell Biochem *109*, 1000-1005.

Guo, W., Chen, W., Yu, W., Huang, W., and Deng, W. (2013). Small interfering RNA-based molecular therapy of cancers. Chinese journal of cancer *32*, 488-493.

Hakomori, S., and Handa, K. (2002). Glycosphingolipid-dependent cross-talk between glycosynapses interfacing tumor cells with their host cells: essential basis to define tumor malignancy. FEBS Lett *531*, 88-92.

Haltiwanger, R., S, Holt, G., D, Hart, G., and W (1990). Enzymatic addition of O-GlcNAc to Nuclear and Cytoplasmic Proteins. J Biol Chem *265*, 2563-2568.

Haltiwanger Rs, B.M.A.H.G. (1992). O-Glycosylation of nuclear and cytosolic proteins. Journal of Biological Chemistry *267*, 9005-9013.

Harley, C.B., Futcher, A.B., and Greider, C.W. (1990). Telomeres shorten during ageing of human fibroblasts. Nature *345*, 458-460.

Harrison, B.C., Huynh, K., Lundgaard, G.L., Helmke, S.M., Perryman, M.B., and McKinsey, T.A. (2010). Protein kinase C-related kinase targets nuclear localization signals in a subset of class IIa histone deacetylases. FEBS Lett *584*, 1103-1110.

Hatakeyama, S. (2011). TRIM proteins and cancer. Nature reviews Cancer *11*, 792-804.

Hattangadi, S.M., Martinez-Morilla, S., Patterson, H.C., Shi, J., Burke, K., Avila-Figueroa, A., Venkatesan, S., Wang, J., Paulsen, K., Gorlich, D*., et al.* (2014). Histones to the cytosol: Exportin 7 is essential for normal terminal erythroid nuclear maturation. Blood.

Hayflick, L. (1965). The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res *37*, 614-636.

Hayflick, L. (1979). Cell biology of aging. Fed Proc *38*, 1847-1850.

Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Experimental cell research *25*, 585-621.

He, J., Kallin, E.M., Tsukada, Y.-I., and Zhang, Y. (2008). The H3K36 demethylase Jhdm1b/Kdm2b regulates cell proliferation and senescence through p15(Ink4b). Nature structural & molecular biology *15*, 1169-1175.

Heidenreich, B., Rachakonda, P.S., Hemminki, K., and Kumar, R. (2014). TERT promoter mutations in cancer development. Current Opinion in Genetics and Development *24*, 30-37.

Helenius, A., and Aebi, M. (2004). Roles of N-linked glycans in the endoplasmic reticulum. Annual review of biochemistry *73*, 1019-1049.

Herbig, U., Ferreira, M., Condel, L., Carey, D., and Sedivy, J.M. (2006). Cellular senescence in aging primates. Science (New York, NY) *311*, 1257-1257.

Herbig, U., Jobling, W.A., Chen, B.P.C., Chen, D.J., and Sedivy, J.M. (2004). of Human Cells through a Pathway Involving ATM , p53 , and p21 CIP1 , but Not p16 INK4a. *14*, 501-513.

Hiroyuki Takai, A.S., and Titia de, L. (2003). DNA Damage Foci at Dysfunctional Telomeres. Current biology : CB *13*, 1549-1556.

Hoare, M., and Narita, M. (2013). Transmitting senescence to the cell neighbourhood. Nature cell biology *15*, 887-889.

Hock, J., and Meister, G. (2008). The Argonaute protein family. Genome Biol *9*, 210.

Hohenauer, T., and Moore, a.W. (2012). The Prdm family: expanding roles in stem cells and development. Development *139*, 2267-2282.

Holts, G.D., and Hart, G.W. (1986). The Subcellular Distribution of Terminal N-Acetylglucosamine Moieties. the Journal of Biological Chemistry *261*, 8049-4305.

Honda, R., and Yasuda, H. (1999). Association of p19 ARF with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. EMBO Journal *18*, 22-27.

Hong, H., Takahashi, K., Ichisaka, T., Aoi, T., Kanagawa, O., Nakagawa, M., Okita, K., and Yamanaka, S. (2009). Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature *460*, 1132-1135.

Hsieh, Y.J., Kundu, T.K., Wang, Z., Kovelman, R., and Roeder, R.G. (1999). The TFIIIC90 subunit of TFIIIC interacts with multiple components of the RNA polymerase III machinery and contains a histone-specific acetyltransferase activity. Molecular and cellular biology *19*, 7697-7704.

Hu, G., Kim, J., Xu, Q., Leng, Y., Orkin, S.H., and Elledge, S.J. (2009). A genome-wide RNAi screen identifies a new transcriptional module required for self-renewal. Genes and Development *23*, 837-848.

Huang, J., Sengupta, R., Espejo, A.B., Lee, M.G., Dorsey, J.a., Richter, M., Opravil, S., Shiekhattar, R., Bedford, M.T., Jenuwein, T.*, et al.* (2007). p53 is regulated by the lysine demethylase LSD1. Nature *449*, 105-108.

Huang, S., Risques, R.A., Martin, G.M., Rabinovitch, P.S., and Oshima, J. (2008). Accelerated telomere shortening and replicative senescence in human fibroblasts overexpressing mutant and wild-type lamin A. Experimental Cell Research *314*, 82-91.

Hubackova, S., Krejcikova, K., Bartek, J., and Hodny, Z. (2012). IL1- and TGFbeta-Nox4 signaling, oxidative stress and DNA damage response are shared features of replicative, oncogene-induced, and drug-induced paracrine 'bystander senescence'. Aging (Albany NY) *4*, 932-951.

Hutvagner, G., and Zamore, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. Science *297*, 2056-2060.

Iakova, P., Wang, G.L., Timchenko, L., Michalak, M., Pereira-Smith, O.M., Smith, J.R., and Timchenko, N.A. (2004). Competition of CUGBP1 and calreticulin for the regulation of p21 translation determines cell fate. EMBO J *23*, 406-417.

Iannello, A., Thompson, T.W., Ardolino, M., Lowe, S.W., and Raulet, D.H. (2013). p53-dependent chemokine production by senescent tumor cells supports NKG2D-dependent tumor elimination by natural killer cells. The Journal of experimental medicine *210*, 2057-2069.

Itkonen, H.M., Minner, S., Guldvik, I.J., Sandmann, M.J., Tsourlakis, M.C., Berge, V., Svindland, A., Schlomm, T., and Mills, I.G. (2013). O-GlcNAc transferase integrates metabolic pathways to regulate the stability of c-MYC in human prostate cancer cells. Cancer Research *73*, 5277-5287.

Itahana, K., Zou, Y., Itahana, Y., Martinez, J.L., Beausejour, C., Jacobs, J.J., Van Lohuizen, M., Band, V., Campisi, J., and Dimri, G.P. (2003). Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1. Mol Cell Biol *23*, 389-401.

Ivanov, A., Pawlikowski, J., Manoharan, I., Tuyn, J.V., Nelson, D.M., Singh Rai, T., Shah, P.P., Hewitt, G., Korolchuk, V.I., Passos, J.F.*, et al.* (2013). Lysosome-mediated processing of chromatin in senescence. Journal of Cell Biology *202*, 129-143.

Ivanov, A.V., Peng, H., Yurchenko, V., Yap, K.L., Negorev, D.G., Schultz, D.C., Psulkowski, E., Fredericks, W.J., White, D.E., Maul, G.G.*, et al.* (2007). PHD domain-mediated E3 ligase activity directs intramolecular sumoylation of an adjacent bromodomain required for gene silencing. Molecular cell *28*, 823-837.

Iyengar, S., and Farnham, P.J. (2011). KAP1 protein: an enigmatic master regulator of the genome. The Journal of biological chemistry *286*, 26267-26276.

Iyer, N.G., Ozdag, H., and Caldas, C. (2004). p300/CBP and cancer. Oncogene *23*, 4225-4231.

Jackson, A.L., Burchard, J., Leake, D., Reynolds, A., Schelter, J., Guo, J., Johnson, J.M., Lim, L., Karpilow, J., Nichols, K.*, et al.* (2006a). Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA *12*, 1197-1205.

Jackson, A.L., Burchard, J., Schelter, J., Chau, B.N., Cleary, M., Lim, L., and Linsley, P.S. (2006b). Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. RNA *12*, 1179-1187.

Jackson, A.L., and Linsley, P.S. (2010). Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. Nature reviews Drug discovery *9*, 57-67.

Jacobs, J.J., and de Lange, T. (2004). Significant role for p16INK4a in p53-independent telomere-directed senescence. Curr Biol *14*, 2302-2308.

Jacobs, J.J., Kieboom, K., Marino, S., DePinho, R.A., and van Lohuizen, M. (1999). The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature *397*, 164-168.

Jani, D., Lutz, S., Hurt, E., Laskey, R.a., Stewart, M., and Wickramasinghe, V.O. (2012). Functional and structural characterization of the mammalian TREX-2 complex that links transcription with nuclear messenger RNA export. Nucleic Acids Research *40*, 4562-4573.

Jean-Philippe, C., Ogier, A., Boese, A., Genovesio, A., Brodin, P., Sommer, P., and Dorval, T. (2012). A novel specific edge effect correction method for RNA interference screenings. Bioinformatics *28*, 261-268.

Jiang, P., Du, W., Mancuso, A., Wellen, K.E., and Yang, X. (2013). Reciprocal regulation of p53 and malic enzymes modulates metabolism and senescence. Nature *493*, 689-693.

Jin, S., Mazzacurati, L., Zhu, X., Tong, T., Song, Y., Shujuan, S., Petrik, K.L., Rajasekaran, B., Wu, M., and Zhan, Q. (2003). Gadd45a contributes to p53 stabilization in response to DNA damage. Oncogene *22*, 8536-8540.

Jin, Y., Zeng, S.X., Dai, M.S., Yang, X.J., and Lu, H. (2002). MDM2 inhibits PCAF (p300/CREB-binding protein-associated factor)-mediated p53 acetylation. Journal of Biological Chemistry *277*, 30838-30843.

Johnson, B.R., Nitta, R.T., Frock, R.L., Mounkes, L., Barbie, D.a., Stewart, C.L., Harlow, E., and Kennedy, B.K. (2004). A-type lamins regulate retinoblastoma protein function by promoting subnuclear localization and preventing proteasomal degradation. Proceedings of the National Academy of Sciences of the United States of America *101*, 9677-9682.

Jones, S., Wang, T.L., Shih Ie, M., Mao, T.L., Nakayama, K., Roden, R., Glas, R., Slamon, D., Diaz, L.A., Jr., Vogelstein, B.*, et al.* (2010). Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. Science *330*, 228-231.

Junttila, M.R., Karnezis, A.N., Garcia, D., Madriles, F., Kortlever, R.M., Rostker, F., Brown Swigart, L., Pham, D.M., Seo, Y., Evan, G.I.*, et al.* (2010). Selective activation of p53-mediated tumour suppression in high-grade tumours. Nature *468*, 567-571.

Kamigaito, T., Okaneya, T., Kawakubo, M., Shimojo, H., Nishizawa, O., and Nakayama, J. (2013). Overexpression of O -GlcNAc by prostate cancer cells is significantly associated with poor prognosis of patients. *17*, 18-22.

Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing, J.R., Ashmun, R.a., Grosveld, G., and Sherr, C.J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell *91*, 649-659.

Kamitani, S., Togi, S., Ikeda, O., Nakasuji, M., Sakauchi, A., Sekine, Y., Muromoto, R., Oritani, K., and Matsuda, T. (2011). Kruppel-associated box-associated protein 1 negatively regulates TNF-alpha-induced NF-kappaB transcriptional activity by influencing the interactions among STAT3, p300, and NF-kappaB/p65. J Immunol *187*, 2476-2483.

Kang, T.-W., Yevsa, T., Woller, N., Hoenicke, L., Wuestefeld, T., Dauch, D., Hohmeyer, A., Gereke, M., Rudalska, R., Potapova, A.*, et al.* (2011).

Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. Nature *479*, 547-551.

Kaplon, J., Zheng, L., Meissl, K., Chaneton, B., Selivanov, V.a., Mackay, G., Der, S.H.V., van der Burg, S.H., Verdegaal, E.M.E., Cascante, M.*, et al.* (2013). A key role for mitochondrial gatekeeper pyruvate dehydrogenase in oncogene-induced senescence. Nature *498*, 109-112.

Kawamura, T., Suzuki, J., Wang, Y.V., Menendez, S., Morera, L.B., Raya, A., Wahl, G.M., and Izpisua Belmonte, J.C. (2009). Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature *460*, 1140-1144.

Kazemi, Z., Chang, H., Haserodt, S., McKen, C., and Zachara, N.E. (2010). O-linked beta-N-acetylglucosamine (O-GlcNAc) regulates stress-induced heat shock protein expression in a GSK-3beta-dependent manner. J Biol Chem *285*, 39096-39107.

Kenneth, N.S., Ramsbottom, B.a., Gomez-Roman, N., Marshall, L., Cole, P.a., and White, R.J. (2007). TRRAP and GCN5 are used by c-Myc to activate RNA polymerase III transcription. Proceedings of the National Academy of Sciences of the United States of America *104*, 14917-14922.

Khursheed, M., Kolla, J.N., Kotapalli, V., Gupta, N., Gowrishankar, S., Uppin, S.G., Sastry, R.A., Koganti, S., Sundaram, C., Pollack, J.R.*, et al.* (2013). ARID1B, a member of the human SWI/SNF chromatin remodeling complex, exhibits tumour-suppressor activities in pancreatic cancer cell lines. British journal of cancer *108*, 2056-2062.

Kim, H.-S., Park, S.Y., Choi, Y.R., Kang, J.G., Joo, H.J., Moon, W.K., and Cho, J.W. (2009). Excessive O-GlcNAcylation of proteins suppresses spontaneous cardiogenesis in ES cells. FEBS letters *583*, 2474-2478.

Kia, S.K., Gorski, M.M., Giannakopoulos, S., and Verrijzer, C.P. (2008). SWI/SNF mediates polycomb eviction and epigenetic reprogramming of the INK4b-ARF-INK4a locus. Molecular and cellular biology *28*, 3457-3464.

Kim, K., Lee, Y.S.I.K., and Carthew, R.W. (2007). Conversion of pre-RISC to holo-RISC by Ago2 during assembly of RNAi complexes. 22-29.

Kim, N.W., Shay, J.W., Piatyszek, M.a., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., and Weinrich, S.L. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011-2015.

Kim, S.M., Kim, J.Y., Choe, N.W., Cho, I.H., Kim, J.R., Kim, D.W., Seol, J.E., Lee, S.E., Kook, H., Nam, K.I.*, et al.* (2010). Regulation of mouse steroidogenesis by WHISTLE and JMJD1C through histone methylation balance. Nucleic Acids Research *38*, 6389-6403.

Kim, W.Y., and Sharpless, N.E. (2006). The Regulation of INK4/ARF in Cancer and Aging. Cell *127*, 265-275.

Kirkland, J.G., Raab, J.R., and Kamakaka, R.T. (2013). TFIIIC bound DNA elements in nuclear organization and insulation. Biochimica et Biophysica Acta - Gene Regulatory Mechanisms *1829*, 418-424.

Kittler, R., Surendranath, V., Heninger, A.K., Slabicki, M., Theis, M., Putz, G., Franke, K., Caldarelli, A., Grabner, H., Kozak, K.*, et al.* (2007). Genome-wide

resources of endoribonuclease-prepared short interfering RNAs for specific loss-of-function studies. Nat Methods *4*, 337-344.

Kiyono, T., Foster, S.a., Koop, J.I., McDougall, J.K., Galloway, D.a., and Klingelhutz, a.J. (1998). Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature *396*, 84-88.

Kleer, C.G., Cao, Q., Varambally, S., Shen, R., Ota, I., Tomlins, S.a., Ghosh, D., Sewalt, R.G.a.B., Otte, A.P., Hayes, D.F.*, et al.* (2003). EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proceedings of the National Academy of Sciences of the United States of America *100*, 11606-11611.

Klinghoffer, R.A., Magnus, J., Schelter, J., Mehaffey, M., Coleman, C., and Cleary, M.A. (2010). Reduced seed region-based off-target activity with lentivirus-mediated RNAi. 879-884.

Klose, R.J., and Zhang, Y. (2007). Regulation of histone methylation by demethylimination and demethylation. Nature reviews Molecular cell biology *8*, 307-318.

Kohler, A., Pascual-Garcia, P., Llopis, A., Zapater, M., Posas, F., Hurt, E., and Rodriguez-Navarro, S. (2006). The mRNA export factor Sus1 is involved in Spt/Ada/Gcn5 acetyltransferase-mediated H2B deubiquitinylation through its interaction with Ubp8 and Sgf11. Mol Biol Cell *17*, 4228-4236.

Kopytova, D.V., Orlova, A.V., Krasnov, A.N., Gurskiy, D.Y., Nikolenko, J.V., Nabirochkina, E.N., Shidlovskii, Y.V., and Georgieva, S.G. (2010). Multifunctional factor ENY2 is associated with the THO complex and promotes its recruitment onto nascent mRNA. Genes & development *24*, 86-96.

Kotake, Y., Zeng, Y., and Xiong, Y. (2009). DDB1-CUL4 and MLL1 mediate oncogene-induced p16INK4a activation. Cancer research *69*, 1809-1814.

Koutelou, E., Hirsch, C.L., and Dent, S.Y.R. (2010). Multiple faces of the SAGA complex. Current opinion in cell biology *22*, 374-382.

Kreppel, L.K., Blomberg, M.A., and Hart, G.W. (1997). Dynamic glycosylation of nuclear and cytosolic proteins. Cloning and characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats. J Biol Chem *272*, 9308-9315.

Krimpenfort, P., Ijpenberg, A., Song, J.-Y., van der Valk, M., Nawijn, M., Zevenhoven, J., and Berns, A. (2007). p15Ink4b is a critical tumour suppressor in the absence of p16Ink4a. Nature *448*, 943-946.

Krizhanovsky, V., Yon, M., Dickins, R.a., Hearn, S., Simon, J., Miething, C., Yee, H., Zender, L., and Lowe, S.W. (2008). Senescence of Activated Stellate Cells Limits Liver Fibrosis. Cell *134*, 657-667.

Krogan, N.J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Ryan, O.W., Golshani, A., Johnston, M.*, et al.* (2003). The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: Linking transcriptional elongation to histone methylation. Molecular Cell *11*, 721-729.

Krtolica, a., Parrinello, S., Lockett, S., Desprez, P.Y., and Campisi, J. (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link

between cancer and aging. Proceedings of the National Academy of Sciences of the United States of America *98*, 12072-12077.

Krzeslak, A., Pomorski, L., and Lipinska, A. (2010). Elevation of nucleocytoplasmic beta-N-acetylglucosaminidase (O-GlcNAcase) activity in thyroid cancers. Int J Mol Med *25*, 643-648.

Kubota, S., Fukumoto, Y., Aoyama, K., Ishibashi, K., Yuki, R., Morinaga, T., Honda, T., Yamaguchi, N., Kuga, T., Tomonaga, T.*, et al.* (2013). Phosphorylation of KRAB-associated protein 1 (KAP1) at Tyr-449, Tyr-458, and Tyr-517 by nuclear tyrosine kinases inhibits the association of KAP1 and heterochromatin protein 1-beta (HP1beta) with heterochromatin. The Journal of biological chemistry *288*, 17871-17883

Kuilman, T., Michaloglou, C., Mooi, W.J., and Peeper, D.S. (2010). The essence of senescence. Genes & development *24*, 2463-2479.

Kuilman, T., Michaloglou, C., Vredeveld, L.C.W., Douma, S., van Doorn, R., Desmet, C.J., Aarden, L.a., Mooi, W.J., and Peeper, D.S. (2008). Oncogene-Induced Senescence Relayed by an Interleukin-Dependent Inflammatory Network. Cell *133*, 1019-1031.

Kuilman, T., and Peeper, D.S. (2009). Senescence-messaging secretome: SMS-ing cellular stress. Nature reviews Cancer *9*, 81-94.

Kundu, T.K., Wang, Z., and Roeder, R.G. (1999). Human TFIIIC relieves chromatin-mediated repression of RNA polymerase III transcription and contains an intrinsic histone acetyltransferase activity. Molecular and cellular biology *19*, 1605-1615.

Kurshakova, M.M., Krasnov, A.N., Kopytova, D.V., Shidlovskii, Y.V., Nikolenko, J.V., Nabirochkina, E.N., Spehner, D., Schultz, P., Tora, L., and Georgieva, S.G. (2007). SAGA and a novel Drosophila export complex anchor efficient transcription and mRNA export to NPC. EMBO J *26*, 4956-4965.

Kutay, U., Bischoff, F.R., Kostka, S., Kraft, R., and Gorlich, D. (1997). Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. Cell *90*, 1061-1071.

Kutay, U., Lipowsky, G., Izaurralde, E., Bischoff, F.R., Schwarzmaier, P., Hartmann, E., and Gorlich, D. (1998). Identification of a tRNA-specific nuclear export receptor. Mol Cell *1*, 359-369.

Kuwabara, T., Warashina, M., Sano, M., Tang, H., Wong-Staal, F., Munekata, E., and Taira, K. (2001). Recognition of engineered tRNAs with an extended 3' end by Exportin-t (Xpo-t) and transport of tRNA-attached ribozymes to the cytoplasm in somatic cells. Biomacromolecules *2*, 1229-1242.

Kwak, I.H., Kim, H.S., Choi, O.R., Ryu, M.S., and Lim, I.K. (2004). Nuclear Accumulation of Globular Actin as a Cellular Senescence Marker. Cancer Research *64*, 572-580.

Lahtela, J., Corson, L.B., Hemmes, A., Brauer, M.J., Koopal, S., Lee, J., Hunsaker, T.L., Jackson, P.K., and Verschuren, E.W. (2013). A high-content cellular senescence screen identifies candidate tumor suppressors, including EPHA3. Cell cycle (Georgetown, Tex) *12*, 625-634.

Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature *304*, 596-602.

Lang, G., Bonnet, J., Umlauf, D., Karmodiya, K., Koffler, J., Stierle, M., Devys, D., and Tora, L.s. (2011). The tightly controlled deubiquitination activity of the human SAGA complex differentially modifies distinct gene regulatory elements. Molecular and cellular biology *31*, 3734-3744.

Lanigan, F., Geraghty, J.G., and Bracken, a.P. (2011). Transcriptional regulation of cellular senescence. Oncogene *30*, 2901-2911.

Lapasset, L., Milhavet, O., Prieur, A., Besnard, E., Babled, A., Ait-Hamou, N., Leschik, J., Pellestor, F., Ramirez, J.M., De Vos, J.*, et al.* (2011). Rejuvenating senescent and centenarian human cells by reprogramming through the pluripotent state. Genes Dev *25*, 2248-2253.

Lazzerini Denchi, E., Attwooll, C., Pasini, D., and Helin, K. (2005). Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. Molecular and cellular biology *25*, 2660-2672.

Lee, A.C., Fenster, B.E., Ito, H., Takeda, K., Bae, N.S., Hirai, T., Yu, Z.X., Ferrans, V.J., Howard, B.H., and Finkel, T. (1999). Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. Journal of Biological Chemistry *274*, 7936-7940.

Lee, B.Y., Han, J.a., Im, J.S., Morrone, A., Johung, K., Goodwin, E.C., Kleijer, W.J., DiMaio, D., and Hwang, E.S. (2006). Senescence-associated β-galactosidase is lysosomal β-galactosidase. Aging Cell *5*, 187-195.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S.*, et al.* (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415-419.

Lee, Y.-K., Thomas, S.N., Yang, A.J., and Ann, D.K. (2007). Doxorubicin down-regulates Kruppel-associated box domain-associated protein 1 sumoylation that relieves its transcription repression on p21WAF1/CIP1 in breast cancer MCF-7 cells. The Journal of biological chemistry *282*, 1595-1606.

Leonard, J.P., Lacasce, A.S., Smith, M.R., Noy, A., Chirieac, L.R., Rodig, S.J., Yu, J.Q., Vallabhajosula, S., Schoder, H., English, P.*, et al.* (2012). Selective Cdk4 / 6 Inhibition With Tumor Responses By Pd0332991 in Patients With Mantle Cell Lymphoma. *119*, 4597-4608.

Levy, M.Z., Allsopp, R.C., Futcher, a.B., Greider, C.W., and Harley, C.B. (1992). Telomere end-replication problem and cell aging. Journal of molecular biology *225*, 951-960.

Li, H., Collado, M., Villasante, A., Strati, K., Ortega, S., Canamero, M., Blasco, M.A., and Serrano, M. (2009). The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature *460*, 1136-1139.

Li, H., Ilin, S., Wang, W., Duncan, E.M., Wysocka, J., Allis, C.D., and Patel, D.J. (2006a). Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. Nature *442*, 91-95.

Li, J., Yang, G., Li, S., Cao, G., Zhao, Q., Liu, X., Fan, M., Shen, B., and Shao, N. (2006b). 3'-Poly(A) tail enhances siRNA activity against exogenous reporter genes in MCF-7 cells. J RNAi Gene Silencing *2*, 195-204.

Li, X., Lee, Y.-K., Jeng, J.-C., Yen, Y., Schultz, D.C., Shih, H.-M., and Ann, D.K. (2007). Role for KAP1 serine 824 phosphorylation and sumoylation/desumoylation switch in regulating KAP1-mediated transcriptional repression. The Journal of biological chemistry *282*, 36177-36189.

Li, X., Lee, Y.-K., Jeng, J.-C., Yen, Y., Schultz, D.C., Shih, H.-M., and Ann, D.K. (2007). Role for KAP1 serine 824 phosphorylation and sumoylation/desumoylation switch in regulating KAP1-mediated transcriptional repression. The Journal of biological chemistry *282*, 36177-36189.

Liang, Q., Deng, H., Li, X., Wu, X., Tang, Q., Chang, T.H., Peng, H., Rauscher, F.J., 3rd, Ozato, K., and Zhu, F. (2011). Tripartite motif-containing protein 28 is a small ubiquitin-related modifier E3 ligase and negative regulator of IFN regulatory factor 7. J Immunol *187*, 4754-4763.

Lin, a.W., Barradas, M., Stone, J.C., van Aelst, L., Serrano, M., and Lowe, S.W. (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes & Development *12*, 3008-3019.

Lin, H.-K., Chen, Z., Wang, G., Nardella, C., Lee, S.-W., Chan, C.-H., Yang, W.-L., Wang, J., Egia, A., Nakayama, K.I*., et al.* (2010). Skp2 targeting suppresses tumorigenesis by Arf-p53-independent cellular senescence. Nature *464*, 374-379.

Lindstrom, D.L., Squazzo, S.L., Muster, N., Burckin, T.A., Wachter, K.C., Emigh, C.A., McCleery, J.A., Yates, J.R., 3rd, and Hartzog, G.A. (2003). Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. Mol Cell Biol *23*, 1368-1378.

Lisowska, E., Hirszfeld, L., and Therapy, E. (2008). Protein Glycosylation , an Overview. Life Sciences, 1-5.

Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G., and Evan, G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic acids research *23*, 1686-1690.

Liu, B., Wang, Z., Ghosh, S., and Zhou, Z. (2013). Defective ATM-Kap-1-mediated chromatin remodeling impairs DNA repair and accelerates senescence in progeria mouse model. Aging cell *12*, 316-318.

Liu, X., Tesfai, J., Evrard, Y.a., Dent, S.Y.R., and Martinez, E. (2003). c-Myc transformation domain recruits the human STAGA complex and requires TRRAP and GCN5 acetylase activity for transcription activation. The Journal of biological chemistry *278*, 20405-20412.

Liu, X., Vorontchikhina, M., Wang, Y.-L., Faiola, F., and Martinez, E. (2008). STAGA recruits Mediator to the MYC oncoprotein to stimulate transcription and cell proliferation. Molecular and cellular biology *28*, 108-121.

Lizardi, P.M., Forloni, M., and Wajapeyee, N. (2011). Genome-wide approaches for cancer gene discovery. Trends Biotechnol *29*, 558-568.

Lopez, M.F., Tollervey, J., Krastins, B., Garces, A., Sarracino, D., Prakash, A., Vogelsang, M., Geesman, G., Valderrama, A., Jordan, I.K.*, et al.* (2012). Depletion of nuclear histone H2A variants is associated with chronic DNA damage signaling upon drug-evoked senescence of human somatic cells. Aging *4*, 823-842.

Love, I.M., Sekaric, P., Shi, D., Grossman, S.R., and Androphy, E.J. (2012). The histone acetyltransferase PCAF regulates p21 transcription through stress-induced acetylation of histone H3. Cell Cycle *11*, 2458-2466.

Lowe, S.W., and Sherr, C.J. (2003). Tumor suppression by Ink4a-Arf: Progress and puzzles. Current Opinion in Genetics and Development *13*, 77-83.

Lujambio, A., Akkari, L., Simon, J., Grace, D., Tschaharganeh, D.F., Bolden, J.E., Zhao, Z., Thapar, V., Joyce, J.a., Krizhanovsky, V.*, et al.* (2013). Non-cell-autonomous tumor suppression by p53. Cell *153*, 449-460.

Luo, J., Emanuele, M.J., Li, D., Creighton, C.J., Schlabach, M.R., Westbrook, T.F., Wong, K.K., and Elledge, S.J. (2009). A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. Cell *137*, 835-848.

Lyden, D., Young, a.Z., Zagzag, D., Yan, W., Gerald, W., O'Reilly, R., Bader, B.L., Hynes, R.O., Zhuang, Y., Manova, K.*, et al.* (1999). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature *401*, 670-677.

Macip, S., Igarashi, M., Fang, L., Chen, A., Pan, Z.Q., Lee, S.W., and Aaronson, S.a. (2002). Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. EMBO Journal *21*, 2180-2188.

MacKeigan, J.P., Murphy, L.O., and Blenis, J. (2005). Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. Nature cell biology *7*, 591-600.

Maehara, K., Takahashi, K., and Saitoh, S. (2010). CENP-A reduction induces a p53-dependent cellular senescence response to protect cells from executing defective mitoses. Molecular and cellular biology *30*, 2090-2104.

Maertens, G.N., El Messaoudi-Aubert, S., Racek, T., Stock, J.K., Nicholls, J., Rodriguez-Niedenfuhr, M., Gil, J., and Peters, G. (2009). Several distinct polycomb complexes regulate and co-localize on the INK4a tumor suppressor locus. PLoS One *4*, e6380.

Mailand, N., Falck, J., Lukas, C., Sylju√¢sen, R.G., Welcker, M., Bartek, J., and Lukas, J. (2000). Rapid destruction of human Cdc25A in response to DNA damage. Science (New York, NY) *288*, 1425-1429.

Malo, N., Hanley, J.a., Cerquozzi, S., Pelletier, J., and Nadon, R. (2006). Statistical practice in high-throughput screening data analysis. Nat Biotechnol *24*, 167-175.

Malumbres, M., Perez De Castro, I., Hernandez, M.I., Jimenez, M., Corral, T., and Pellicer, A. (2000). Cellular response to oncogenic ras involves induction of the Cdk4 and Cdk6 inhibitor p15(INK4b). Mol Cell Biol *20*, 2915-2925.

Malz, M., Weber, A., Singer, S., Riehmer, V., Bissinger, M., Riener, M.O., Longerich, T., Soll, C., Vogel, A., Angel, P.*, et al.* (2009). Overexpression of

far upstream element binding proteins: a mechanism regulating proliferation and migration in liver cancer cells. Hepatology *50*, 1130-1139.

Mao, Z., Ke, Z., Gorbunova, V., and Seluanov, A. (2012). Replicatively senescent cells are arrested in G1 and G2 phases. Aging *4*, 431-435.

Marion, R.M., Strati, K., Li, H., Murga, M., Blanco, R., Ortega, S., Fernandez-Capetillo, O., Serrano, M., and Blasco, M.A. (2009). A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature *460*, 1149-1153.

Martin, N., Beach, D., and Gil, J.s. (2014). Ageing as developmental decay: insights from p16INK4a. Trends in Molecular Medicine *20*, 667-674.

Martin, N., Popov, N., Aguilo, F., Loghlen, A.O.R., Raguz, S., Snijders, A.P., Dharmalingam, G., Li, S., Thymiakou, E., Carroll, T.*, et al.* (2013a). Interplay between Homeobox proteins and Polycomb repressive complexes in p16INK4a regulation. The EMBO Journal *32*, 982-995.

Martin, N., Raguz, S., Dharmalingam, G., and Gil, J.s. (2013b). Co-regulation of senescence-associated genes by oncogenic homeobox proteins and polycomb repressive complexes. Cell Cycle *12*, 2194-2199.

Mason, D.X., Jackson, T.J., and Lin, A.W. (2004). Molecular signature of oncogenic ras-induced senescence. Oncogene *23*, 9238-9246.

Matranga, C., Tomari, Y., Shin, C., Bartel, D.P., and Zamore, P.D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell *123*, 607-620.

Mauger, E., and Scott, P.H. (2004). Mitogenic stimulation of transcription by RNA polymerase III. Biochemical Society transactions *32*, 976-977.

McCormick, M.a., Mason, A.G., Guyenet, S.J., Dang, W., Garza, R.M., Ting, M.K., Moller, R.M., Berger, S.L., Kaeberlein, M., Pillus, L.*, et al.* (2014). The SAGA Histone Deubiquitinase Module Controls Yeast Replicative Lifespan via Sir2 Interaction. Cell reports *8*, 477-486.

McMahon, S.B., Van Buskirk, H.a., Dugan, K.a., Copeland, T.D., and Cole, M.D. (1998). The novel ATM-related protein TRRAP is an essential cofactor for the c- Myc and E2F oncoproteins. Cell *94*, 363-374.

Meacham, C.E., Ho, E.E., Dubrovsky, E., Gertler, F.B., and Hemann, M.T. (2009). In vivo RNAi screening identifies regulators of actin dynamics as key determinants of lymphoma progression. Nature genetics *41*, 1133-1137.

Michaloglou, C., Vredeveld, L.C., Soengas, M.S., Denoyelle, C., Kuilman, T., van der Horst, C.M., Majoor, D.M., Shay, J.W., Mooi, W.J., and Peeper, D.S. (2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature *436*, 720-724.

Miura, Y., Sakurai, Y., and Endo, T. (2012). O-GlcNAc modification affects the ATM-mediated DNA damage response. Biochimica et biophysica acta *1820*, 1678-1685.

Mizushima, N., and Komatsu, M. (2011). Autophagy: Renovation of cells and tissues. Cell *147*, 728-741.

Mohammad, H.P., Cai, Y., McGarvey, K.M., Easwaran, H., Van Neste, L., Ohm, J.E., O'Hagan, H.M., and Baylin, S.B. (2009). Polycomb CBX7

promotes initiation of heritable repression of genes frequently silenced with cancer-specific DNA hypermethylation. Cancer Research *69*, 6322-6330.

Mohr, S.E., and Perrimon, N. (2012). RNAi screening: new approaches, understandings, and organisms. Wiley Interdiscip Rev RNA *3*, 145-158.

Moiseeva, O., Bourdeau, V.r., Roux, A., Desch√™nes-Simard, X., and Ferbeyre, G. (2009). Mitochondrial dysfunction contributes to oncogene-induced senescence. Molecular and cellular biology *29*, 4495-4507.

Morin, G.B. (1989). The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell *59*, 521-529.

Morin, R.D., Johnson, N.a., Severson, T.M., Mungall, A.J., An, J., Goya, R., Paul, J.E., Boyle, M., Woolcock, B.W., Kuchenbauer, F.*, et al.* (2010). Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nature genetics *42*, 181-185.

Motallebipour, M., Ameur, A., Reddy Bysani, M.S., Patra, K., Wallerman, O., Mangion, J., Barker, M.A., McKernan, K.J., Komorowski, J., and Wadelius, C. (2009). Differential binding and co-binding pattern of FOXA1 and FOXA3 and their relation to H3K4me3 in HepG2 cells revealed by ChIP-seq. Genome Biol *10*, R129.

Moyzis, R.K., Buckingham, J.M., Cram, L.S., Dani, M., Deaven, L.L., Jones, M.D., Meyne, J., Ratliff, R.L., and Wu, J.R. (1988). A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proceedings of the National Academy of Sciences of the United States of America *85*, 6622-6626.

Mummaneni, P., and Shord, S.S. (2014). Epigenetics and oncology. Pharmacotherapy *34*, 495-505.

Munoz-Espin, D., Canamero, M., Maraver, A., Gomez-Lopez, G., Contreras, J., Murillo-Cuesta, S., Rodriguez-Baeza, A., Varela-Nieto, I., Ruberte, J., Collado, M.*, et al.* (2013). Programmed cell senescence during mammalian embryonic development. Cell *155*, 1104-1118.

Munoz-Espin, D., and Serrano, M. (2014). Cellular senescence: from physiology to pathology. Nature reviews Molecular cell biology *15*, 482-496.

Murchison, E.P., Partridge, J.F., Tam, O.H., Cheloufi, S., and Hannon, G.J. (2005). Characterization of Dicer-deficient murine embryonic stem cells. Proc Natl Acad Sci U S A *102*, 12135-12140.

Myers, S.a., Panning, B., and Burlingame, A.L. (2011). Polycomb repressive complex 2 is necessary for the normal site-specific O-GlcNAc distribution in mouse embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America *108*, 9490-9495.

Nacher, V., Carretero, A., Navarro, M., Armengol, C., Llombart, C., Rodr√≠guez, A., Herrero-Fresneda, I., Ayuso, E., and Ruberte, J.s. (2006). The quail mesonephros: A new model for renal senescence? Journal of Vascular Research *43*, 581-586.

Nandakumar, J., and Cech, T.R. (2013). Finding the end: recruitment of telomerase to telomeres. Nature reviews Molecular cell biology *14*, 69-82.

Nardella, C., Clohessy, J.G., Alimonti, A., and Pandolfi, P.P. (2011). Pro-senescence therapy for cancer treatment. Nature reviews Cancer *11*, 503-511.

Narita, M. (2007). Cellular senescence and chromatin organisation. British journal of cancer *96*, 686-691.

Narita, M. (2010). Quality and quantity control of proteins in senescence. Aging *2*, 311-314.

Narita, M., Narita, M., Krizhanovsky, V., Nunez, S., Chicas, A., Hearn, S.a., Myers, M.P., and Lowe, S.W. (2006). A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. Cell *126*, 503-514.

Narita, M., Nun, S., Heard, E., Narita, M., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., Lowe, S.W., Brook, S.*, et al.* (2003). Rb-Mediated Heterochromatin Formation and Silencing of E2F Target Genes during Cellular Senescence State University of New York at Stony Brook. *113*, 703-716.

Narita, M., Young, A.R.J., Arakawa, S., Samarajiwa, S.A., Nakashima, T., Yoshida, S., Hong, S., Berry, L.S., Reichelt, S., Ferreira, M.*, et al.* (2011). Spatial coupling of mTOR and autophagy augments secretory phenotypes. Science (New York, NY) *332*, 966-970.

Negishi, M., Saraya, A., Mochizuki, S., Helin, K., Koseki, H., and Iwama, A. (2010). A novel zinc finger protein Zfp277 mediates transcriptional repression of the ink4a/Arf locus through polycomb repressive complex 1. PLoS ONE *5*, 1-14.

Nelson, D.M., McBryan, T., Jeyapalan, J.C., Sedivy, J.M., and Adams, P.D. (2014). A comparison of oncogene-induced senescence and replicative senescence: Implications for tumor suppression and aging. Age *36*, 1049-1065.

Nicolas, F.J., De Bosscher, K., Schmierer, B., and Hill, C.S. (2004). Analysis of Smad nucleocytoplasmic shuttling in living cells. J Cell Sci *117*, 4113-4125.

Nielsen, a.L., Ortiz, J.a., You, J., Oulad-Abdelghani, M., Khechumian, R., Gansmuller, a., Chambon, P., and Losson, R. (1999). Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. The EMBO journal *18*, 6385-6395.

Nishimura, K., Kumazawa, T., Kuroda, T., Katagiri, N., Tsuchiya, M., Goto, N., Furumai, R., Murayama, A., Yanagisawa, J., and Kimura, K. (2015). Perturbation of Ribosome Biogenesis Drives Cells into Senescence through 5S RNP-Mediated p53 Activation. Cell Reports *10*, 1310-1323.

Nishio, K., and Inoue, A. (2005). Senescence-associated alterations of cytoskeleton: Extraordinary production of vimentin that anchors cytoplasmic p53 in senescent human fibroblasts. Histochemistry and Cell Biology *123*, 263-273.

Nishio, K., Inoue, A., Qiao, S., Kondo, H., and Mimura, A. (2001). Senescence and cytoskeleton: Overproduction of vimentin induces senescent-like morphology in human fibroblasts. Histochemistry and Cell Biology *116*, 321-327.

Noma, K.I., Cam, H.P., Maraia, R.J., and Grewal, S.I.S. (2006). A Role for TFIIIC Transcription Factor Complex in Genome Organization. Cell *125*, 859-872.

Nose, K., and Okamoto, H. (1980). Transcriptional activity of nuclei from WI-38 cells at various passages. J Cell Physiol *102*, 51-54.

O'Connor, O.a., Heaney, M.L., Schwartz, L., Richardson, S., Willim, R., MacGregor-Cortelli, B., Curly, T., Moskowitz, C., Portlock, C., Horwitz, S.*, et al.* (2006). Clinical experience with intravenous and oral formulations of the novel histone deacetylase inhibitor suberoylanilide hydroxamic acid in patients with advanced hematologic malignancies. Journal of Clinical Oncology *24*, 166-173.

O'Sullivan, R.J., and Karlseder, J. (2012). The great unravelling: chromatin as a modulator of the aging process. Trends Biochem Sci *37*, 466-476.

O'Sullivan, R.J., Kubicek, S., Schreiber, S.L., and Karlseder, J. (2010). Reduced histone biosynthesis and chromatin changes arising from a damage signal at telomeres. Nature structural & molecular biology *17*, 1218-1225.

Ogryzko, V.V., Kotani, T., Zhang, X., Schiltz, R.L., Howard, T., Yang, X.-j., Howard, B.H., Qin, J., and Nakatani, Y. (1998). Histone-like TAFs within the PCAF Histone Acetylase Complex. *94*, 35-44.

Ohanna, M.l., Giuliano, S., Bonet, C., Imbert, V.r., Hofman, V.r., Zangari, J.p., Bille, K., Robert, C., Bressac-de Paillerets, B., Hofman, P.*, et al.* (2011). Senescent cells develop a PARP-1 and nuclear factor-{kappa}B-associated secretome (PNAS). Genes & development *25*, 1245-1261.

Ohtani, N., Zebedee, Z., Huot, T.J., Stinson, J.a., Sugimoto, M., Ohashi, Y., Sharrocks, a.D., Peters, G., and Hara, E. (2001). Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. Nature *409*, 1067-1070.

Ohtsubo, K., and Marth, J.D. (2006). Glycosylation in cellular mechanisms of health and disease. Cell *126*, 855-867.

Okada, N., Ishigami, Y., Suzuki, T., Kaneko, A., Yasui, K., Fukutomi, R., and Isemura, M. (2008). Importins and exportins in cellular differentiation. J Cell Mol Med *12*, 1863-1871.

Okamoto, a., Demetrick, D.J., Spillare, E.a., Hagiwara, K., Hussain, S.P., Bennett, W.P., Forrester, K., Gerwin, B., Serrano, M., and Beach, D.H. (1994). Mutations and altered expression of p16INK4 in human cancer. Proceedings of the National Academy of Sciences of the United States of America *91*, 11045-11049.

Okamoto, K., Kitabayashi, I., and Taya, Y. (2006). KAP1 dictates p53 response induced by chemotherapeutic agents via Mdm2 interaction. Biochemical and biophysical research communications *351*, 216-222.

Orjalo, A.V., Bhaumik, D., Gengler, B.K., Scott, G.K., and Campisi, J. (2009). Cell surface-bound IL-1alpha is an upstream regulator of the senescence-associated IL-6/IL-8 cytokine network. Proceedings of the National Academy of Sciences of the United States of America *106*, 17031-17036.

Oruetxebarria, I., Venturini, F., Kekarainen, T., Houweling, A., Zuijderduijn, L.M.P., Mohd-Sarip, A., Vries, R.G.J., Hoeben, R.C., and Verrijzer, C.P.

(2004). p16INK4a Is Required for hSNF5 Chromatin Remodeler-induced Cellular Senescence in Malignant Rhabdoid Tumor Cells. Journal of Biological Chemistry *279*, 3807-3816.

Palmero, I., Pantoja, C., and Serrano, M. (1998). p19ARF links the tumour suppressor p53 to Ras. Nature *395*, 125-126.

Pang, J.H., and Chen, K.Y. (1994). Global change of gene expression at late G1/S boundary may occur in human IMR-90 diploid fibroblasts during senescence. J Cell Physiol *160*, 531-538.

Pantoja, C., and Serrano, M. (1999). Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras. Oncogene *18*, 4974-4982.

Parrinello, S., Coppe, J.-P., Krtolica, A., and Campisi, J. (2005). Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. Journal of cell science *118*, 485-496.

Passos, J.o.F., Nelson, G., Wang, C., Richter, T., Simillion, C., Proctor, C.J., Miwa, S., Olijslagers, S., Hallinan, J., Wipat, A.*, et al.* (2010). Feedback between p21 and reactive oxygen production is necessary for cell senescence. Molecular systems biology *6*, 347-347.

Passos, J.o.F., Saretzki, G., Ahmed, S., Nelson, G., Richter, T., Peters, H., Wappler, I., Birket, M.J., Harold, G., Schaeuble, K.*, et al.* (2007a). Mitochondrial dysfunction accounts for the stochastic heterogeneity in telomere-dependent senescence. PLoS Biology *5*, 1138-1151.

Passos, J.o.F., Saretzki, G., and Von Zglinicki, T. (2007b). DNA damage in telomeres and mitochondria during cellular senescence: Is there a connection? Nucleic Acids Research *35*, 7505-7513.

Pawlowski, R., Muhl, S.M., Sulser, T., Krek, W., Moch, H., and Schraml, P. (2013). Loss of PBRM1 expression is associated with renal cell carcinoma progression. Int J Cancer *132*, E11-17.

Pearson, M., and Pelicci, P.G. (2001). PML interaction with p53 and its role in apoptosis and replicative senescence. Oncogene *20*, 7250-7256.

Pedeux, R., Sengupta, S., Shen, J.C., Demidov, O.N., Saito, S.i., Onogi, H., Kumamoto, K., Wincovitch, S., Garfield, S.H., McMenamin, M.*, et al.* (2005). ING2 regulates the onset of replicative senescence by induction of p300-dependent p53 acetylation. Molecular and cellular biology *25*, 6639-6648.

Pelz, O., Gilsdorf, M., and Boutros, M. (2010). web cellHTS2: a web-application for the analysis of high-throughput screening data. BMC bioinformatics *11*, 185-185.

Pepperkok, R., and Ellenberg, J. (2006). microscopy for systems biology. *7*, 690-696.

Perez-Mancera, P.a., Young, A.R.J., and Narita, M. (2014). Inside and out: the activities of senescence in cancer. Nature Reviews Cancer *14*, 547-558.

Peters, G. (2008). An INKlination for epigenetic control of senescence. Nature structural & molecular biology *15*, 1133-1134.

Piekarz, R.L., Frye, R., Turner, M., Wright, J.J., Allen, S.L., Kirschbaum, M.H., Zain, J., Prince, H.M., Leonard, J.P., Geskin, L.J.*, et al.* (2009). Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as

monotherapy for patients with cutaneous T-cell lymphoma. Journal of Clinical Oncology *27*, 5410-5417.

Pina, B., and Suau, P. (1987). Changes in the proportions of histone H1 subtypes in brain cortical neurons. FEBS Lett *210*, 161-164.

Pomerantz, J., Schreiber-agus, N., Lie, N.J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H.-w.*, et al.* (1998). The Ink4a Tumor Suppressor Gene Product , p19 Arf , Interacts with MDM2 and Neutralizes MDM2 ‚Äô s Inhibition of p53. Cell *92*, 713-723.

Popov, N., and Gil, J. (2010). Epigenetic regulation of the INK4b-ARF-INK4a locus: in sickness and in health. Epigenetics *5*, 685-690.

Pribluda, A., Elyada, E., Wiener, Z., Hamza, H., Goldstein, R., Biton, M., Burstain, I., Morgenstern, Y., Brachya, G., Billauer, H.*, et al.* (2013). A Senescence-inflammatory switch from cancer-inhibitory to cancer-promoting mechanism. Cancer Cell *24*, 242-256.

Prieur, A., Besnard, E., Babled, A.l., and Lemaitre, J.-M. (2011). p53 and p16(INK4A) independent induction of senescence by chromatin-dependent alteration of S-phase progression. Nature communications *2*, 473-473.

Prowse, K.R., and Greider, C.W. (1995). Developmental and tissue-specific regulation of mouse telomerase and telomere length. Proceedings of the National Academy of Sciences of the United States of America *92*, 4818-4822.

Purcell, M., Kruger, A., and Tainsky, M.a. (2015). Gene expression profiling of replicative and induced senescence. Cell Cycle *13*, 3927-3937.

Qian, Y., and Chen, X. (2013). Senescence regulation by the p53 protein family. Methods Mol Biol *965*, 37-61.

Quelle, D.E., Zindy, F., Ashmun, R.A., and Sherr, C.J. (1995). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell *83*, 993-1000.

Quijano, C., Cao, L., Fergusson, M.M., Romero, H., Liu, J., Gutkind, S., Rovira, I.I., Mohney, R.P., Karoly, E.D., and Finkel, T. (2012). Oncogene-induced senescence results in marked metabolic and bioenergetic alterations. Cell Cycle *11*, 1383-1392.

Rabenhorst, U., Beinoraviciute-Kellner, R., Brezniceanu, M.L., Joos, S., Devens, F., Lichter, P., Rieker, R.J., Trojan, J.r., Chung, H.J., Levens, D.L.*, et al.* (2009). Overexpression of the far upstream element binding protein 1 in hepatocellular carcinoma is required for tumor growth. Hepatology *50*, 1121-1129.

Rai, T.S., and Adams, P.D. (2012). Lessons from senescence: Chromatin maintenance in non-proliferating cells. Biochimica et Biophysica Acta - Gene Regulatory Mechanisms *1819*, 322-331.

Rakhra, K., Bachireddy, P., Zabuawala, T., Zeiser, R., Xu, L., Kopelman, A., Fan, A.C., Yang, Q., Braunstein, L., Crosby, E.*, et al.* (2010). CD4(+) T cells contribute to the remodeling of the microenvironment required for sustained tumor regression upon oncogene inactivation. Cancer Cell *18*, 485-498.

Rand, T.A., Petersen, S., Du, F., and Wang, X. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell *123*, 621-629.

Ranuncolo, S.M., Ghosh, S., Hanover, J.a., Hart, G.W., and Lewis, B.a. (2012). Evidence of the involvement of O-GlcNAc-modified human RNA polymerase II CTD in transcription in vitro and in vivo. Journal of Biological Chemistry *287*, 23549-23561.

Rao, D.D., Senzer, N., Cleary, M.a., and Nemunaitis, J. (2009a). Comparative assessment of siRNA and shRNA off target effects: what is slowing clinical development. Cancer gene therapy *16*, 807-809.

Rao, D.D., Vorhies, J.S., Senzer, N., and Nemunaitis, J. (2009b). siRNA vs. shRNA: similarities and differences. Adv Drug Deliv Rev *61*, 746-759.

Reeves, W.M., and Hahn, S. (2005). Targets of the Gal4 transcription activator in functional transcription complexes. Molecular and cellular biology *25*, 9092-9102.

Reimann, M., Lee, S., Loddenkemper, C., Dorr, J.R., Tabor, V., Aichele, P., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C.a. (2010). Tumor Stroma-Derived TGF-beta Limits Myc-Driven Lymphomagenesis via Suv39h1-Dependent Senescence. Cancer Cell *17*, 262-272.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105-111.

Rinn, J.L., and Huarte, M. (2011). To repress or not to repress: this is the guardian's question. Trends in cell biology *21*, 344-353.

Rodier, F., Copp√©, J.-P., Patil, C.K., Hoeijmakers, W.a.M., Mu√±oz, D.P., Raza, S.R., Freund, A., Campeau, E., Davalos, A.R., and Campisi, J. (2009). Persistent DNA damage signaling triggers senescence-associated inflammatory cytokine secretion. Nature cell biology *11*, 973-979.

Rodriguez-Navarro, S., Fischer, T., Luo, M.J., Antunez, O., Brettschneider, S., Lechner, J., Perez-Ortin, J.E., Reed, R., and Hurt, E. (2004). Sus1, a functional component of the SAGA histone acetylase complex and the nuclear pore-associated mRNA export machinery. Cell *116*, 75-86.

Rodriguez-Paredes, M., and Esteller, M. (2011a). Cancer epigenetics reaches mainstream oncology. Nat Med *17*, 330-339.

Rodriguez-Paredes, M., and Esteller, M. (2011b). A combined epigenetic therapy equals the efficacy of conventional chemotherapy in refractory advanced non-small cell lung cancer. Cancer Discov *1*, 557-559.

Rovillain, E., Mansfield, L., Caetano, C., Alvarez-Fernandez, M., Caballero, O.L., Medema, R.H., Hummerich, H., and Jat, P.S. (2011). Activation of nuclear factor-kappa B signaling promotes cellular senescence. Oncogene *30*, 2356-2366.

Ruiz-Canada, C., Kelleher, D.J., and Gilmore, R. (2009). Cotranslational and Posttranslational N-Glycosylation of Polypeptides by Distinct Mammalian OST Isoforms. Cell *136*, 272-283.

Sadaie, M., Salama, R., Carroll, T., Tomimatsu, K., Chandra, T., Young, A.R.J., Narita, M., Perez-Mancera, P.a., Bennett, D.C., Chong, H.*, et al.* (2013). Redistribution of the Lamin B1 genomic binding profile affects rearrangement of heterochromatic domains and SAHF formation during senescence. Genes & development *27*, 1800-1808.

Safina, A., Garcia, H., Commane, M., Guryanova, O., Degan, S., Kolesnikova, K., and Gurova, K.V. (2013). Complex mutual regulation of facilitates chromatin transcription (FACT) subunits on both mRNA and protein levels in human cells. Cell Cycle *12*, 2423-2434.

Sakabe, K., Wang, Z., and Hart, G.W. (2010). Beta-N-acetylglucosamine (O-GlcNAc) is part of the histone code. Proceedings of the National Academy of Sciences of the United States of America *107*, 19915-19920.

Salama, R., Sadaie, M., Hoare, M., and Narita, M. (2014). Cellular senescence and its effector programs. Genes & development *28*, 99-114.

Samara, N.L., and Wolberger, C. (2011). A new chapter in the transcription SAGA. Current opinion in structural biology *21*, 767-774.

Sarkisian, C.J., Keister, B.a., Stairs, D.B., Boxer, R.B., Moody, S.E., and Chodosh, L.a. (2007). Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis. Nature cell biology *9*, 493-505.

Sasaki, M., Miyakoshi, M., Sato, Y., and Nakanuma, Y. (2010). Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. Laboratory investigation; a journal of technical methods and pathology *90*, 835-843.

Sato, Y., and Endo, T. (2010). Alteration of brain glycoproteins during aging. Geriatr Gerontol Int *10 Suppl 1*, S32-40.

Schlabach, M.R., Luo, J., Solimini, N.L., Hu, G., Xu, Q., Li, M.Z., Zhao, Z., Smogorzewska, A., Sowa, M.E., Ang, X.L.*, et al.* (2008). Cancer proliferation gene discovery through functional genomics. Science (New York, NY) *319*, 620-624.

Schmitt, C.A., Fridman, J.S., Yang, M., Lee, S., Baranov, E., Hoffman, R.M., Lowe, S.W., and Diego, S. (2002). A Senescence Program Controlled by p53 and p16 INK4a Contributes to the Outcome of Cancer Therapy. *109*, 335-346.

Schmitz, K.M., Schmitt, N., Hoffmann-Rohrer, U., Schafer, A., Grummt, I., and Mayer, C. (2009). TAF12 recruits Gadd45a and the nucleotide excision repair complex to the promoter of rRNA genes leading to active DNA demethylation. Mol Cell *33*, 344-353.

Schramm, L., and Hernandez, N. (2002). Recruitment of RNA polymerase III to its target promoters. Genes & development *16*, 2593-2620.

Schultz, D.C., Ayyanathan, K., Negorev, D., Maul, G.G., and Rauscher, F.J. (2002). SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. Genes & development *16*, 919-932

Schultz, D.C., Friedman, J.R., and Rauscher, F.J. (2001). Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. Genes & development *15*, 428-443.

Schultz, M.C. (2003). DNA damage regulation of the RNA components of the translational apparatus: new biology and mechanisms. IUBMB life *55*, 243-247.

Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P.D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell *115*, 199-208.

Seluanov, A., Gorbunova, V., Falcovitz, A., Sigal, A., Milyavsky, M., Zurer, I., Shohat, G., Goldfinger, N., and Rotter, V. (2001). Change of the death pathway in senescent human fibroblasts in response to DNA damage is caused by an inability to stabilize p53. Mol Cell Biol *21*, 1552-1564.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Seshadri, T., and Campisi, J. (1990). Repression of c-fos transcription and an altered genetic program in senescent human fibroblasts. Science *247*, 205-209.

Shah, P.P., Donahue, G., Otte, G.L., Capell, B.C., Nelson, D.M., Cao, K., Aggarwala, V., Cruickshanks, H.a., Rai, T.S., McBryan, T.*, et al.* (2013). Lamin B1 depletion in senescent cells triggers large-scale changes in gene expression and the chromatin landscape. Genes and Development *27*, 1787-1799.

Shamma, A., Takegami, Y., Miki, T., Kitajima, S., Noda, M., Obara, T., Okamoto, T., and Takahashi, C. (2009). Rb Regulates DNA Damage Response and Cellular Senescence through E2F-Dependent Suppression of N-Ras Isoprenylation. Cancer Cell *15*, 255-269.

Sharma, V.M., Li, B., and Reese, J.C. (2003). SWI / SNF-dependent chromatin remodeling of RNR3 requires TAF II s and the general transcription machinery. Genes & Development, 502-515.

Sharpless, N.E. (2005). INK4a/ARF: A multifunctional tumor suppressor locus. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis *576*, 22-38.

Shay, J.W., and Wright, W.E. (2000). Hayflick, his limit, and cellular ageing. Nature reviews Molecular cell biology *1*, 72-76.

Shelton, D.N., Chang, E., Whittier, P.S., Choi, D., and Funk, W.D. (1999). Microarray analysis of replicative senescence. Current biology : CB *9*, 939-945.

Shen, H., and Laird, P.W. (2013). Interplay between the cancer genome and epigenome. Cell *153*, 38-55.

Sherr, C.J., and DePinho, R.a. (2000). Cellular senescence: mitotic clock or culture shock? Cell *102*, 407-410.

Shi, F.T., Kim, H., Lu, W., He, Q., Liu, D., Goodell, M.a., Wan, M., and Songyang, Z. (2013). Ten-eleven translocation 1 (Tet1) is regulated by o-linked n-acetylglucosamine transferase (ogt) for target gene repression in mouse embryonic stem cells. Journal of Biological Chemistry *288*, 20776-20784.

Shimi, T., Butin-Israeli, V., Adam, S.A., Hamanaka, R.B., Goldman, A.E., Lucas, C.A., Shumaker, D.K., Kosak, S.T., Chandel, N.S., and Goldman, R.D. (2011). The role of nuclear lamin B1 in cell proliferation and senescence. Genes and Development *25*, 2579-2593.

Shumaker, D.K., Dechat, T., Kohlmaier, A., Adam, S.A., Bozovsky, M.R., Erdos, M.R., Eriksson, M., Goldman, A.E., Khuon, S., Collins, F.S.*, et al.* (2006). Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging. Proceedings of the National Academy of Sciences of the United States of America *103*, 8703-8708.

Silva, J.M., Li, M.Z., Chang, K., Ge, W., Golding, M.C., Rickles, R.J., Siolas, D., Hu, G., Paddison, P.J., Schlabach, M.R.*, et al.* (2005). Second-generation shRNA libraries covering the mouse and human genomes. Nature genetics *37*, 1281-1288.

Siolas, D., Lerner, C., Burchard, J., Ge, W., Linsley, P.S., Paddison, P.J., Hannon, G.J., and Cleary, M.a. (2005). Synthetic shRNAs as potent RNAi triggers. Nature biotechnology *23*, 227-231.

Sloan, K.E., Bohnsack, M.T., and Watkins, N.J. (2013). The 5S RNP Couples p53 Homeostasis to Ribosome Biogenesis and Nucleolar Stress. Cell Reports *5*, 237-247.

Slawson, C., Pidala, J., and Potter, R. (2001). Increased N-acetyl-beta-glucosaminidase activity in primary breast carcinomas corresponds to a decrease in N-acetylglucosamine containing proteins. Biochim Biophys Acta *1537*, 147-157.

Slawson, C., Zachara, N.E., Vosseller, K., Cheung, W.D., Lane, M.D., and Hart, G.W. (2005). Perturbations in O-linked beta-N-acetylglucosamine protein modification cause severe defects in mitotic progression and cytokinesis. J Biol Chem *280*, 32944-32956.

Spitkovsky, D., Schulze, a., Boye, B., and Jansen-D√ºrr, P. (1997). Down-regulation of cyclin A gene expression upon genotoxic stress correlates with reduced binding of free E2F to the promoter. Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research *8*, 699-710.

Sporn, J.C., Kustatscher, G., Hothorn, T., Collado, M., Serrano, M., Muley, T., Schnabel, P., and Ladurner, A.G. (2009). Histone macroH2A isoforms predict the risk of lung cancer recurrence. Oncogene *28*, 3423-3428.

Stein, G.H., Drullinger, L.F., Robetorye, R.S., Pereira-Smith, O.M., and Smith, J.R. (1991). Senescent cells fail to express cdc2, cycA, and cycB in response to mitogen stimulation. Proc Natl Acad Sci U S A *88*, 11012-11016.

Steward, M.M., Lee, J.-S., O'Donovan, A., Wyatt, M., Bernstein, B.E., and Shilatifard, A. (2006). Molecular regulation of H3K4 trimethylation by ASH2L, a shared subunit of MLL complexes. Nature structural & molecular biology *13*, 852-854.

Storer, M., Mas, A., Robert-Moreno, A., Pecoraro, M., Ortells, M.C., Di Giacomo, V., Yosef, R., Pilpel, N., Krizhanovsky, V., Sharpe, J.*, et al.* (2013). Senescence is a developmental mechanism that contributes to embryonic growth and patterning. Cell *155*, 1119-1130.

Sulli, G., Di Micco, R., and d'Adda di Fagagna, F. (2012). Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer. Nature reviews Cancer *12*, 709-720.

Sun, P., Yoshizuka, N., New, L., Moser, B.a., Li, Y., Liao, R., Xie, C., Chen, J., Deng, Q., Yamout, M.*, et al.* (2007). PRAK Is Essential for ras-Induced Senescence and Tumor Suppression. Cell *128*, 295-308.

Sun, Y., Campisi, J., Higano, C., Beer, T.M., Porter, P., Coleman, I., True, L., and Nelson, P.S. (2012). Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B. Nature Medicine *18*, 1359-1368.

Swanson, E.C., Manning, B., Zhang, H., and Lawrence, J.B. (2013). Higher-order unfolding of satellite heterochromatin is a consistent and early event in cell senescence. Journal of Cell Biology *203*, 929-942.

Tachibana, M., Ueda, J., Fukuda, M., Takeda, N., Ohta, T., Iwanari, H., Sakihama, T., Kodama, T., Hamakubo, T., and Shinkai, Y. (2005). Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9. Genes and Development *19*, 815-826.

Tai, C.-J., Hsu, C.-H., Shen, S.-C., Lee, W.-R., and Jiang, M.-C. (2010). Cellular apoptosis susceptibility (CSE1L/CAS) protein in cancer metastasis and chemotherapeutic drug-induced apoptosis. Journal of experimental & clinical cancer research : CR *29*, 110-110.

Takahashi, A., Imai, Y., Yamakoshi, K., Kuninaka, S., Ohtani, N., Yoshimoto, S., Hori, S., Tachibana, M., Anderton, E., Takeuchi, T.*, et al.* (2012). DNA damage signaling triggers degradation of histone methyltransferases through APC/C Cdh1 in senescent cells. Molecular Cell *45*, 123-131.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663-676.

Takeuchi, S., Takahashi, A., Motoi, N., Yoshimoto, S., Tajima, T., Yamakoshi, K., Hirao, A., Yanagi, S., Fukami, K., Ishikawa, Y.*, et al.* (2010). Intrinsic cooperation between p16INK4a and p21Waf1/Cip1 in the onset of cellular senescence and tumor suppression in vivo. Cancer research *70*, 9381-9390.

Tallent, M.K., Varghis, N., Skorobogatko, Y., Hernandez-Cuebas, L., Whelan, K., Vocadlo, D.J., and Vosseller, K. (2009). In vivo modulation of O-GlcNAc levels regulates hippocampal synaptic plasticity through interplay with phosphorylation. The Journal of biological chemistry *284*, 174-181.

Tao, W., and Levine, a.J. (1999). P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. Proceedings of the National Academy of Sciences of the United States of America *96*, 6937-6941.

Tarutani, M., Cai, T., Dajee, M., and Khavari, P.a. (2003). Inducible activation of Ras and Raf in adult epidermis. Cancer research *63*, 319-323.

Tomari, Y., and Zamore, P.D. (2005). Perspective : machines for RNAi. 517-529.

Torres, C.R., and Hart, G.W. (1984). Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. Journal of Biological Chemistry *259*, 3308-3317.

Tsuruma, R., Ohbayashi, N., Kamitani, S., Ikeda, O., Sato, N., Muromoto, R., Sekine, Y., Oritani, K., and Matsuda, T. (2008). Physical and functional interactions between STAT3 and KAP1. Oncogene *27*, 3054-3059.

Tu, Z., Zhuang, X., Yao, Y.-G., and Zhang, R. (2013). BRG1 is required for formation of senescence-associated heterochromatin foci induced by oncogenic RAS or BRCA1 loss. Molecular and cellular biology *33*, 1819-1829.

Utikal, J., Polo, J.M., Stadtfeld, M., Maherali, N., Kulalert, W., Walsh, R.M., Khalil, A., Rheinwald, J.G., and Hochedlinger, K. (2009). Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. Nature *460*, 1145-1148.

Umlauf, D., Bonnet, J., Waharte, F.o., Fournier, M., Stierle, M., Fischer, B., Brino, L., Devys, D., and Tora, L.s. (2013). The human TREX-2 complex is stably associated with the nuclear pore basket. Journal of cell science *126*, 2656-2667.

van Deursen, J.M. (2014). The role of senescent cells in ageing. Nature *509*, 439-446.

Vanhooren, V., Dewaele, S., Kuro, O.M., Taniguchi, N., Dolle, L., van Grunsven, L.A., Makrantonaki, E., Zouboulis, C.C., Chen, C.C., and Libert, C. (2011). Alteration in N-glycomics during mouse aging: a role for FUT8. Aging Cell *10*, 1056-1066.

Varambally, S., Dhanasekaran, S.M., Zhou, M., Barrette, T.R., Kumar-Sinha, C., Sanda, M.G., Ghosh, D., Pienta, K.J., Sewalt, R.G.a.B., Otte, A.P.*, et al.* (2002). The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature *419*, 624-629.

Varela, I., Cadinanos, J., Pendas, A.M., Gutierrez-Fernandez, A., Folgueras, A.R., Sanchez, L.M., Zhou, Z., Rodriguez, F.J., Stewart, C.L., Vega, J.A.*, et al.* (2005). Accelerated ageing in mice deficient in Zmpste24 protease is linked to p53 signaling activation. Nature *437*, 564-568.

Varela, I., Tarpey, P., Raine, K., Huang, D., Ong, C.K., Stephens, P., Davies, H., Jones, D., Lin, M.L., Teague, J.*, et al.* (2011). Exome sequencing identifies frequent mutation of the SWI/SNF Complex Gene PBRM1 in renal carcinoma. Journal of Urology *186*, 1150-1150.

Vassilev, a., Yamauchi, J., Kotani, T., Prives, C., Avantaggiati, M.L., Qin, J., and Nakatani, Y. (1998). The 400 kDa subunit of the PCAF histone acetylase complex belongs to the ATM superfamily. Molecular cell *2*, 869-875.

Vella, P., Scelfo, A., Jammula, S., Chiacchiera, F., Williams, K., Cuomo, A., Roberto, A., Christensen, J., Bonaldi, T., Helin, K.*, et al.* (2013). Tet proteins connect the O-linked N-acetylglucosamine transferase Ogt to chromatin in embryonic stem cells. Molecular cell *49*, 645-656.

Vernier, M., Bourdeau, V.r., Gaumont-Leclerc, M.F., Moiseeva, O., B√©gin, V., Saad, F., Mes-Masson, A.M., and Ferbeyre, G. (2011). Regulation of E2Fs and senescence by PML nuclear bodies. Genes and Development *25*, 41-50.

Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vanderwinden, J.M.*, et al.* (2006). The Polycomb group protein EZH2 directly controls DNA methylation. Nature *439*, 871-874.

Vlcek, S., and Foisner, R. (2007). A-type lamin networks in light of laminopathic diseases. Biochim Biophys Acta *1773*, 661-674.

Vocadlo, D.J. (2012). O-GlcNAc processing enzymes: Catalytic mechanisms, substrate specificity, and enzyme regulation. Current Opinion in Chemical Biology *16*, 488-497.

Voorhoeve, P.M., and Agami, R. (2003). The tumor-suppressive functions of the human INK4A locus. Cancer Cell *4*, 311-319.

Voulgari, A., Voskou, S., Tora, L.s., Davidson, I., Sasazuki, T., Shirasawa, S., and Pintzas, A. (2008). TATA box-binding protein-associated factor 12 is important for RAS-induced transformation properties of colorectal cancer cells. Molecular cancer research : MCR *6*, 1071-1083.

Wang, C., Ivanov, A., Chen, L., Fredericks, W.J., Seto, E., Rauscher, F.J., and Chen, J. (2005). MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. The EMBO journal *24*, 3279-3290.

Wang, W. (2012). Regulatory RNA-binding proteins in senescence. Ageing Res Rev *11*, 485-490.

Wang, W., Yang, X., Cristofalo, V.J., Holbrook, N.J., and Gorospe, M. (2001). Loss of HuR is linked to reduced expression of proliferative genes during replicative senescence. Mol Cell Biol *21*, 5889-5898.

Wang, Y., Sharpless, N., and Chang, S. (2013). p16 INK4a protects against dysfunctional telomere-induced ATR-dependent DNA damage responses. The Journal of clinical investigation *123*, 4489-4501.

Wang, Z., Udeshi, N.D., Slawson, C., Compton, P.D., Sakabe, K., Cheung, W.D., Shabanowitz, J., Hunt, D.F., and Hart, G.W. (2010). Extensive crosstalk between O-GlcNAcylation and phosphorylation regulates cytokinesis. Sci Signal *3*, ra2.

Watson, J.D. (1972). Origin of concatemeric T7 DNA. Nature: New biology *239*, 197-201.

Weake, V.M., and Workman, J.L. (2012). SAGA function in tissue-specific gene expression. Trends in cell biology *22*, 177-184.

Weber, A., Kristiansen, I., Johannsen, M., Oelrich, B., Scholmann, K., Gunia, S., May, M., Meyer, H.A., Behnke, S., Moch, H.*, et al.* (2008). The FUSE binding proteins FBP1 and FBP3 are potential c-myc regulators in renal, but not in prostate and bladder cancer. BMC cancer *8*, 369.

Wei, S., Wei, W., and Sedivy, J.M. (1999). Expression of catalytically active telomerase does not prevent premature senescence caused by overexpression of oncogenic Ha-Ras in normal human fibroblasts. Cancer Research *59*, 1539-1543.

Wei, W., Hemmer, R.M., and Sedivy, J.M. (2001). Role of p14 ARF in Replicative and Induced Senescence of Human Fibroblasts. Molecular and Cellular Biology *21*, 6748-6757.

Weinberg, R.a. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

Wiles, A.M., Ravi, D., Bhavani, S., and Bishop, A.J. (2008). An analysis of normalization methods for Drosophila RNAi genomic screens and development of a robust validation scheme. J Biomol Screen *13*, 777-784.

White, D., Rafalska-Metcalf, I.U., Ivanov, A.V., Corsinotti, A., Peng, H., Lee, S.C., Trono, D., Janicki, S.M., and Rauscher, F.J., 3rd (2012). The ATM substrate KAP1 controls DNA repair in heterochromatin: regulation by HP1 proteins and serine 473/824 phosphorylation. Mol Cancer Res *10*, 401-414.

Wiegand, K.C., Shah, S.P., Al-Agha, O.M., Zhao, Y., Tse, K., Zeng, T., Senz, J., McConechy, M.K., Anglesio, M.S., Kalloger, S.E.*, et al.* (2010). ARID1A mutations in endometriosis-associated ovarian carcinomas. N Engl J Med *363*, 1532-1543.

Kipling, D., and Cooke, H.J. (1990). Hypervariable ultra-long telomeres in mice. Nature *347*, 400-402.

Wilson, B.G., and Roberts, C.W.M. (2011). SWI/SNF nucleosome remodellers and cancer. Nature reviews Cancer *11*, 481-492.

Wilson, B.G., Wang, X., Shen, X., McKenna, E.S., Lemieux, M.E., Cho, Y.J., Koellhoffer, E.C., Pomeroy, S.L., Orkin, S.H., and Roberts, C.W. (2010). Epigenetic antagonism between polycomb and SWI/SNF complexes during oncogenic transformation. Cancer Cell *18*, 316-328.

Wu, J., Prindle, M.J., Dressler, G.R., and Yu, X. (2009). PTIP regulates 53BP1 and SMC1 at the DNA damage sites. Journal of Biological Chemistry *284*, 18078-18084.

Wu, P.Y., Ruhlmann, C., Winston, F., and Schultz, P. (2004). Molecular architecture of the S. cerevisiae SAGA complex. Mol Cell *15*, 199-208.

Wuestefeld, T., Pesic, M., Rudalska, R., Dauch, D., Longerich, T., Kang, T.-W., Yevsa, T., Heinzmann, F., Hoenicke, L., Hohmeyer, A.*, et al.* (2013). A Direct in vivo RNAi screen identifies MKK4 as a key regulator of liver regeneration. Cell *153*, 389-401.

Wyce, A., Xiao, T., Whelan, K.A., Kosman, C., Walter, W., Eick, D., Hughes, T.R., Krogan, N.J., Strahl, B.D., and Berger, S.L. (2007). H2B ubiquitylation acts as a barrier to Ctk1 nucleosomal recruitment prior to removal by Ubp8 within a SAGA-related complex. Mol Cell *27*, 275-288.

Xiao, X., Moreno-Moral, A., Rotival, M., Bottolo, L., and Petretto, E. (2014). Multi-tissue Analysis of Co-expression Networks by Higher-Order Generalized Singular Value Decomposition Identifies Functionally Coherent Transcriptional Modules. PLoS Genetics *10*.

Xu, Y. (2003). Regulation of p53 responses by post-translational modifications. Cell death and differentiation *10*, 400-403.

Xue, W., Zender, L., Miething, C., Dickins, R.a., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature *445*, 656-660.

Yamakoshi, K., Takahashi, A., Hirota, F., Nakayama, R., Ishimaru, N., Kubo, Y., Mann, D.J., Ohmura, M., Hirao, A., Saya, H.*, et al.* (2009). Real-time in vivo imaging of p16Ink4a reveals cross talk with p53. Journal of Cell Biology *186*, 393-407.

Yan, G., Eller, M.S., Elm, C., Larocca, C.a., Ryu, B., Panova, I.P., Dancy, B.M., Bowers, E.M., Meyers, D., Lareau, L.*, et al.* (2013). Selective inhibition of p300 HAT blocks cell cycle progression, induces cellular senescence, and

inhibits the DNA damage response in melanoma cells. The Journal of investigative dermatology *133*, 2444-2452.

Yang, M.H., Hsu, D.S., Wang, H.W., Wang, H.J., Lan, H.Y., Yang, W.H., Huang, C.H., Kao, S.Y., Tzeng, C.H., Tai, S.K.*, et al.* (2010). Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition. Nat Cell Biol *12*, 982-992.

Yang, W.H., Kim, J.E., Nam, H.W., Ju, J.W., Kim, H.S., Kim, Y.S., and Cho, J.W. (2006). Modification of p53 with O-linked N-acetylglucosamine regulates p53 activity and stability. Nature cell biology *8*, 1074-1083.

Yang, X., Zhang, F., and Kudlow, J.E. (2002). Recruitment of O-GlcNAc transferase to promoters by corepressor mSin3A: Coupling protein O-GlcNAcylation to transcriptional repression. Cell *110*, 69-80.

Yap, K.L., Li, S., Munoz-Cabello, A.M., Raguz, S., Zeng, L., Mujtaba, S., Gil, J., Walsh, M.J., and Zhou, M.M. (2010). Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell *38*, 662-674.

Ye, X., Zerlanko, B., Zhang, R., Somaiah, N., Lipinski, M., Salomoni, P., and Adams, P.D. (2007). Definition of pRB- and p53-dependent and -independent steps in HIRA/ASF1a-mediated formation of senescence-associated heterochromatin foci. Molecular and cellular biology *27*, 2452-2465.

Yekta, S., Shih, I.H., and Bartel, D.P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. Science *304*, 594-596.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev *17*, 3011-3016.

Yoon, I.K., Kim, H.K., Kim, Y.K., Song, I.-H., Kim, W., Kim, S., Baek, S.-H., Kim, J.H., and Kim, J.-R. (2004). Exploration of replicative senescence-associated genes in human dermal fibroblasts by cDNA microarray technology. Experimental gerontology *39*, 1369-1378.

Young, A.P., Schlisio, S., Minamishima, Y.A., Zhang, Q., Li, L., Grisanzio, C., Signoretti, S., and Kaelin, W.G. (2008). VHL loss actuates a HIF-independent senescence programme mediated by Rb and p400. Nature cell biology *10*, 361-369.

Young, A.R.J., Narita, M., Ferreira, M., Kirschner, K., Sadaie, M., Darot, J.F.J., Tavare, S., Arakawa, S., Shimizu, S., Watt, F.M.*, et al.* (2009). Autophagy mediates the mitotic senescence transition. Genes and Development *23*, 798-803.

Zanella, F., Lorens, J.B., and Link, W. (2010). High content screening: seeing is believing. Trends in biotechnology *28*, 237-245.

Zender, L., Xue, W., Zuber, J., Semighini, C.P., Krasnitz, A., Ma, B., Zender, P., Kubicka, S., Luk, J.M., Schirmacher, P.*, et al.* (2008). An Oncogenomics-Based In Vivo RNAi Screen Identifies Tumor Suppressors in Liver Cancer. Cell *135*, 852-864.

Zeng, L., Yap, K.L., Ivanov, A.V., Wang, X., Mujtaba, S., Plotnikova, O., Rauscher, F.J., 3rd, and Zhou, M.M. (2008). Structural insights into human

KAP1 PHD finger-bromodomain and its role in gene silencing. Nat Struct Mol Biol *15*, 626-633.

Zeng, Y., Yi, R., and Cullen, B.R. (2005). Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha. EMBO J *24*, 138-148.

Zhan, Q. (2005). Gadd45a, a p53- and BRCA1-regulated stress protein, in cellular response to DNA damage. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis *569*, 133-143.

Zhan, Q., Antinore, M.J., Wang, X.W., Carrier, F., Smith, M.L., Harris, C.C., and Fornace, a.J. (1999). Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene *18*, 2892-2900.

Zhang, F., Tian, Q., and Wang, Y. (2013). Far upstream element-binding protein 1 (FUBP1) is overexpressed in human gastric cancer tissue compared to non-cancerous tissue. Onkologie *36*, 650-655.

Zhang, H., Kolb, F.A., Brondani, V., Billy, E., and Filipowicz, W. (2002). Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. EMBO J *21*, 5875-5885.

Zhang, H., Pan, K.-H., and Cohen, S.N. (2003). Senescence-specific gene expression fingerprints reveal cell-type-dependent physical clustering of up-regulated chromosomal loci. Proceedings of the National Academy of Sciences of the United States of America *100*, 3251-3256.

Zhang, J., and Chen, Q.M. (2013). Far upstream element binding protein 1: a commander of transcription, translation and beyond. Oncogene *32*, 2907-2916.

Zhang, P., Furukawa, K., Opresko, P.L., Xu, X., Bohr, V.a., and Mattson, M.P. (2006). TRF2 dysfunction elicits DNA damage responses associated with senescence in proliferating neural cells and differentiation of neurons. Journal of Neurochemistry *97*, 567-581.

Zhang, Q., Zhong, Q., Evans, a.G., Levy, D., and Zhong, S. (2011a). Phosphorylation of histone H3 serine 28 modulates RNA polymerase III-dependent transcription. Oncogene *30*, 3943-3952.

Zhang, R., Chen, W., and Adams, P.D. (2007). Molecular dissection of formation of senescence-associated heterochromatin foci. Molecular and cellular biology *27*, 2343-2358.

Zhang, R., Poustovoitov, M.V., Ye, X., Santos, H.A., Chen, W., Daganzo, S.M., Erzberger, J.P., Serebriiskii, I.G., Canutescu, A.A., Dunbrack, R.L.*, et al.* (2005). Formation of macroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Developmental Cell *8*, 19-30.

Zhang, S., Roche, K., Nasheuer, H.P., and Lowndes, N.F. (2011b). Modification of histones by sugar beta-N-acetylglucosamine (GlcNAc) occurs on multiple residues, including histone H3 serine 10, and is cell cycle-regulated. J Biol Chem *286*, 37483-37495.

Zhang, X.-Y., Varthi, M., Sykes, S.M., Phillips, C., Warzecha, C., Zhu, W., Wyce, A., Thorne, A.W., Berger, S.L., and McMahon, S.B. (2008). The

putative cancer stem cell marker USP22 is a subunit of the human SAGA complex required for activated transcription and cell-cycle progression. Molecular cell *29*, 102-111.

Zhang, Y., Xiong, Y., and Yarbrough, W.G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell *92*, 725-734.

Zhao, Y., Lang, G., Ito, S., Bonnet, J., Metzger, E., Sawatsubashi, S., Suzuki, E., Le Guezennec, X., Stunnenberg, H.G., Krasnov, A.*, et al.* (2008). A TFTC/STAGA module mediates histone H2A and H2B deubiquitination, coactivates nuclear receptors, and counteracts heterochromatin silencing. Molecular cell *29*, 92-101.

Zhao, Y., Lang, G., Ito, S., Bonnet, J., Metzger, E., Sawatsubashi, S., Suzuki, E., Le Guezennec, X., Stunnenberg, H.G., Krasnov, A.*, et al.* (2008). A TFTC/STAGA module mediates histone H2A and H2B deubiquitination, coactivates nuclear receptors, and counteracts heterochromatin silencing. Molecular cell *29*, 92-101.

Zhou, J., Ma, J., Zhang, B.C., Li, X.L., Shen, S.R., Zhu, S.G., Xiong, W., Liu, H.Y., Huang, H., Zhou, M.*, et al.* (2004). BRD7, a novel bromodomain gene, inhibits G1-S progression by transcriptionally regulating some important molecules involved in ras/MEK/ERK and Rb/E2F pathways. Journal of Cellular Physiology *200*, 89-98.

Zhu, J., Woods, D., McMahon, M., and Bishop, J.M. (1998). Senescence of human fibroblasts induced by oncogenic Raf Senescence of human fibroblasts induced by oncogenic Raf. Genes & Development, 2997-3007.

Zindy, F., Quelle, D.E., Roussel, M.F., and Sherr, C.J. (1997). Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. Oncogene *15*, 203-211.

Ziv, Y., Bielopolski, D., Galanty, Y., Lukas, C., Taya, Y., Schultz, D.C., Lukas, J., Bekker-Jensen, S., Bartek, J., and Shiloh, Y. (2006). Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. Nature cell biology *8*, 870-876.

Appendix

Table A1. Antibodies

Target	Clone	Company	Cat. No.	Source	Application (dilution)
Anti-Mouse IgG	Alexa Fluor® 488	Invitrogen	A11029	Goat	IF
Anti-Mouse IgG	Alexa Fluor® 594	Invitrogen	A11032	Goat	IF
Anti-Rabbit IgG	Alexa Fluor® 594	Invitrogen	A1103	Goat	IF
Anti-Goat IgG	Alexa Fluor® 594	Invitrogen	A11058	Donkey	IF
Anti Rat IgG	Alexa Fluor® 488	Invitrogen	A11006	Goat	IF
Anti Rat IgG	Alexa Fluor® 594	Invitrogen	A11007	Goat	IF
BrdU	BU1/75(ICR1)	Abcam	ab6326	Rat	IF (1:1000)
CXCL8/IL8	Polyclonal	R&D Systems, Inc	AF-208-NA	Goat	IF (1:100)
Histone H3	mAb10799	Abcam	mAb10799	Mouse	WB (1:1000)
OGT	Polyclonal	Abcam	ab50270	Rabbit	IF (1:100) WB (1:1000)
O-GlcNAc	RL2	Abcam	ab2739	Mouse	IF (1:100)
p16	JC8	Santa Cruz Biotechnology,Inc	sc56330	Mouse	IF (1:500)
p21	CP74	Sigma Aldrich	p1484	Mouse	WB (1:100)
p53	DO-1	Santa Cruz Biotechnology,In	sc-126	Mouse	IF (1:100)
PABP2	EP3001Y	GeneTex	GTX62070	Rabbit	WB (1:10000)
P-TRIM28 Ser824)	Polyclonal	Bethyl Laboratories	A300-767A	Rabbit	IF (1:1000)
TRIM28	Polyconal	Abcam	ab10484	Rabbit	IF (1:1000) WB (1:1000)
α-Tubulin	B-5-1-2	Sigma Aldrich	T6074	Mouse	WB (1:10000)
53BP1	Polyclonal	Novus Biologicals	NB100-304	Rabbit	IF (1:1000)

Table A2. Constructs

Vector	Insert	Antibiotic resistance
pGIPZ	Empty vector/shRNA	Puromycin
LXSN	Empty vector	Neomycin
pLNC	Empty vector	Neomycin
pLNC	ER:RAS	Neomycin
pLNC	MEK:ER	Neomycin
pBABE	Cherry	Puromycin

Table A3. shRNA target sequences

Construct (pGIPZ)	Target sequence
shENY2_1	TACTGAAAACTGTATACCA
shENY2_2	TAATCGCTGCTCTCATCTG
shENY2_3	TTAATTACCTCTTTACAGT
shGTF3C4_1	TGAACTGAAGATTCCAGGA
shGTF3C4_2	AACCTCTTAATCCAGTCGG
shOGT_1	AAATTGATATAAGCATCCA
shOGT_2	ATTCTTCTCTAACTGGTCA
shp53	TCTCTTCCTCTGTGCGCCG
shTAF12_1	TCTCCAAATACATTGCTGT
shTAF10_1	TAGAGTGTACTTGCGGTCC
shTRIM28_1	AAGGTTGTAGTCCTCAGTG
shTRIM28_2	TTCACACCTGACACATGGG

Table A4. RT-qPCR Primers

Target gene	Sequence
CDKN1a	5'-CCTGTCACTGTCTTGTACCCT-3' 5'-GCGTTTGGAGTGGTAGAAATCT-3'
CCL20	5'-GGCGAATCAGAAGCAGCAAGCAAC-3 5'-ATTGGCCAGCTGCCGTGTGAA-3'
CXCL1	5'-GAAAGCTTGCCTCAATCCTG-3' 5'-CACCAGTGAGCTTCCTCCTC-3'
ENY2	5'-CAAGGGTCATTTCGTCGCTG-3' 5'- AATCGCTGCTCTCATCTGCG-3'
GTF3C4	5'- CCCGCGGACTCTGTAACTTT-3' 5'- GGAGCCAAATGTGGCCATTG-3'
IL1β	5'-TGCACGCTCCGGGACTCACA-3' 5'-CATGGAGAACACCACTTGTTGCTCC-3'
IL6	5'-CATGGAGAACACCACTTGTTGCTCC-3' 5'-CCCAGGGAGAAGGCAACTG-3',
IL8	5'-GAGTGGACCACACTGCGCCA-3' 5'-TCCACAACCCTCTGCACCCAGT-3'
INK4a	5'-CGGTCGGAGGCCGATCCAG-3' 5'-GCGCCGTGGAGCAGCAGCAGCT-3'
OGT	5'- TTTCCTTCCAAGGGTTAGCTG-3' 5'- CCAGAGTGCTAAAGTGAGCAGA-3'
RPS14	5'-CTGCGAGTGCTGTCAGAGG-3 5'-TCACCGCCCTACACATCAAACT-3'
TAF10	5'- GCCTCAGACCCACGCATAAT-3' 5'- GTACTTGCGGTCCTTGCTCT-3'
TAF12	5'-GAGCAGGATTATGAGGACCCG-3' 5'- CTGAGGGGCCAAACTGGTTC-3'
TRIM28	5'-AGCGGAAATGTGAGCGTGTA-3' 5'-CACGTCTGCCTTGTCCTCAG-3'

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TRIM28/KAP1 regulates senescence

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ABSTRACT

Senescence is a highly stable cell cycle arrest which limits the replication of cells with damaged genomes. The senescence program is activated during aging or in response to insults like DNA damage or oncogenic signaling. Upon induction of senescence, cells undergo profound changes on their transcription program, chromatin organization, and they secrete a complex mixture of mainly pro-inflammatory components termed the senescence-associated secretory phenotype (SASP). The SASP mediates multiple effects, including reinforcing senescence and activating immune surveillance responses. Given the important role that senescence has in aging, cancer and other pathologies, identifying mechanisms regulating senescence has therapeutic potential. Here we describe a role for TRIM28 (also known as KRAB-associated protein 1, KAP1) on mediating oncogene-induced senescence (OIS). TRIM28 accumulates during OIS becoming phosphorylated on serine 824. To investigate the role of TRIM28, we knocked down its expression and observed that the depletion of TRIM28 partially prevented cell arrest during OIS. While induction of p53 and p21 during OIS, was not affected by TRIM28 depletion, $p16^{INK4a}$ induction was partially prevented. Finally, we observed that the induction of IL8, IL6 and other SASP components were strongly suppressed upon TRIM28 depletion. In conclusion, the above-described results show that TRIM28 regulates senescence and affects the induction of the senescence-associated secretory phenotype.

1. Introduction

Senescence is a highly stable cell cycle arrest triggered in old cells and in response to a range of insults including oncogenic signaling, DNA damage, irradiation or exposure to chemotherapeutic agents [1]. In addition to the stable arrest, senescent cells undergo profound phenotypic changes in their transcriptional program, chromatin organization, metabolism and cell morphology [2]. Senescent cells also produce a complex mix of secreted factors, known as the senescence-associated secretory phenotype (SASP) or the senescence-messaging secretome (SMS) [3,4]. The SASP influences the tumor microenvironment and the homeostasis of aged tissues [3,4].

Epigenetic control and chromatin remodeling are two critical layers on senescence regulation. During senescence, cells reorganize their chromatin establishing new heterochromatin domains termed senescence-associated heterochromatin foci (SAHF) [5]. SAHFs comprise redistributed non-overlapping chromatin regions with histone H3K9me3 and H3K27me3 marks [6]. Interestingly, heterochomatin has been suggested to restrain the DNA damage response (DDR) observed during senescence [7]. In addition to the global chromatin reorganization on senescence, several epigenetic mechanisms also contribute to regulate senescence. For example, the *INK4/ARF* locus, that encodes for the critical effectors of senescence $p16^{INK4a}$ and ARF is strongly repressed by Polycomb repressive complexes, and regulated by other epigenetic modifiers including histone demethylases or chromatin remodeling complexes [8].

TRIM28 (also known as KAP1 or TIF1β) belongs to the Tripartite Motif (TRIM) family of E3 ligases characterized by the presence of a RING-finger motif, zinc-binding motifs and a coiled–coil region. [9]. TRIM28 functions mainly as a co-repressor for the KRAB-ZFPs transcriptional regulators by interacting with HP1 proteins and chromatin repressive complexes such as SETDB1, NurD and HDACs leading to the formation of heterochromatin [10–12].

The activity of TRIM28 is regulated post-translationally via phosphorylation on serine (Ser 473 and 824) and tyrosine (Tyr 499, 458 and 517) residues. The most widely studied TRIM28 modification is its phosphorylation on serine 824 by ATM upon DNA damage [13]. Phosphorylation of TRIM28 leads to the release of HP1 from the chromatin and therefore the relaxation of the DNA, allowing the assembly of the repair machinery onto the double-strand breaks

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sites [14]. TRIM28 can also be modified by sumoylation [15,16], what facilitates its interaction with SETDB1 contributing to gene repression [17].

TRIM28 regulates multiple cellular processes, including pluripotency, proliferation, differentiation and apoptosis [17]. The role of TRIM28 in tumorigenesis is complex. TRIM28 can behave as an oncogene and it is frequently overexpressed in tumors [9]. Indeed TRIM28 is a negative regulator of p53 and $p21^{CIP1}$ expression [13,18–20]. On the other hand, the knock down of TRIM28 in breast and lung cancer cell lines results in increased proliferation. As higher expression of TRIM28 in early-stage lung tumors also increases overall survival of patients, TRIM28 has been categorized as a tumor suppressor in some genetic contexts [21]. Potentially contributing to this role as a tumor suppressor, a recent study suggested that the knock down of TRIM28 abrogated senescence in a mouse model of progeria [22].

Here, we aim to clarify the role of TRIM28 in senescence by using a defined, inducible system to study oncogene-induced senescence (OIS). In our experiments, knock down of TRIM28 expression partially prevented senescence, suggesting that TRIM28 could be a mediator of OIS. Intriguingly TRIM28 expression resulted in impaired $p16^{INK4a}$ induction without affecting p53 or $p21^{CIP1}$ expression. TRIM28 depletion also resulted in defective SASP induction. Overall, the results presented here show a role for TRIM28 in mediating senescence and suggest a mechanistic explanation to its functions in tumor suppression and during aging.

2. Materials and methods

2.1. Cell culture and retroviral and lentiviral infection

HEK-293T and IMR-90 cells were obtained from the ATCC. Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 1% antibiotic-antimycotic solution (Invitrogen). Cell number and viability measurements were determined using the Guava Viacount reagent (Millipore) and the Guava Cytometer (Millipore). Retroviral and lentiviral infections were performed as described [23].

2.2. Plasmids

pLNC-ER:RAS and pLXSN and pGIPZ-based shRNA vector targeting p53 have been described previously [24]. Lentiviral pGIPZ-based shRNA vectors targeting TRIM28 (shT.1, AAGGTTGTAGTCCTCAGTG and shT.2, TTCACACCTGACACATGGG) were from SIGMA.

2.3. Antibodies

The following antibodies were used for the immunofluorescence and blotting analysis shown in this manuscript: rat Anti-BrdU (ab6326, Abcam), mouse anti-p16 (sc56330, Santa Cruz Biotechnology, Inc), mouse anti-p53 (sc-126, Santa Cruz Biotechnology, Inc), mouse anti-$p21^{WAF1/Cip1}$ (p1484, Sigma Aldrich), goat anti-CXCL8/IL8 (AF-208-NA, R&D Systems, Inc), rabbit anti-53BP1 (NB100-304, Novus Biologicals), rabbit anti-TRIM28 (ab10484, Abcam), rabbit anti-phospho-TRIM28 (S824) (A300-767A, Bethyl Laboratories, Inc).

2.4. BrdU incorporation, senescence-associated β-galactosidase and crystal violet staining

These assays were performed as previously described [23,24].

2.5. Gene expression analysis

Total RNA was extracted using Trizol reagent (Invitrogen) and the RNeasy isolation kit (Qiagen). DNAs were generated using SuperScript II reverse transcriptase (Invitrogen), dNTPs and Random Hexamers. PCR reactions were performed in a Real-Time PCR Detection System (BioRad) using Power SYBR Green Master Mix (Applied Biosystems). Expression was normalized to ribosomal protein S14 (*RPS14*) expression. The following primer sets were used: CCL20 5′-GGCGAATCAGAAGCAGCAAGCAAC-3′ and 5′-ATTGGCCAGCTGCCGTGTGAA-3′, CXCL1 5′-GAAAGCTTGCCTCAATCCTG-3′ and 5′-CACCAGTGAGCTTCCTCCTC-3′, IL1β 5′-TGCACGCTCCGGGACTCACA-3′ and 5′-CATGGAGAACACCACTTGTTGCTCC-3′, IL6 5′-CATGGAGAACACCACTTGTTGCTCC-3′ and 5′-CCCAGGGAGAAGGCAACTG-3′, IL8 5′-GAGTGGACCACACTGCGCCA-3′ and 5′-TCCACAACCCTCTGCACCCAGT-3′, INK4a 5′-CGGTCGGAGGCCGATCCAG-3′ and 5′-GCGCCGTGGAGCAGCAGCAGCT-3′, CDKN1a 5′-CCTGTCACTGTCTTGTACCCT-3′ and 5′-GCGTTTGGAGTGGTAGAAATCT-3′, RPS14 5′-CTGCGAGTGCTGTCAGAGG-3′ and 5′-TCACCGCCCTACACATCAAACT-3′, TRIM28 5′-AGCGGAAATGTGAGCGTGTA-3′ and 5′-CACGTCTGCCTTGTCCTCAG-3′.

2.6. Immunofluorescence and high content analysis

Immunofluorescence (IF) was performed as previously described [25] using the antibodies listed above. Images were acquired using an automated high throughput microscope (IN Cell Analyzer 2000, GE Healthcare). A minimum of 1000 cells were acquired for each sample per duplicate. High content analysis (HCA) was performed using the IN Cell Investigator software (v 3.2; GE Healthcare), as described elsewhere [24,25]. Briefly, DAPI staining of the nuclei was used to identify cells. The nuclei were segmented using top-hat segmentation, specifying a minimum nucleus area of 100 μm^2. To define the cell area, a collar segmentation approach was used with a border of 1 μm around DAPI staining or alternatively, multiscale top-hat was used to detect cytoplasmic intensity for a given staining. Each cell was assigned a nuclear intensity value (and cell intensity value when applicable) for the specific protein being studied. A histogram of the intensity values of all cells was produced and used to set a threshold filter to determine positive and negative expressing cells. Alternatively, intensity values were plotted. The antibodies used were validated with robust controls (including shRNAs inhibition) to assess their specificity as shown before [24].

2.7. Western blot analysis

Protein extracts were processed and Western blot performed as previously described [24], using the antibodies described above.

3. Results

3.1. TRIM28 is phosphorylated on Serine 824 during oncogene-induced senescence

To investigate the role of TRIM28 during OIS, we took advantage of IMR90 ER:RAS cells. IMR90 ER:RAS are human primary fibroblasts expressing a chimeric fusion protein between the ligand binding motif of the estrogen receptor and oncogenic RAS G12V [24,26]. The ER:RAS fusion can be activated by 4-hydroxytamoxifen (4OHT). Once RAS is activated a full senescence response is triggered, making it an ideal system to study OIS. After 4OHT treatment, IMR90 ER:RAS cells slowed down their proliferation, as assessed by BrdU incorporation (Fig. 1A). In addition, the induction of ER:RAS results in signs of senescence, such as an increase in the percentage of cells positive for senescence-associated β-galactosidase

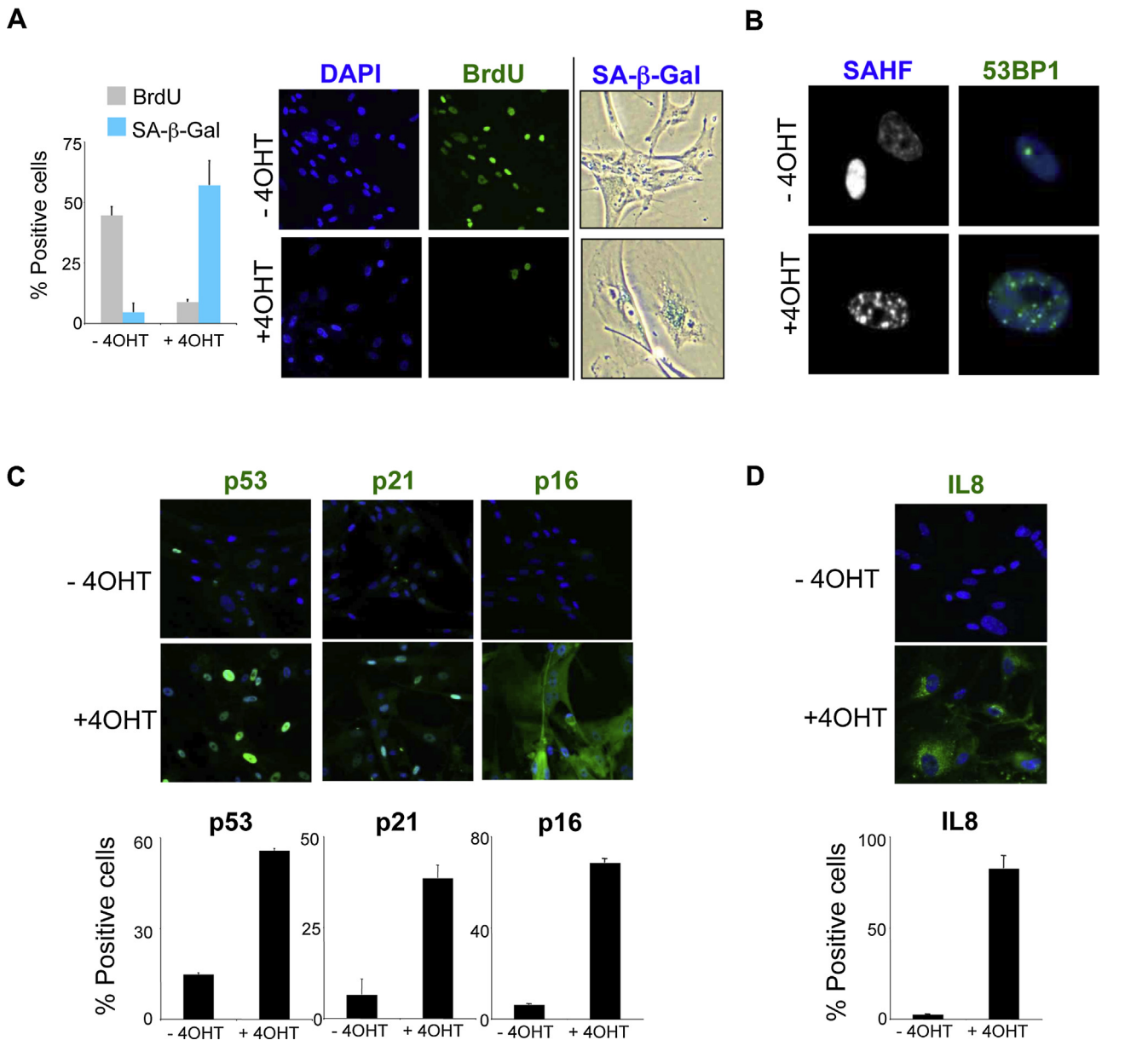

Fig. 1. IMR90 ER:RAS as a cell system to study oncogene-induced senescence. IMR90 ER: RAS cells were treated with 200 nM 4OHT to activate ER:RAS. Cells were fixed at 6 or 8 days post induction for further analysis. **(A)** BrdU incorporation and SA-β-Gal assays shows growth arrest and increase in SA-β-Gal positive cells after induction of OIS. Quantification (left) and representative pictures, right are shown). **(B)** Accumulation of cells with senescence-associated heterochromatin foci (SAHF, left) and DNA damage (as evaluated by 53BP1 IF staining, right) is observed upon induction of OIS. **(C)** Cells undergoing OIS upon ER:RAS activation induce the expression of p53, p21 and p16. Immunofluorescence was performed using the appropriate antibodies. Representative pictures (top) and quantification of the percentage of positive cells (bottom) is shown. **(D)** OIS results in the induction of the SASP component IL8. Immunofluorescence was performed in IMR90 ER:RAS cells 8 days after 4OHT treatment. Representative pictures (top) and quantification of cells positive for IL8 (bottom) are shown.

(SA-β-Gal, Fig. 1A) and cells showing senescence-associated heterochromatin foci (SAHFs) or presenting a DNA damage response (as exemplified by 53BP1 staining) (Fig. 1B). The senescence program is implemented by a parallel and coordinated activation of the p53/p21 and $p16^{INK4a}$/Rb tumor suppressor networks [1]. Consistent with this, we observed in IMR90 ER:RAS cells an induction of p53, p21 and p16 tumor suppressors levels upon 4OHT treatment, as assessed using quantitative immunofluorescence (Fig. 1C). Finally, one of the most striking characteristics of senescence cells, with profound functional implications is the expression of a complex mix of secreted factors known as the SASP [3,4]. We have previously used IMR90 ER:RAS cells to interrogate the SASP function and dissect its composition [24]. We assessed the expression of IL8, one of the prototypic SASP components 8 days after 4OHT induction, and observed that RAS activation strongly induced IL8 during OIS (Fig. 1D).

As an initial experiment to assess the role of TRIM28 during senescence, we analyzed the overall TRIM28 levels and its phosphorylation on Serine 824 upon senescence induced by RAS activation (Fig. 2). We observed that upon RAS activation, the levels of TRIM28 increased slightly (Fig. 2A). Interestingly, the most striking observation was that RAS activation induced the phosphorylation of TRIM28 at serine 824 (Fig. 2B). It has been shown that TRIM28 gets phosphorylated upon DNA damage by either ATM/CHK2 or ATR/CHK1 complexes. Since the activation of a persistent DNA damage response (DDR) is one of the hallmarks of senescence ([27]

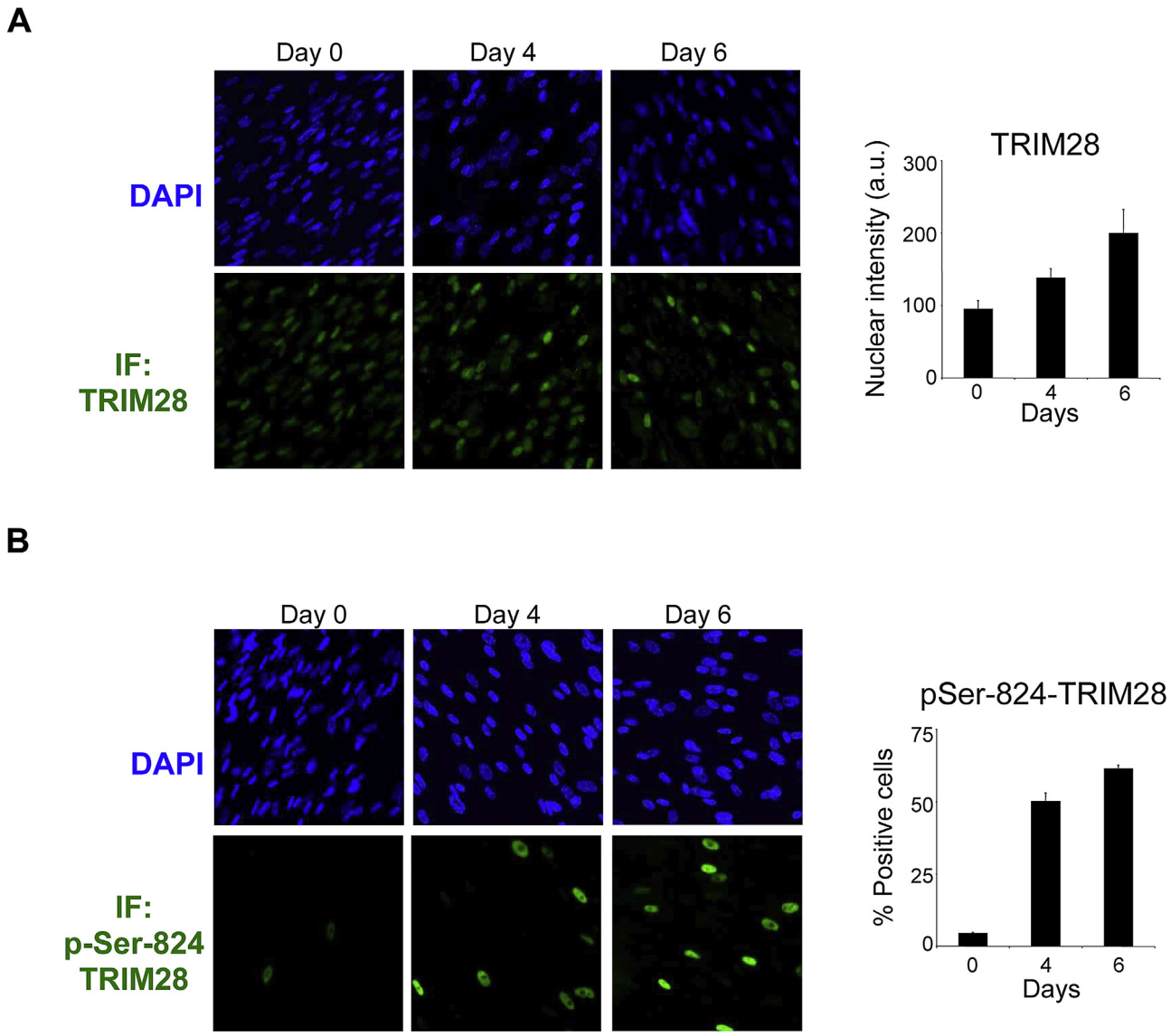

Fig. 2. TRIM28 is phosphorylated on serine 824 during OIS. IMR90 ER:RAS cells were treated with 200 nM 4OHT to induce RAS. Cells were fixed at the indicated times and immunofluorescence performed using antibodies recognizing total TRIM28 **(A)** or TRIM28 phosphorylated on serine 824 **(B)**. Representative images (left) and quantification of the immunofluorescence as described in materials and methods (right) are shown. For total TRIM28 (A), nuclear intensity was plotted, while for phospho-TRIM28 (B), the percentage of the positive cells was plotted.

and Fig. 1B), it is logic to expect the phosphorylation of TRIM28. This result presented the question of whether TRIM28 has a role on senescence and what that role might be.

3.2. Depletion of TRIM28 delays senescence

To investigate the role of TRIM28 in OIS, we tested multiple shRNAs targeting TRIM28. From the tested shRNAs we chose shTRIM28.1 and shTRIM28.2 as they were the most efficient. Indeed, when we infected IMR90 cells and analyzed TRIM28 expression by immunofluorescence and western blot, observing that both resulted in a significant knockdown of TRIM28 levels (Fig. 3A and B).

Next, we analyzed what effect knocking down TRIM28 had on OIS. IMR90 ER:RAS cells were infected with the viruses expressing shRNAs against TRIM28 and the effects on cell arrest were first evaluated by culturing cells at low density and staining with crystal violet. We observed that TRIM28 depletion resulted in increased cell growth upon OIS induction, similar to what was observed upon p53 knockdown (Fig. 3B). A higher percentage of cells with depleted TRIM28 levels incorporated BrdU 6 days upon 4OHT induction, suggesting that depletion of TRIM28 partially prevented the effects of OIS (Fig. 3C). In addition, TRIM28 knockdown also resulted in less cells presenting features characteristic of senescence such as senescence-associated heterochromatic foci (SAHF, Fig. 3D). Overall, knocking down TRIM28 partially prevents the effects of OIS, implying that TRIM28 might have a role in mediating OIS.

3.3. Depletion of TRIM28 affects the induction of $p16^{INK4a}$ during senescence

To understand how the depletion of TRIM28 could affect senescence, we examined the effect that TRIM28 knockdown has over the induction of the key tumor suppressors involved in the implementation of senescence, p53, $p21^{CIP}$ and $p16^{INK4a}$. To this end, IMR90 ER:RAS cells were infected with shRNAs targeting TRIM28 and OIS induced by treating with 200 nM 4OHT. While the induction of p53 and $p21^{CIP}$ during OIS was unaffected, $p16^{INK4a}$ levels were lower

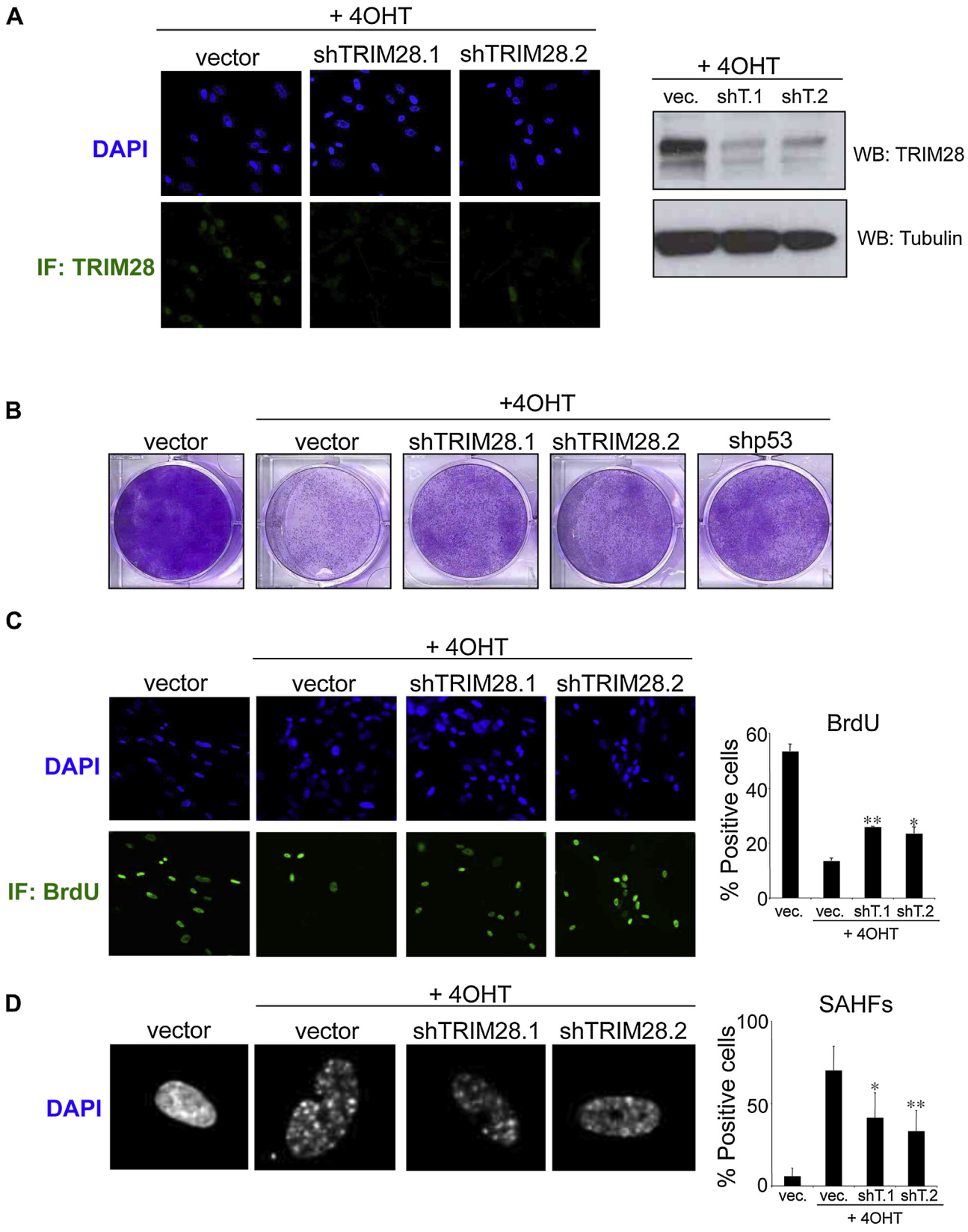

Fig. 3. Depletion of TRIM28 delays senescence. **(A)** IMR90 cells were infected with lentiviruses expressing shRNAs targeting TRIM28 (shTRIM28.1 and shTRIM28.2). Knock-down efficiency was assessed by immunofluorescence (left) and Western blot analysis (right). **(B)–(D)** Depletion of TRIM28 partially prevents OIS. IMR90 ER:RAS cells were infected with control vectors or shRNAs targeting TRIM28 or p53 as indicated. The effect on cell proliferation was assessed by crystal violet staining **(B)** and BrdU incorporation **(C)**. **(D)** IMR90 ER:RAS cells infected with the indicated vectors were stained with DAPI. At least 100 cells on each condition were counted. Representative pictures (left) and quantification of cells with senescence-associated heterochromatin foci (right) are shown. For (C) and (D) the significance of the difference between vector +4OHT and shTIM28.1 o shTRIM28.2 was assessed used a non-parametric test (Mann Whitney). $^{*}p < 0.05$; $^{**}p < 0.01$.

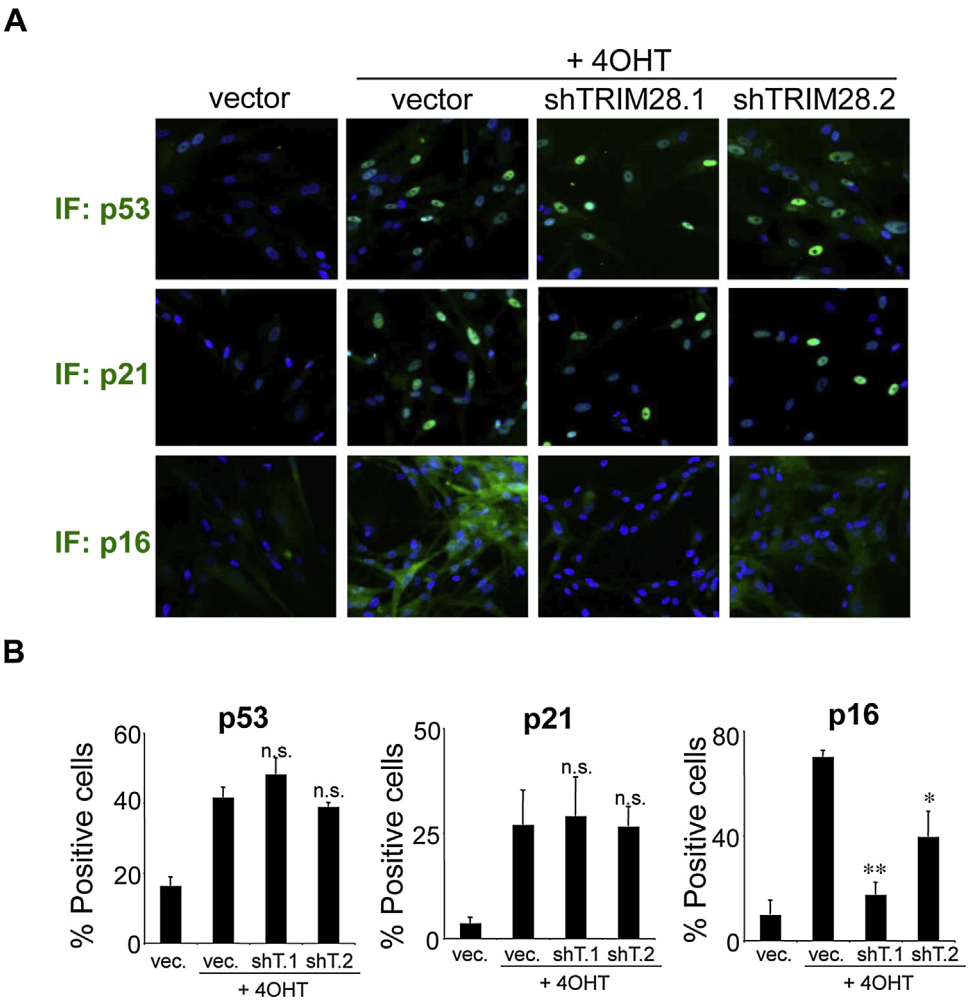

Fig. 4. Depletion of TRIM28 prevents $p16^{INK4a}$ expression during OIS. IMR90 ER:RAS were infected with the indicated vectors. RAS expression was induced using 200 nM 4OHT and 6 days after induction cells were prepared for analysis. Immunofluorescence analysis of the expression of p53, p21 and p16 during OIS. Representative pictures **(A)** and quantification **(B)** is shown. The significance of the difference between vector +4OHT and shTIM28.1 o shTRIM28.2 was assessed used a non-parametric test (Mann Whitney). n.s. $p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$.

upon knock down of TRIM28 (Fig. 4A and B). These results suggested that TRIM28 could regulate, directly or indirectly, $p16^{INK4a}$ expression to control senescence.

3.4. Depletion of TRIM28 partially prevents the induction of the SASP

Senescent cells secrete a plethora of mainly pro-inflammatory factors often referred as the senescence-associated secretory phenotype (SASP) [28]. Through the SASP senescent cells are able to influence their surrounding microenvironment, and therefore the SASP is an important contributor that defines the overall outcome of senescence. For example, the SASP enables senescent cells to influence, activate and recruit both the innate and adaptive immunities [29,30].

As we have shown that TRIM28 influences senescence, we decided to investigate how its depletion affects SASP production during OIS. To this end we infected IMR90 ER:RAS cells with shRNAs targeting TRIM28, and first analyzed the expression of the prototypic SASP component IL8, 8 days upon RAS activation with 4OHT. Consistent with our previous observations (Fig. 1D and [24]), IL8 was induced during OIS. Interestingly, depletion of TRIM28 strongly suppressed IL8 induction during OIS as assessed by IF (Fig. 5A and B).

To extend this observation, we analyzed the expression of IL8 and additional SASP components by qRT-PCR in IMR90 ER:RAS cells in which TRIM28 expression had been depleted using shRNAS. In addition to IL8, we analyzed the expression of IL1b, CCL20, IL6 and CXCL1, factors that we and others have previously shown to be part of the SASP and play an important role in SASP functions [24,28,31,32]. We observed that TRIM28 expression partially suppressed the induction of these factors during OIS (Fig. 5C-G). Overall, these results suggest that depletion of TRIM28 depletion prevent the induction of the SASP during OIS.

4. Discussion

Senescence is associated with aging and an increasing list of pathologies such as cancer and fibrosis. Pioneering work has shown that triggering senescence or ablating senescent cells are two potential strategies that could be exploited for treating cancer and age-associated pathologies [33,34]. Therefore there is mounting interest in understanding the molecular pathways that control senescence, with the eventual aim of identifying liabilities that could be targeted on senescent cells.

Epigenetic factors are a particularly attractive target, as senescent cells undergo profound chromatin reorganization [1,35] and epigenetic modifications have the potential to be reverted. TRIM28

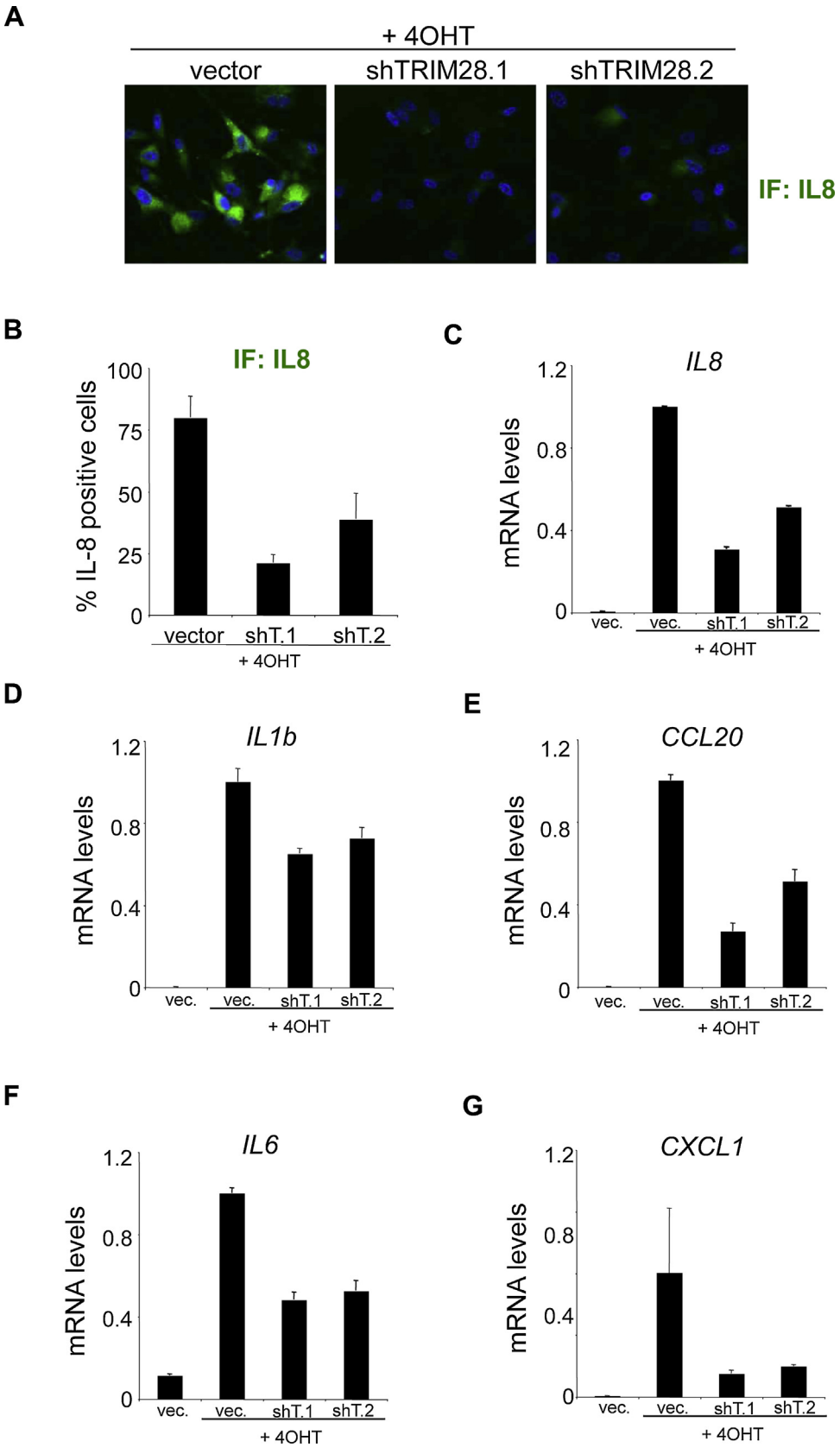

Fig. 5. Depletion of TRIM28 prevents SASP induction during OIS. **(A) and (B) TR**IM28 knockdown prevents IL8 induction as observed by IF. IMR90 ER:RAS cells were infected with the indicated vectors. RAS:ER was induced using 200 nM 4OHT and 8 days after induction cells were analyzed by immunofluorescence with a specific antibody against IL8. Quantification (A) and representative images (B) are shown. **(C)–(G)** Depletion of TRIM28 prevents SASP induction during OIS. IMR90 ER:RAS cells were infected with the indicated vectors. RAS:ER was induced using 200 nM 4OHT and 6 days after induction, samples were subjected to qRT-PCR. Expression of IL8 (C), IL1b (D), CCL20 (E), IL6 (F) and CXCL1 (G) is shown.

is a member of a family of around 60 human genes containing a tripartite motif (TRIM) [17]. TRIM28 works as a co-repressor for the KRAB-ZFP transcriptional regulators, and has been involved in regulating multiple processes such as gene repression, cell growth and differentiation, stem cell self-renewal, oncogenic transformation, apoptosis or DNA repair [17]. In addition, a recent study has linked TRIM28 with regulating DNA repair and affecting senescence in a model of progeria [22]. *Zmpste24*$^{-}$/$^{-}$ mice undergo accelerated aging linked to p53 signaling activation [36], and mouse embryonic fibroblasts (MEFs) derived from *Zmpste24*$^{-}$/$^{-}$ mice undergo premature senescence. Interestingly, this senescence response can be rescued by knocking down TRIM28 [22], but how exactly TRIM28 does that and what are the mechanisms or implications remained unexplored. Here, using a model for OIS, we extent those observation and showed that TRIM28 regulates OIS. TRIM28 knock down is able to partially prevent OIS, and depletion of TRIM28 results in a prevention of p16^{INK4a} induction, without affecting to the activation of the p53/p21 pathway.

Whether the effect of TRIM28 on p16^{INK4a} induction is direct, indirect or just a consequence of TRIM28 inhibiting senescence is not clear yet but will be worth investigating. Since the main function of TRIM28 is acting as a transcriptional repressor, a possible explanation could be that TRIM28 represses a negative regulator of p16^{INK4a}. Indeed, the expression of p16^{INK4a}, and by extension the *INK4/ARF* locus, is subjected to strict transcriptional and epigenetic control [37].

Interestingly, we observed that TRIM28 is phosphorylated on serine 824 during OIS. TRIM 28 is phosphorylated on serine 824 in response to the activation of the DNA damage response [13]. Phosphorylation has been shown to interfere with TRIM28 repressive abilities. It is not clear how to reconcile this observation with the effects that TRIM28 has on p16^{INK4a} expression during senescence. As TRIM28 activity is subjected to regulation by multiple phosphorylation and SUMOylation events further work would be needed to understand the precise mechanism(s) by which TRIM28 controls senescence.

TRIM28 connects the DNA damage response with heterochromatin organization. A DNA damage response is one of the key triggers initiating OIS [38] and profound chromatin reorganization, that includes chromatin redistribution in SAHFs also occur during senescence [5]. It has been shown that the chromatin reorganization observed during senescence contributes to limit the DNA damage response [7]. Whether TRIM28 is a key factor coordinating these responses during OIS remains unknown but it is an attractive hypothesis.

TRIM28 depletion also results in suppression of the SASP. Given that depletion of TRIM28 partially prevents senescence, the easiest explanation is that decreased senescence results in decreased SASP. However, it would be interesting to investigate whether TRIM28 directly regulates the SASP. Interestingly, besides its role in controlling chromatin formation, TRIM28 directly binds, and inhibit proteins of the IRF family involved in inflammatory signaling [39,40]. Although it is not clear how that could be reconciled with our observations, the relation between TRIM28 and IRFs opens additional avenues to explain how TRIM28 could control the SASP.

The role of TRIM28 in cancer is controversial, and probably context dependent [9]. Our data would suggest that, by mediating senescence, TRIM28 could have tumor suppressive functions. Overall, here we have described a role for TRIM28 in regulating senescence that joins the list of other cellular processes regulated by TRIM28. Given the unique position of TRIM28 in the coordination of the DNA damage response and heterochromatin formation, understanding fully the role of TRIM28 in senescence will be important to further evaluate the possibility of targeting TRIM28 in disease.

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References

[1] Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. Genes Dev 2010;24:2463–79.
[2] Sadaie M, Salama R, Carroll T, Tomimatsu K, Chandra T, Young AR, et al. Redistribution of the Lamin B1 genomic binding profile affects rearrangement of heterochromatic domains and SAHF formation during senescence. Genes Dev 2013;27:1800–8.
[3] Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol 2010;5:99–118.
[4] Kuilman T, Peeper DS. Senescence-messaging secretome: SMS-ing cellular stress. Nat Rev Cancer 2009;9:81–94.
[5] Narita M, Nunez S, Heard E, Lin AW, Hearn SA, Spector DL, et al. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 2003;113:703–16.
[6] Chandra T, Kirschner K, Thuret JY, Pope BD, Ryba T, Newman S, et al. Independence of repressive histone marks and chromatin compaction during senescent heterochromatic layer formation. Mol Cell 2012;47:203–14.
[7] Di Micco R, Sulli G, Dobreva M, Liontos M, Botrugno OA, Gargiulo G, et al. Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. Nat Cell Biol 2011;13:292–302.
[8] Popov N, Gil J. Epigenetic regulation of the INK4b-ARF-INK4a locus: in sickness and in health. Epigenetics 2010;5:685–90.
[9] Hatakeyama S. TRIM proteins and cancer. Nat Rev Cancer 2011;11:792–804.
[10] Schultz DC, Ayyanathan K, Negorev D, Maul GG, Rauscher 3rd FJ. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. Genes Dev 2002;16:919–32.
[11] Schultz DC, Friedman JR, Rauscher 3rd FJ. Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. Genes Dev 2001;15:428–43.
[12] Nielsen AL, Ortiz JA, You J, Oulad-Abdelghani M, Khechumian R, Gansmuller A, et al. Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. EMBO J 1999;18:6385–95.
[13] Li X, Lee YK, Jeng JC, Yen Y, Schultz DC, Shih HM, et al. Role for KAP1 serine 824 phosphorylation and sumoylation/desumoylation switch in regulating KAP1-mediated transcriptional repression. J Biol Chem 2007;282:36177–89.
[14] Goodarzi AA, Kurka T, Jeggo PA. KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response. Nat Struct Mol Biol 2011;18:831–9.
[15] Zeng L, Yap KL, Ivanov AV, Wang X, Mujtaba S, Plotnikova O, et al. Structural insights into human KAP1 PHD finger-bromodomain and its role in gene silencing. Nat Struct Mol Biol 2008;15:626–33.
[16] Ivanov AV, Peng H, Yurchenko V, Yap KL, Negorev DG, Schultz DC, et al. PHD domain-mediated E3 ligase activity directs intramolecular sumoylation of an adjacent bromodomain required for gene silencing. Mol Cell 2007;28:823–37.
[17] Iyengar S, Farnham PJ. KAP1 protein: an enigmatic master regulator of the genome. J Biol Chem 2011;286:26267–76.
[18] Wang C, Ivanov A, Chen L, Fredericks WJ, Seto E, Rauscher 3rd FJ, et al. MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. EMBO J 2005;24:3279–90.
[19] Okamoto K, Kitabayashi I, Taya Y. KAP1 dictates p53 response induced by chemotherapeutic agents via Mdm2 interaction. Biochem Biophys Res Commun 2006;351:216–22.
[20] Lee YK, Thomas SN, Yang AJ, Ann DK. Doxorubicin down-regulates Kruppel-associated box domain-associated protein 1 sumoylation that relieves its transcription repression on p21WAF1/CIP1 in breast cancer MCF-7 cells. J Biol Chem 2007;282:1595–606.
[21] Chen L, Chen DT, Kurtyka C, Rawal B, Fulp WJ, Haura EB, et al. Tripartite motif containing 28 (Trim28) can regulate cell proliferation by bridging HDAC1/E2F interactions. J Biol Chem 2012;287:40106–18.
[22] Liu B, Wang Z, Ghosh S, Zhou Z. Defective ATM-Kap-1-mediated chromatin remodeling impairs DNA repair and accelerates senescence in progeria mouse model. Aging Cell 2013;12:316–8.
[23] Banito A, Gil J. Induced pluripotent stem cells and senescence: learning the biology to improve the technology. EMBO Rep 2010;11:353–9.
[24] Acosta JC, Banito A, Wuestefeld T, Georgilis A, Janich P, Morton JP, et al. A complex secretory program orchestrated by the inflammasome controls paracrine senescence. Nat Cell Biol 2013;15:978–90.
[25] Banito A, Rashid ST, Acosta JC, Li S, Pereira CF, Geti I, et al. Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev 2009;23:2134–9.
[26] Barradas M, Anderton E, Acosta JC, Li S, Banito A, Rodriguez-Niedenfuhr M, et al. Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS. Genes Dev 2009;23:1177–82.

[27] d'Adda di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer 2008;8:512–22.
[28] Coppe JP, Patil CK, Rodier F, Sun Y, Munoz DP, Goldstein J, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol 2008;6:2853–68.
[29] Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V, et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature 2007;445:656–60.
[30] Kang TW, Yevsa T, Woller N, Hoenicke L, Wuestefeld T, Dauch D, et al. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. Nature 2011.
[31] Acosta JC, O'Loghlen A, Banito A, Guijarro MV, Augert A, Raguz S, et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell 2008;133:1006–18.
[32] Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell 2008;133:1019–31.
[33] Acosta JC, Gil J. Senescence: a new weapon for cancer therapy. Trends Cell Biol 2012;22:211–9.
[34] Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, van de Sluis B, et al. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature 2011;479:232–6.
[35] Salama R, Sadaie M, Hoare M, Narita M. Cellular senescence and its effector programs. Genes Dev 2014;28:99–114.
[36] Varela I, Cadinanos J, Pendas AM, Gutierrez-Fernandez A, Folgueras AR, Sanchez LM, et al. Accelerated ageing in mice deficient in Zmpste24 protease is linked to p53 signalling activation. Nature 2005;437:564–8.
[37] Gil J, Peters G. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol 2006;7:667–77.
[38] Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, et al. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 2006;444:638–42.
[39] Eames HL, Saliba DG, Krausgruber T, Lanfrancotti A, Ryzhakov G, Udalova IA. KAP1/TRIM28: an inhibitor of IRF5 function in inflammatory macrophages. Immunobiology 2012;217:1315–24.
[40] Liang Q, Deng H, Li X, Wu X, Tang Q, Chang TH, et al. Tripartite motif-containing protein 28 is a small ubiquitin-related modifier E3 ligase and negative regulator of IFN regulatory factor 7. J Immunol 2011;187:4754–63.