

NEW ROLES FOR OLD PROTEINS: MDM2 AND ATRX DRIVE THE TRANSITION
FROM QUIESCENCE TO SENESENCE

By

Marta Kovatcheva

A Dissertation

Presented to the Faculty of the Louis V. Gerstner, Jr.
Graduate School of Biomedical Sciences,
Memorial Sloan Kettering Cancer Center
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

New York, NY

December, 2016

Andrew Koff, PhD

Date

Dissertation Mentor

© Marta Kovatcheva 2016

ALL RIGHTS RESERVED

DEDICATION

I would like to dedicate this thesis to my parents, Silvia and George Kovatchev. They instilled in me a passion for having a passion, a spirit of perseverance, and the belief that failure is always an opportunity to open yet another door that may itself lead to success. Without these values, science would be an impossible undertaking; with them, it is a fun one.

ABSTRACT

Senescence is a form of irreversible growth arrest with important implications in development, aging and disease. Notably, it is a barrier to tumor progression and can contribute to the efficacy of cancer therapies. However, senescence remains poorly understood at the molecular level. There is a paucity of reliable, specific markers of senescent cells and the gene products that drive a cell from a readily reversible cell cycle exit – that is, quiescence – into the more permanent form of growth arrest – that is, senescence – remain poorly understood. Here, I used a new class of small molecule drugs that specifically inhibit CDK4 (CDK4i) to investigate therapy induced senescence in transformed cell lines.

I began my studies in well differentiated/de-differentiated liposarcoma (WD/DDLS), a disease characterized by amplification of CDK4 and MDM2. It was known that in a panel of patient-derived cell lines, some cell lines underwent quiescence in response to CDK4i, while others underwent senescence. I found that this was dependent on the post-translational turnover of MDM2 and that reducing MDM2 in quiescent cells could drive them into senescence, regardless of the nature of their response to CDK4i and independent of CDK4i treatment. This indicated that quiescent cells were not impervious to senescence; on the contrary, quiescence could be converted into senescence simply by knocking down MDM2. Surprisingly, this was independent of MDM2's role in regulating p53 but was dependent on an intact E3 ubiquitin ligase domain, suggesting there may be an alternative MDM2 substrate that is important in regulating senescence. In a small pilot study of 7 WD/DDLS patients in a phase II clinical trial at MSKCC using the CDK4i drug Palbociclib, patients in which MDM2 was downregulated following treatment had a more favorable outcome as measured by progression free survival as compared to those patients whose MDM2 levels were unchanged. Collectively, this work

describes a previously unidentified, p53-independent, clinically relevant role for MDM2 in senescence.

In trying to understand how cells undergo senescence in response to CDK4i, I identified that the chromatin remodeling protein ATRX has a role in regulating MDM2 turnover. Moreover, I found that ATRX has a more general role in senescence. ATRX is required for senescence downstream of MDM2 both in CDK4i therapy induced senescence, and in senescence triggered by the DNA damaging agent doxorubicin, which is independent of MDM2. Without ATRX, cells still underwent growth arrest, indicating that ATRX is important for the transition from quiescence to senescence.

I found that ATRX accumulates in nuclear foci specifically in senescent cells but not in quiescent, autophagic or differentiated cells; this accumulation was also observed in untransformed normal human diploid fibroblasts undergoing senescence triggered by a variety of stimuli. Introducing ATRX into the ATRX deficient cell line U2OS could facilitate senescence in response to CDK4i and this was dependent on the interaction of ATRX with the HP1 proteins, H3K9Me3 modified histones and an intact helicase domain. ATRX was required for the formation and maintenance of senescence associated heterochromatic foci (SAHF), which are involved in silencing E2F target genes in senescence.

Chromatin immunoprecipitation followed by sequencing revealed that ATRX bound a number of genes in a senescence-specific manner. Notably, expression of one of these genes – *HRAS* – was suppressed in an ATRX-dependent fashion in senescent cells. Reducing *HRAS* levels in quiescent cells was sufficient to drive them into senescence, identifying at least one manner in which ATRX functions to promote senescence. Thus, I have identified a novel role for ATRX in promoting the transition

from quiescence into senescence that works – at least in some circumstances – through its ability to reduce *HRAS*.

ACKNOWLEDGEMENTS

I would first and foremost like to express my gratitude to my dissertation mentor, Andrew Koff. You have been more inspirational, motivational, supportive and influential than I ever could have imagined. The five years I have spent in your lab feel like the blink of an eye, and yet when I look back at the scientist I was when I first walked into your office door, I barely recognize myself. I am endlessly grateful for all of the hours, meetings, meandering chats, white board sketches, piles of paper with indiscernible scribbles, beers, coffees, conferences, trips, jokes, wisdoms and insights you have shared with me. Through all this, you have not only taught me how to be a good scientist, but to have the confidence in myself to believe that I am actually a good scientist. I cannot express in words how much I cherish my experiences in the Koff Lab and how grateful I am for everything you do to make it the Koff Lab. Thank you.

The members of the Koff Lab have been an evolving and amazing bunch of talented, outgoing, generous and smart individuals I have had the privilege to call my colleagues and my friends. To Ellen Hukkelhoven – thank you for encouraging me to rotate in the Koff lab and for showing me what a stimulating and fun environment this lab can be. To David Liu and Aimee Crago – thank you for starting this project, and for allowing me to get involved back when I was a wide-eyed first year student who was disillusioned with microglia. To Aimee – thank you for all the time and effort you have put into my clinical mentorship and all the opportunities and experiences you have afforded me through it. I am a much more well-rounded and educated scientist because of it. To Nancy Yeh – thank you for your boundless enthusiasm and your perseverance in cloning, without which a lot of this project would have been impossible. To Jossie Yasshinskie – thank you for taking over Nancy's cloning duties, for convincing Andy to digitize ordering, and for keeping me smiling and laughing every day at work. To Mary

Klein – thank you for all the hours we have spent back-to-back in our bay; it has been my daily lab meeting, my sounding board, my psychiatrist's office and my karaoke lounge. I can't imagine graduate school without you. To everyone else through the years – Daniel Ciznadija, Radhika Suresh, Fatima Wilder, Annalee Tai, Ashira Mawji and Hannah Millimet – thank you for all of your help, guidance, laughter and time. To the newcomer, Caroline Gleason – the lab is in good hands ☺.

I would like to thank my thesis committee, John Petrini and Scott Lowe. Thank you for so many hours of your limited time, your insights, advice, reagents, protocols, lab members, contacts, invitations to meetings, career guidance and everything in between. I am so fortunate to have as my personal mentors two scientific greats that most graduate students will only ever dream of chatting with for a few moments at a poster session. Your input has been instrumental in shaping not only my thesis work but also who I am as a scientist. Thank you to Emily Bernstein for agreeing to be my external examiner and also for the ATRX reagents and insights. Thank you to Prasad Jallepalli for agreeing to chair my defense committee.

I would like to thank our fantastic collaborators, without whom much of this work would have been impossible. Thank you to Samuel Singer for your cell lines, patient samples, clinical insight, shared lab meetings and qPCR machine, and to Rachael O'Connor for teaching me how to use it. Thank you to Mark Dickson, William Tap and Gary Schwartz for all of your insights and support. Thank you to Hediye Erdjument-Bromage for your incredibly patient help with mass spectrometry. Thank you to William Liao for your above-and-beyond bioinformatic support and interest in the project. Thank you to Akiko Inagaki and Beth Ashbridge for your help with the ALT assays. Thank you to Richard Gibbons and Hsiao Voon for your patience and detailed help with the ATRX ChIP-seq protocol. Thank you to Amaia Lujambio (and your lab) for all of the joint lab

meetings and insights in our work. Thank you to Daniel Durocher – my undergraduate thesis advisor – not only for the NEDDylator reagents but also for being a source of support and guidance for me even many years after I left your lab, and for the original enthusiasm for science you instilled in me.

I would like to thank the Gerstner Sloan Kettering Graduate School, which is composed of the most dedicated and caring individuals. Thank you to Ken Marians for your work in creating the graduate school and for ensuring – on an individual basis – that its students succeed. Thank you to the office for your boundless support and help with every detail, no matter how small. Maria Torres and Ivan Gerena – we have lost two irreplaceable foundations of the graduate school this year, but I wish you both the utmost success in your new careers. Your contributions to the graduate school and in the lives and happiness of the graduate students will truly be life-long. The hours I personally have spent laughing in the office with you have been a therapeutic escape from work. Thank you to Linda Burnely, Iwona Abramek, Ady Schneider, Katherine Gentile, Alexandria Woodside and David McDonagh for your hard work and dedication to the students. And very importantly, thank you to all of the graduate students who make GSK such a collaborative, stimulating and fun journey. I really would not change my graduate school choices or experiences for anything. A special thank you to Neel Shah for unlimited sonicator use and for all the Bioinformatics for Dummies lessons you taught me; to Jens Hamann for coming up to the 9th floor and changing my media when I was out of lab for too long; to Scott Callahan for all the “sample size” of reagents I wasn’t sure if I wanted to buy yet; and to Hannah Johnsen for one antibody aliquot after another, without asking any questions.

I would like to thank all my friends – both here in NYC and farther away – for every last experience we have shared together. You keep me laughing, eating, traveling and drinking, even when the science fails or my wisdom teeth have been pulled out.

To Anthony Kuey – thank you for patiently bearing every single stressful moment I have lived through during this PhD, even when you were 500 miles away. You are the calmest, most supportive person I have ever met and your unwavering belief in my success has encouraged me to keep pushing more times than you know. Thank you.

To my family, Mom, Dad and Lili – you are the most inspiring, supportive, compassionate, brilliant, loving, hilarious and amazing individuals I have ever met and I will never stop thinking I hit the jackpot just by being born into this family. Thank you for supporting me at every step of my academic life, for reminding me that life goes on (even when your paper gets rejected) and for being way prouder of me than I deserve. Dad – thank you for your *pro bono* computational services, you saved my butt yet again. Maybe one day I will grow up and stop needing you guys so much but – for now – thanks for still being there for me.

Table of Contents

LIST OF TABLES	XV
LIST OF FIGURES.....	XVI
LIST OF ABBREVIATIONS	XXI
1. INTRODUCTION	1
1.1 Consequences of cell cycle exit	1
1.2 Senescence: many roads in, but how many destinations?	3
1.3 Assaying senescence: a collection of associated hallmarks, but no <i>bona fide</i> markers	6
1.4 Physiologic consequences of senescence	10
1.5 Well differentiated/dedifferentiated liposarcoma as a system in which to study senescence	15
1.6 Thesis objectives.....	16
2. MATERIALS AND METHODS.....	19
Cell culture.....	19
Gene expression analysis	19
Gene targeting by shRNA	19
Antibodies	20
Patient Samples	21
Senescence analyses.....	21

Clonogenic growth assays	22
Generation of ATRX mutants	22
Mutational analysis in U2OS cell lines	22
Chromatin immunoprecipitation followed by sequencing (ChIP-seq)	22
RNA sequencing.....	23
RNA-sequencing gene list derivation	24
GO profiling, transcription factor profiling and GSEA.....	24
Real time quantitative qPCR (RT-qPCR)	25
Senescence associated secretory phenotype (SASP) cytokine array	25

3. MDM2 REGULATES THE SENESCENCE RESPONSE TO CDK4

INHIBITION 28

3.1 Introduction: CDK4 inhibition as a targeted cancer therapy 28

3.2 Results..... 32

3.2.1 Reducing MDM2 is sufficient to drive senescence in WD/DDLS 32

3.2.2 MDM2 is reduced in a post-translational manner following CDK4 inhibition 33

3.2.3 Senescence is dependent on functional E3 ligase activity of MDM2 but independent of p53 regulation 37

3.2.4 Amplification of MDM2 is not required for CDK4 inhibitor induced senescence..... 44

3.2.5 MDM2 turnover is correlated with a clinically favorable response to PD0332991 in WD/DDLS patients 48

3.3 Discussion 53

4. ATRX IS A REGULATOR OF THERAPY INDUCED SENESCENCE

REQUIRED FOR FORMATION AND MAINTENANCE OF THE SAHF 58

4.1 Introduction..... 58

4.1.1 ATRX is commonly mutated in soft-tissue sarcoma.....	59
4.2 Results.....	62
4.2.1 ATRX is required for therapy induced senescence.....	62
4.2.2 The number of ATRX foci increases in senescent cells.....	70
4.2.3 The ability of ATRX to support senescence requires HP1 and H3K9me3 binding sites.....	85
4.2.4 ATRX is involved in the establishment and maintenance of SAHF	89
4.2.5 ATRX contributes to E2F target repression in senescence	98
4.3 Discussion	103
5. ATRX DIRECTLY REGULATES <i>HRAS</i> EXPRESSION TO DRIVE GEROCONVERSION IN THERAPY INDUCED SENESCENCE	107
5.1 Introduction: A role for ATRX beyond the SAHF	107
5.2 Results.....	107
5.2.1 ATRX regulates EZH2 targets in CDK4 inhibitor induced senescence.....	107
5.2.2 ATRX directly binds to and represses <i>HRAS</i> in senescent cells	108
5.2.3 <i>HRAS</i> repression drives geroconversion: the transition from quiescence into senescence	119
5.3 Discussion	130
6. DISCUSSION & FUTURE DIRECTIONS.....	133
6.1 Overview.....	133
6.2 Clinical Implications.....	135
6.2.1 A prognostic biomarker for CDK4i.....	136

6.2.2 The MDM2 substrate	137
6.2.3 Combinatorial therapies	138
6.3 ATRX foci as a tool.....	140
6.4 The molecular and temporal evolution of senescence	144
REFERENCES.....	147

LIST OF TABLES

Table 1: List of primers	26
Table 2: Enrichr analysis of GO Biological Processes that are enriched in the differentially expressed genes following PD0332991 treatment	102
Table 3: RNA expression of genes in which ATRX binding was enriched (within the promoter or gene body) in senescent cells	112

LIST OF FIGURES

Figure 1.01 The molecular and temporal evolution of senescence.....	18
Figure 3.01 Transducing WD/DDLS cell lines with shRNA targeting MDM2 reduces MDM2 protein levels.....	34
Figure 3.02 Loss of MDM2 can trigger senescence in WD/DDLS.	35
Figure 3.03 Reducing MDM2 can convert quiescence into senescence.....	36
Figure 3.04 MDM2 transcripts are similarly reduced in all the WD/DDLS cells after treatment with PD0332991 regardless of the outcome of this arrest.	38
Figure 3.05 MDM2 is regulated post-translationally.	39
Figure 3.06 MDM turnover following CDK4 inhibition is proteasome-dependent. ...	40
Figure 3.07 MDM turnover following CDK inhibition is dependent on a functional E3 ligase domain.	41
Figure 3.08 Enforcing MDM2 expression does not prevent growth arrest in WD/DDLS cells treated with PD0332991.....	42
Figure 3.09 Enforcing MDM2 expression prevents senescence in WD/DDLS cells treated with PD0332991 in an E3 ligase-dependent manner.	45
Figure 3.10 Reducing p53 does not affect PD0332991 or MDM2 knockdown induced senescence in LS8817 cells.	46
Figure 3.11 CDK4 inhibitor induced senescence causes reduction in the DNA damage markers 53BP1 and γ H2AX in the liposarcoma cell line LS8817.....	47
Figure 3.12 PD0332991 induces senescence in the glioma-derived SNB19 cell line.	49
Figure 3.13 Enforced MDM2 expression can bypass PD032991-induced senescence in an E3 ligase-dependent manner in SNB19 cells.	50
Figure 3.14 MDM2 knockdown can induce senescence in SNB19 cells.....	51

Figure 3.15 MDM2 loss is associated with patient response to Palbociclib.	52
Figure 4.01 SAHF components are functionally associated with ATRX.....	61
Figure 4.02 Senescence response to CDK4 inhibition does not associate with the ALT status of the cell lines.....	63
Figure 4.03 Senescence response to CKD4 inhibition correlates with ATRX reactivity to a Bethyl antibody.	64
Figure 4.04 ATRX is required for PD0332991 induced down-regulation of MDM2. .	66
Figure 4.05 ATRX is required for SA-β-gal induction and elaboration of the SASP in response to CDK4 inhibition in WD/DLS cells.....	67
Figure 4.06 ATRX is required for MDM2 knockdown induced senescence in WD/DDLS cells.....	68
Figure 4.07 : Loss of ATRX does not affect the DNA damage response to doxorubicin.....	69
Figure 4.08 ATRX is required for doxorubicin-induced senescence in WD/DDLS cells.....	71
Figure 4.09 Loss of ATRX does not affect cell cycle growth kinetics of LS8817 cells.	72
Figure 4.10 ATRX accumulates in nuclear foci in senescent but not quiescent WD/DDLS cells.....	73
Figure 4.11 Kinetics of ATRX foci formation following PD0332991 treatment in WD/DDLS cells that undergo senescence.	74
Figure 4.12 ATRX foci accumulate in glioma-derived SNB19 cells that undergo CDK4i induced senescence.....	75
Figure 4.13 ATRX foci accumulate in breast cancer-derived MCF7 cells that undergo CDK4i induced senescence.....	76

Figure 4.14 ATRX foci accumulate in transformed lung cancer cells that undergo CDK4i induced senescence but not during quiescence.	77
Figure 4.15 ATRX foci accumulate in WD/DDLS cells induced to senesce via MDM2 knockdown.	79
Figure 4.16 ATRX foci accumulate in WD/DDLS cells induced to senescence via DNA damage.....	80
Figure 4.17 ATRX foci do not accumulate in quiescent LS8817 cells.....	81
Figure 4.18 ATRX foci do not accumulate in MCF7 cells undergoing autophagy. ...	82
Figure 4.19 ATRX foci do not accumulate in WD/DDLS cells induced to differentiate.	83
Figure 4.20 ATRX facilitates senescence in response to CDK4 inhibition in the ATRX-deficient cell line U2OS.....	84
Figure 4.21 The ability of ATRX to support senescence requires HP1 and H3K9me3 binding sites.	86
Figure 4.22 ATRX expression and capacity of PD0332991 to induce accumulation of unphosphorylated Rb is not affected by ATRX mutation status.	87
Figure 4.23 ATRX foci accumulation depends on H3K9Me3 and HP1 interactions.	88
Figure 4.24 PML foci begin to accumulate within one day of PD0332991 treatment.	90
Figure 4.25 Temporal accumulation of various senescence hallmarks.....	91
Figure 4.26 ATRX co-localizes with SAHF and PML in senescent cells.....	92
Figure 4.27 ATRX is required for establishment of HP1 γ SAHF but not PML foci in senescent cells.....	93
Figure 4.28 ATRX is required for maintenance of HP1 γ SAHF in senescent cells...	94
Figure 4.29 ATRX foci are dispensable for senescence maintenance.....	95

Figure 4.30 Loss of ATRX does not affect PML foci.....	96
Figure 4.31 ATRX foci accumulate in normal human diploid fibroblasts induced to undergo premature senescence.	99
Figure 4.32 ATRX foci accumulate in normal human fibroblasts undergoing replicative senescence.	100
Figure 4.33 ATRX represses E2F4 target genes in senescent cells.....	101
Figure 5.01 ATRX affects EZH2 target gene expression in senescent cells.....	109
Figure 5.02 ATRX binding sites in senescent cells.....	111
Figure 5.03 Validation of genes potentially bound to and down-regulated by ATRX in senescence.	114
Figure 5.04 Validation of genes potentially bound to and up-regulated by ATRX in senescence.	115
Figure 5.05 ATRX is required for efficient repression of <i>HRAS</i> in LS8817 cells. ...	116
Figure 5.06 ATRX is required for efficient repression of <i>HRAS</i> in U2OS cells.....	117
Figure 5.07 ATRX accumulates at genomic DNA of the <i>HRAS</i> locus in senescent but not quiescent WD/DDLS cells.....	118
Figure 5.08 <i>HRAS</i> is repressed in a variety of different transformed cell lines undergoing senescence.	120
Figure 5.09 ATRX is enriched at the <i>HRAS</i> locus in senescent as compared to quiescent LS8817 cells.....	121
Figure 5.10 ATRX does not accumulate at genomic <i>HRAS</i> in LS8817 cells until 7 days following PD0332991 treatment.	122
Figure 5.11 ATRX binding to <i>HRAS</i> following PD0332991 treatment in U2OS cells requires H3K9Me3 and HP1 interactions.....	123
Figure 5.12 ATRX repression of <i>HRAS</i> following PD0332991 treatment in U2OS cells requires H3K9Me3 and HP1 interactions.....	124

Figure 5.13 shRNA reduces <i>HRAS</i> to levels comparable to CD4k inhibition in LS8817 cells.....	126
Figure 5.14 Reduction of <i>HRAS</i> is sufficient to drive geroconversion: the transition from quiescence into senescence.....	127
Figure 5.15 Reduction of HRAS is sufficient to drive geroconversion: the transition from quiescence into senescence.....	128
Figure 5.16 Enforcing expression of HRAS in LS8817 can prevent senescence induced by CDK4 inhibition.....	129
Figure 6.01 MDM2, ATRX and HRAS drive the transition from quiescence to senescence.....	134

LIST OF ABBREVIATIONS

53BP1	p53-binding protein 1
5-FU	fluorouracil
ALT	Alternative lengthening of telomeres
ARF	Alternative reading frame
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
ATRX	Alpha Thalassemia/Mental Retardation Syndrome X-Linked
BrdU	5-bromo-2'-deoxyuridine
CDK	Cyclin dependent kinase
CDK4i	Cyclin dependent kinase 4 inhibitor
ChIP	Chromatin immunoprecipitation
COPD	Chronic obstructive pulmonary disease
CT	Computed tomography
CXCL1	C-X-C motif chemokine ligand 1
DAPI	4',6-diamidino-2-phenylindole
DAXX	Death-domain associated protein
DME-HG	Dulbecco's Modified Eagle's medium-high glucose
DNA	Deoxyribonucleic acid
doxo	doxorubicin
EDTA	Ethylenediaminetetraacetic acid
EZH2	Enhancer of zeste homolog 2
FBS	Fetal bovine serum
FDA	Food and drug administration
FDR	False discovery rate
FISH	Fluorescence <i>in situ</i> hybridization
FTI	Farnesyltransferase inhibitor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GEO	Gene expression omnibus
GFP	Green fluorescent protein
GSEA	Gene set enrichment analysis
GTP	Guanosine-5'-triphosphate
H3K4me0	Unmodified histone H3K4
H3K9me3	Trimethylated histone H3K9
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HDAC	Histone deacetylase
HDF	Human diploid fibroblast
HGPS	Hutchinson–Gilford Progeria Syndrome
HIRA	Histone cell cycle regulation defective homolog A
HLI-373	Hdm2 E3 ligase inhibitor
HMGA	High mobility group A
HP1	Heterochromatin protein 1
IDR	Irreproducible discovery rate
IL-6	Interleukin-6
IL-8	Interleukin-8
INK4	Inhibitor of cyclin dependent kinase 4
MAPK	Mitogen-activated protein kinase
MCM2	Mini-chromosome maintenance protein 2
MDM2	Mouse double minute 2 homolog

MEK	Mitogen-activated protein/extracellular signal-regulated kinase kinase
mH2A	Macro H2A
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8
NF-κB	Nuclear factor-kappaB
OIS	Oncogene induced senescence
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PML	Progressive multifocal leukoencephalopathy
PML-NBs	Progressive multifocal leukoencephalopathy-nuclear bodies
PMSF	Phenylmethane sulfonyl fluoride
PRC2	Polycomb repressive complex 2
qPCR	Quantitative polymerase chain reaction
Rb	Retinoblastoma
RECIST	Response evaluation criteria in solid tumors
RFP	Red fluorescent protein
RING	Really interesting new gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RS	Replicative senescence
RTK	Receptor tyrosine kinase
SAHF	Senescence associated heterochromatic foci
SASP	Senescence associated secretory phenotype
SA-β-gal	Senescence associated β-galactosidase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
seq	Sequencing
SIRT	Sirtuin
SuvH1	Suppressor of variegation 3-9 homolog 1
SUZ12	Suppressor of zeste 12
TF	Transcription factor
TIS	Therapy induced senescence
TR	Tandem repeat
WD/DDLS	Well differentiated/dedifferentiated liposarcoma
XCI	X-chromosome inactivation

1. INTRODUCTION

1.1 Consequences of cell cycle exit

Cyclin-dependent kinases (CDKs) are the major regulators governing progression through the mitotic cell cycle. These kinases are responsible for the commitment of cells to the cell cycle in G1 phase, they participate in firing the DNA replication origin machinery and ensuring that each origin is fired once and only once per cell cycle, and they coordinate the myriad number of events required for the correct distribution of the genome and other cellular components to the two daughter cells (Hochegger et al. 2008; Satyanarayana and Kaldis 2009; Rhind and Russell 2012).

Failure to activate the CDKs in G1 prevents cells from engaging into the mitotic cycle. Cells that have exited the cell cycle can adopt one of multiple fates (Blomen and Boonstra 2007; Blagosklonny 2003; Massague 2004). Quiescent cells are those that can return to the cell cycle once mitogenic conditions are favorable for CDK activation (Oki et al. 2014; Iyer et al. 1999). In some quiescent cells, particularly those driven by nutrient limitation, autophagic processes can be activated to ensure the cell's survival while awaiting appropriate conditions to activate CDKs (Kaur and Debnath 2015). Other growth arrested cells will not return to the cycle regardless of whether or not conditions become favorable for CDK activation. For example, differentiated muscle or neuron cells undergo reprogramming that commits them to a tissue-specific function and irreversibly locks them out of cell cycle (Hobert 2011; Bentzinger et al. 2012; Yun and Wold 1996). Other differentiated cells are more 'plastic' and transformation may drive them back into cell cycle or the needs of the organism can allow their expansion and recovery (Ziv et al. 2013; Schmolka et al. 2013). Senescence is another state of

irreversible cell cycle exit, usually triggered by stress, in which cells are refractory to mitogenic stimuli (Kuilman et al. 2010; Blagosklonny 2011).

Dysregulation of the cell cycle is characteristic of virtually all cancers. Transformation of normal diploid cells is accompanied by sustained proliferative signaling, the loss of growth suppressors, replicative immortality and a resistance to cell death; each of these hallmarks can occur to varying degrees (Hanahan and Weinberg 2011, 2000). Thus, it has long been thought that cancer therapies designed to curb unrestrained proliferation, either by inducing growth arrest (i.e. cytostatic) or inducing cell death (i.e. cytotoxic), may be valuable in controlling or curing cancer (Kummar et al. 2006; Millar and Lynch 2003).

Consistent with the idea that cells can adopt many different cell fates upon cell cycle exit, exposure to cancer therapies can drive a number of different fates. DNA damage inducing chemotherapies including 5-fluorouracil (5-FU), paclitaxel, docetaxel, cisplatin, carboplatin and doxorubicin can induce quiescence, apoptosis or senescence *in vitro* (Joyner et al. 2006; Longley et al. 2003; Hernandez-Vargas et al. 2007b, a; Gatti et al. 2002; Childs et al. 2014). The type of response depends on the concentration of drug and the cell type that is exposed to the therapeutic agent and may be dictated by the duration and nature of the p53 response (Purvis et al. 2012). Histone deacetylase (HDAC) inhibitors like SAHA can induce apoptosis, quiescence and differentiation (Li et al. 2011). Small molecules that target CDKs can induce apoptosis, differentiation or senescence, depending on the disease type (Baughn et al. 2006; Michaud et al. 2010; Choi et al. 2012; Sawai et al. 2012).

From a cancer therapeutic perspective some of these cell states are more favorable than others. Worst is probably quiescence, as this is a fully reversible state from which cells may re-enter the cell cycle and proliferate again. Best is probably apoptosis as the cell is eliminated from the organism. Terminal differentiation and senescence may be positive since these cells, although not immediately eliminated, are brought into a non-cycling yet stable state. Senescence may be even more preferential as senescent cells often elaborate a secretory program that induces recruitment of the innate immune system and may lead to their elimination (Ewald et al. 2010; Zeuner et al. 2014; Nardella et al. 2011) (see **1.4 Physiologic consequences of senescence**).

1.2 Senescence: many roads in, but how many destinations?

Senescence was first described by Hayflick and Moorhead in the 1960's as a cellular state following population doubling exhaustion due to telomere erosion (Hayflick 1965). Senescence refers to a metabolically active state in which cells are impervious to the cell-cycle promoting effects of extracellular growth signals. Since this initial description, senescence has become appreciated as a critical *in vivo* phenomenon with consequences for development, aging ((van Deursen 2014; Munoz-Espin and Serrano 2014) and references therein) and disease (either promoting it or suppressing it) ((Rodier and Campisi 2011)and references therein); see **1.4 Physiologic consequences of senescence**.

Perhaps two of the best studied inducers of senescence in normal, untransformed cells are replicative senescence (RS) and oncogene induced senescence (OIS). RS is induced by cumulative telomere attrition as a result of continued passaging in cells that lack active mechanisms of telomere maintenance (Campisi 1997b). Telomeres are bound by a multiprotein complex known as shelterin (Palm and de Lange 2008). Mutations in shelterin components lead to premature telomere loss and senescence.

Once a critically short telomere length is reached, the exposed telomere ends are perceived as double stranded breaks and trigger a p53-dependent DNA damage response mechanism that can induce senescence (Fumagalli et al. 2012; Hewitt et al. 2012).

OIS occurs in the absence of telomere attrition in response to aberrant oncogenic signaling. Senescence typically follows an initial wave of proliferation, followed by an increase in p16 and/or p53 protein levels, which elicit cell cycle exit and senescence. Bypass of OIS can occur if p53 and/or the retinoblastoma (Rb) tumor suppressor genes are inactivated (Courtois-Cox et al. 2006). OIS is thought to have evolved as a tumor suppressive mechanism that promotes tissue homeostasis in higher organisms (Rodier and Campisi 2011). The sensitivity of a cell type to oncogenic stress is variable, and not all untransformed cells will undergo OIS (Courtois-Cox et al. 2008; Sansom et al. 2006).

Beyond OIS and RS, there are numerous examples of conditions that can induce premature cellular senescence in untransformed cells. These include but are not limited to accumulation of reactive oxygen species (ROS), DNA damage, and telomere maintenance defects (Vigneron and Vousden 2010; Kuilman et al. 2010; Blasco et al. 1997). Collectively these may fall under the classification of DNA damage/genotoxic stressors. Many of these can also elicit senescence in transformed cells; this is referred to as therapy induced senescence (TIS) (Ewald et al. 2010). However, not all TIS is associated with DNA damage or p53/p16. For example, CDK4 inhibitor drugs and targeted therapies like p300 inhibitors and BRAF inhibitors induce senescence with little evidence that DNA damage occurs or that telomeres are lost (Priour et al. 2011; Yoshida et al. 2016; Haferkamp et al. 2013).

Despite this extensive and growing characterization of triggers that can induce senescence, the details of the molecular events that drive senescence remain elusive. It seems as though almost all senescence pathways converge on the p53 signaling axis, the Rb signaling axis, or some combination and/or crosstalk thereof (Courtois-Cox et al. 2008; Naylor et al. 2013). However, most of the pioneering studies that have documented genes and proteins involved in senescence were not designed to segregate the prerequisite that the cell must first exit the cell cycle, from the decision to undergo senescence. Thus, there is an extensive literature on the importance of cell cycle regulators for senescence such as p21, p16, p27 and p53 (promote senescence), or Skp2 (prevents senescence) ((Ahmad et al. 2011; Lin et al. 2010; Flores et al. 2014; Munoz-Espin and Serrano 2014; Campisi and d'Adda di Fagagna 2007; Stein et al. 1999; Rufini et al. 2013; Xue et al. 2007; Rayess et al. 2012; Baker et al. 2011; Lin et al. 1998; Bugs et al. 2011) and references therein). Such reports suggest that these gene products are key players for senescence, but critically one might ask whether these proteins were involved in senescence, or were they simply important for cell cycle exit, upon which senescence depends.

Formally, it is reasonable to consider that other gene products are responsible for the initiation and maintenance of the senescent state, but they can only act after a cell exits the cell cycle. The decision to enter into senescence may be dictated by the duration or nature of the cell cycle inhibitory signal (Purvis et al. 2012). Recent evidence also suggests that the signaling environment of the cell impinges on the senescence decision. It was reported that sustained growth factor signaling – namely via the mTOR pathway – in the presence of high p16 levels results in a period of “futile attempted growth” and it is this milieu of conflicting signals that ultimately converts quiescence into

senescence; this transition has been termed geroconversion (Leontieva et al. 2011; Blagosklonny 2014).

It is also unclear how many different terminal senescent states might exist. Senescent cells are generally characterized by a number of hallmarks, but whether any of these are unique to or definitive of senescent cells is debatable (see section **1.3 Assaying senescence: a collection of associated hallmarks but no *bona fide* markers**). This raises the question of whether senescence describes a single cellular state, or a collection of unique states that share some common properties. Much like variable environments with respect to cytokines, growth factors, chemicals and mechanotransduction can dictate what a stem cell will differentiate into, there may be numerous types of senescence that are unique, depending on the inducer, cell type and microenvironment (Yim and Sheetz 2012; Geginat et al. 2001; Bachmann et al. 2006; Stavridis et al. 2010). Furthermore, they may be different stages or “depths” of senescence that mature over time and give rise to evolving senescence hallmarks (Baker and Sedivy 2013). Thus, it is important to define not only context-specific pathways to senescence, but also which – if any – components of the senescence machinery constitute a “common core” shared by all senescent cells. It is only through such a detailed, mechanistic understanding of the triggers, entry into, deepening, maturation and maintenance of senescence that we may hope to rationally interfere with this complex process.

1.3 Assaying senescence: a collection of associated hallmarks, but no *bona fide* markers

Despite the “laundry list” of cellular events that drive senescence, a *bona fide* characterization of a senescent cell remains elusive. Indeed, the gold standard is the

permanence of the growth arrest; that is, an inability to return to cell cycle and generate a clonogenic outgrowth once the inducer has been removed or mitogenic signals that would promote proliferation in reversibly arrested quiescent cells have been restored (Childs et al. 2015). However, this is difficult to assay *in vitro* and often impossible in the context of tissue *in vivo*. Moreover, it is challenging to determine whether any outgrowth of cells in such an experiment is driven by a subpopulation of cells that were quiescent, or a true escape from senescence due to further genetic alterations (Lin et al. 1998; Beausejour et al. 2003).

Thus, the senescence community has attempted to define a senescent cell by a collection of associated hallmarks including senescence associated β -galactosidase (SA- β -gal), senescence associated heterochromatic foci (SAHF), the senescence associated secretory phenotype (SASP) and high levels of cell cycle inhibitory proteins like p16 or p21 (described in detail below). Frustratingly, these are neither unique to senescent cells, nor strictly required, making it nearly impossible to unequivocally define a cell as senescent with these hallmarks (Sharpless and Sherr 2015). Although rather arbitrary, the most commonly used approach to define a senescent state is to (1) establish growth arrest (i.e. by means of a deficiency of BrdU incorporation, flow cytometric analysis of DNA content, a loss of proliferation markers like Ki67, an accumulation of hypo-phosphorylated Rb, etc) and then (2) supplement it with “at least two additional senescence markers, the choice of which may vary for different settings” (Kuilman et al. 2010). The characteristics and implications of such associated markers are discussed below.

One of the first phenotypes that was attributed a senescent cell was SA- β -gal. The detection of SA- β -gal reflects the accumulation of the lysosomal β -galactosidase enzyme that can be assayed at a sub-optimal, more neutral pH (pH 6.0) (Dimri et al. 1995).

When this enzyme is highly expressed, like in a senescent cell, the optimal acidic pH (i.e. pH 4.0-4.5) is not necessary to detect its activity (Kurz, J Cell Science 2000). This lysosomal activity measurement remains the most widely used marker of senescence and without the presence of SA- β -gal, a cell is unlikely to be defined as senescent. However, this marker is not unique to a senescent cell; many non-senescent cells with high lysosomal content will stain positively, including autophagic cells (Young and Narita 2010), active phagocytes such as macrophages or resident microglia (Bursucker et al. 1982) and even cells that have achieved quiescence due to prolonged contact inhibition (Itahana et al. 2007). Moreover, senescence, defined by other markers, can occur even without detectable levels of SA- β -gal at pH 6.0 (Kurz et al. 2000).

Another commonly used marker of senescent cells is elevated expression of the cell cycle inhibitor protein p16, which is the gene product of the *INK4A* locus. p16 potently inhibits progression of the cell cycle by binding to and inhibiting the CDK4/6 kinases (Roussel 1999). Although there is clearly evidence that p16 plays a direct role in some senescence (Overhoff et al. 2014; Sousa-Victor et al. 2014), it is difficult to separate this from its role regulating the cell cycle. Indeed, many reversibly arrested cells will upregulate p16 including in the contexts of DNA damage (Stone et al. 1996) and wound healing (Demaria et al. 2014). Moreover, there are a growing number of examples in which p16 activation is dispensable for senescence induction (Prieur et al. 2011; Beausejour et al. 2003; Rheinwald et al. 2002; Herbig et al. 2004). Although p16 is probably the most commonly used of the cell cycle inhibitors as a marker of senescence, additional cell cycle inhibitory proteins that have been implicated in senescence – like p21, p53 or p27 – suffer from the same complications.

Senescent cells also undergo dramatic changes in chromatin structure and gene expression. They tend to acquire an increase in heterochromatic DNA with the

accumulation of repressive histone marks like H3K9me3, repressive proteins like the heterochromatin protein family (HP1) and repressive histone variants like macro H2A (mH2A) (Zhang et al. 2005; Zhang et al. 2007; Adams 2007; Narita et al. 2006). These proteins, along with Rb, can be found at the promoters of E2F target genes and contribute to their repression (Narita et al. 2003). In human fibroblasts, the heterochromatin is readily visualized as dense 4'-6-Diamidino-2-phenylindole (DAPI)-staining regions known as senescence associated heterochromatic foci (SAHF), but this is not the case in mouse cells or in examples of transformed human cells undergoing senescence (Kennedy et al. 2010; Aird and Zhang 2013). In both mouse and human cells, SAHF can be seen via the accumulation of HP1 foci via immunofluorescence; however, there are also clear examples in which SAHF are dispensable for senescence so these too are a context-specific marker of senescence (Kosar et al. 2011; Di Micco et al. 2011). Moreover, there is evidence for the evolution of the composition of the SAHF over time, with a marked decrease in histone components in the DNA of cells as they progress into mature or “deep” senescence (Baker and Sedivy 2013). Thus, our understanding of chromatin changes that mark senescent cells and how they drive the senescent state is immature.

Finally, senescent cells secrete a panel of chemokines and cytokines, growth factors and matrix remodeling proteases collectively referred to as the senescence associated secretory phenotype (SASP) (Coppe et al. 2010; Perez-Mancera et al. 2014). The SASP is largely thought to be driven by NF- κ B signaling (Chien et al. 2011) and has many physiologic implications including wound healing, tumor suppression or promotion, development and age-associated diseases (Munoz-Espin and Serrano 2014; Coppe et al. 2010). However the SASP is not only cell type and context specific like all of the other senescent hallmarks, but also seems to change with time (Tchkonia et al. 2013).

Furthermore, the level of activation may be at secretion rather than gene expression, making it difficult to analyze *in vivo* (Rodier 2013). Many immune cells, including those recruited to senescence areas express SASP factors, often to much higher levels than those observed in the senescent cells themselves, further complicating the analysis of the SASP *in vivo* (Arango Duque and Descoteaux 2014). Thus, although the cytokines IL-6 and IL-8 are the most often assayed when referring to the SASP, the milieu of SASP factors is not definitive and varies depending on context-specific cues including cell type, senescence inducer, time and tissue microenvironment.

In addition to these frequently assayed hallmarks of senescence, there exist a number of other features of senescent cells. These include, but are not limited to, an enlarged, flattened cell morphology (particularly in adherent cultured cells) (Chen et al. 2000), changes in metabolic profiles (Gey and Seeger 2013) and resistance to apoptosis (Wang 1995). Evidently, the means to unequivocally identify a senescent cell is still lacking because of the deficiency in our understanding of what is required for senescence itself, rather than for cell cycle exit. Ultimately, a molecular understanding of the context-specific nuances of senescence and whether or not there exists a core machinery will afford us the ability to define specific and unique senescence markers. As discussed below, such a toolkit has far-reaching implications for physiology and disease.

1.4 Physiologic consequences of senescence

Senescence has been described as a “double edged” sword because it, like inflammation, has both beneficial and detrimental physiologic implications (Ohtani et al. 2012; Campisi 1997a). Beneficially, senescence is thought to have evolved as a tumor suppressor in multicellular organisms to promote tissue homeostasis (Rodier and Campisi 2011). Conceptually, this is consistent with the evidence that harmful, potentially transforming events such as DNA damage, ROS and aberrant oncogene

activation can trigger senescence. It would be favorable for an organism to limit the proliferative capacity of such cells until they can be eliminated by the innate immune system, in order to maintain tissue structure and function. Indeed, this is supported by overwhelming physiologic evidence from a number of different mouse model systems and by correlative studies in human tissue (Braig et al. 2005; Chen et al. 2005; Collado et al. 2005; Courtois-Cox et al. 2006; Xue et al. 2007; Krizhanovsky et al. 2008b). For example, benign nevi express activated B Raf^{V600E}, which induces the expression of the cell cycle inhibitory protein p16 and the accumulation of SA- β -gal, whereas malignant melanoma cells lack these features (Michaloglou et al. 2005; Dhomen et al. 2009).

The SASP also recruits both innate and adaptive immune cells to clear senescent tumor cells, thereby restricting tumor progression via direct removal of premalignant or transformed cells. Mouse models of liver carcinoma indicate that both a competent immune system and senescence activation in the perturbed cells themselves are required for tumor clearance (Xue et al. 2007; Krizhanovsky et al. 2008b). The SASP can also alleviate tumor burden in a non-cell autonomous manner, by directing macrophage polarization and activity. In the context of chronic liver damage, the SASP can polarize invading macrophages such that they limit the transformation of adjacent epithelial cells into carcinoma (Lujambio et al. 2013).

There is evidence that another beneficial role of senescence is in limiting tissue fibrosis in the liver through the SASP. Senescent cells accumulate during chronic liver damage and the associated fibrosis and cirrhosis they pose are limited because of recruitment of immune cells that clear the damaged, senescent cells (Krizhanovsky et al. 2008b; Kong et al. 2012; Lujambio et al. 2013; Kim et al. 2013). Similarly, senescent cells promote wound healing by recruitment of immune cells and limit tissue fibrosis that causes skin scarring (Jun and Lau 2010; Demaria et al. 2014). Beneficial roles of the

SASP have also been hypothesized in tissue repair following cardiac infarct, protection against atherosclerosis and pulmonary hypertension. Such ideas remain largely based on genetic inference rather than direct correlation with senescence; mice deficient in proteins associated with senescence (i.e. Arf, p53, p21, p27) have an increased tendency to develop atherosclerosis whereas mice with increased *Trp53* are protected (Diez-Juan and Andres 2001; Mercer and Bennett 2006; Mercer et al. 2005; Gonzalez-Navarro et al. 2010; Khanna 2009; Sanz-Gonzalez et al. 2007). Recent data indicates that senescence also has a functional role in development. Senescent cells have been found across a variety of embryonic structures in numerous vertebrate organisms, where they promote cell clearance via macrophage recruitment and are required for proper tissue morphology (Storer et al. 2013; Munoz-Espin et al. 2013). Thus, senescence is not just a stress response but also has an important role in normal developmental contexts.

However, senescent cells and their related SASP can also have negative ramifications. In the context of cancer, the SASP can also be pro-tumorigenic; it has been shown to stimulate the proliferation of neighboring cells, remodel the extracellular matrix to promote invasion and contribute to dedifferentiation of cancer cells, which is associated with worse prognosis (Laberge et al. 2015; Lujambio 2016). Chemotherapies can also elicit senescence in untransformed cells within the microenvironment; this can contribute to an overall cancer-promoting inflammation, decreased tissue function and perhaps contributes to the increased propensity to develop secondary cancer years after therapy (Naylor et al. 2013; Bent et al. 2016).

Senescence may also underlie a number of age-associated pathologies. Indeed, the number of p16-positive and SA- β -gal-positive cells increase across many different tissues during aging in both rodents and humans (Sharpless 2004; Liu et al. 2009;

Ressler et al. 2006; Geng et al. 2010; Baker et al. 2016; Baker et al. 2011). Causal links have been established between telomere loss, senescence and organismal aging. Mice lacking telomerase develop many age-related pathologies like diabetes and degeneration of numerous organs including the brain, liver and intestines (Bernardes de Jesus et al. 2012; Jaskelioff et al. 2011). Strikingly, these effects can be reversed by reactivating telomerase, indicating a causal role for telomere crisis and the development of such age-associated phenotypes (Jaskelioff et al. 2011). Additionally patients with the premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS) have shortened or mutated telomeres and develop lethal age-related pathologies such as heart disease and stroke at very early stages in life (Hennekam 2006). It is important to bear in mind the striking differences between mouse and human telomeres and aging biology, including the observation that while human telomeres are ~5-10 times shorter than those of mice, human lifespan is on the order of 70-80 years, compared to a mere 18-24 months for mice (Calado and Dumitriu 2013). Thus, although there is clear evidence for a relationship between telomere attrition and aging, the biology is complex and not well understood.

In another mouse model of premature aging, BubR1 insufficiency, Van Deursen and colleagues showed that clearance of p16-positive cells in aged mice could prevent or slow the onset of numerous age-associated defects including sarcopenia and the loss of adipose tissue (if clearance of such cells was started early in life) or attenuate their progression (if clearance was initiated late in life) (Baker et al. 2011). These pioneering studies suggested that senescent cells might actually be causative in age-related pathologies. Such experiments were repeated in normally aging mice, in which early clearance of p16-positive cells extended median lifespan, decreased tumorigenesis and attenuated the age-associated deterioration of a number of tissues including the kidney,

heart and fat (Baker et al. 2016). Given the limitations of p16 and SA- β -gal as senescence markers, these studies likely only begin to uncover the far-reaching implications senescent cells may have on an aging organism. Indeed, senescence is thought to underlie not just the aforementioned pathologies, but also may also be a driver of obesity, type 2 diabetes, cataracts, glaucoma, macular degeneration, Parkinson's disease, Alzheimer's disease, COPD, hypertension and general age-associated frailty (Reviewed in (Naylor et al. 2013; Munoz-Espin and Serrano 2014)).

How exactly senescence contributes to so many age-related pathologies is still unclear. Based on what is known about senescence biology, there are a number of reasonable hypotheses. One is simply via the depletion of tissue function, as the number of senescent cells increases with age. Moreover, senescence may exhaust stem cell pools and the ability to replace damaged, defective, or normal cells becomes limited (Sharpless and DePinho 2004). Sustained SASP can stimulate a chronic "sterile inflammation" that can lead to further tissue degeneration and inflammatory, non-functional microenvironments (Freund et al. 2010). There is evidence to suggest that a defective immune system, which is associated with aging, perhaps due to senescence of immune cells themselves, results in a failure of resident senescent cells in other tissues to be cleared. This can lead to chronic local inflammation, an increase in the concentration of inflammatory cytokines in the blood and perhaps all of the aforementioned age-associated defects (Lord et al. 2001; Mocchegiani and Malavolta 2004; Franceschi et al. 2007).

1.5 Well differentiated/dedifferentiated liposarcoma as a system in which to study senescence

Evidently, senescence has far-reaching implications in both normal and diseased tissue and is a complicated, multi-factorial process. However, a detailed molecular understanding of senescence is still lacking. Frustratingly, many of the gene products that have been identified and studied as regulators of senescence including p53, p16, and p21 are direct cell cycle regulators. Thus, it becomes difficult to distinguish the decision to exit the cell cycle from the decision to commit irreversibly to senescence. When I began my thesis work, the Koff lab was investigating the efficacy of a novel small molecule inhibitor – PD0332991 or Palbociclib – that specifically targets the CDK4/6 kinases as a cancer therapeutic. In particular, they were exploring this drug as a treatment option in well differentiated/dedifferentiated liposarcoma (WD/DDLS).

Liposarcoma is the most common of the histologically diverse class of soft tissue sarcomas; of the further distinguishable liposarcoma subtypes, WD/DDLS is the most common and the most lethal (Crago and Singer 2011). More than 90% of WD/DDLS tumors are characterized by amplification of chromosome 12q13-15, which contains the oncogenes *CDK4* and *MDM2* as well as additional genes that may contribute to the disease (Meis-Kindblom et al. 2001). The well differentiated component of the tumor tends to be locally aggressive and non-metastasizing; however, tumors can present with or evolve to acquire a dedifferentiated component, which is associated with metastatic potential and poor patient prognosis. The primary course of treatment for WD/DDLS is surgical resection due to the lack of efficacy of both chemotherapy and radiation therapy (Crago and Singer 2011). Local disease burden due to tumor size, coalescence of tumor with normal tissue precluding surgical resection, as well as distant metastases from the DDLS component all contribute to patient mortality. Due to the prevalence of the *CDK4*

amplification, the chemo- and radio-resistance of the disease, as well as the Rb-positive status of the majority of tumors, WD/DDLS became a candidate for single agent CDK4 inhibitor (CDK4i) clinical trial and *in vitro* studies to understand the mechanisms of action and patient response to this class of drugs (for further discussion, see **3.1 CDK4 inhibition as a targeted cancer therapy**).

Using a panel of seven Rb-positive, patient-derived WD/DDLS cell lines positive for both *CDK4* and *MDM2* amplification, the Koff lab had identified that two different outcomes were possible when these cells were treated with PD0332991 *in vitro*: cells could undergo either quiescence or senescence (Kovatcheva et al. 2015). Furthermore, they had identified that the down-regulation of MDM2 was associated with the outcome: cells that underwent senescence had reduced levels of MDM2 following drug treatment, while those cells that underwent quiescence maintained constant MDM2 levels in response to PD0332991. This was the first time that anyone had described a cellular system in which both quiescence and senescence had been observed as a response to (CDK4i) treatment in a single disease type, thus illuminating new possibilities to address how senescence is uniquely related to or derived from quiescence.

1.6 Thesis objectives

Although we know much about the conditions that can induce senescence, the regulatory proteins involved in cell cycle exit, and the hallmarks we use to assay senescence, the molecular details between these events are essentially a black box (Figure 1.01). It is also unclear whether what we collectively refer to as senescence is indeed a single cellular outcome, or whether it is a collection of various end states that share some common phenotypes. In order to solve this, it is imperative not only to characterize individual inducers in individual cell types, but to ask whether a core senescence machinery and *bona fide* markers of senescent cells can be identified

through comparative studies. I saw the distinction between quiescence and senescence in WD/DDLS in response to CDK4i as an opportunity to characterize the molecular events that underlie these two cellular fates and how they relate to one another.

The objective of my thesis project was to understand how senescence occurs in response to CDK4i therapy, first in WD/DDLS and then to investigate whether these pathways are conserved in other cell types; whether senescence underlies clinical benefit; and – more generally – to interrogate the molecular mechanisms of senescence and how they may be distinguished from quiescence. In Chapter 3, I detail the molecular events relating to MDM2 that drive quiescence into senescence, how these relate to WD/DDLS patient outcome and potential biomarkers for CDK4i efficacy *in vivo*. In Chapter 4, I investigate the role of ATRX, a chromatin remodeling protein, in senescence. I propose that ATRX regulates the SAHF and that the accumulation of ATRX nuclear foci may serve as a novel and specific marker of cells that have embarked on the path of irreversible growth arrest that we refer to as senescence, regardless of the cell type or inducer. In Chapter 5, I investigate the TIS-specific role of ATRX in regulating gene expression, and identify *HRAS* as a target gene that is bound to and repressed by ATRX. I demonstrate that repression of *HRAS* drives the transition from quiescence to senescence.

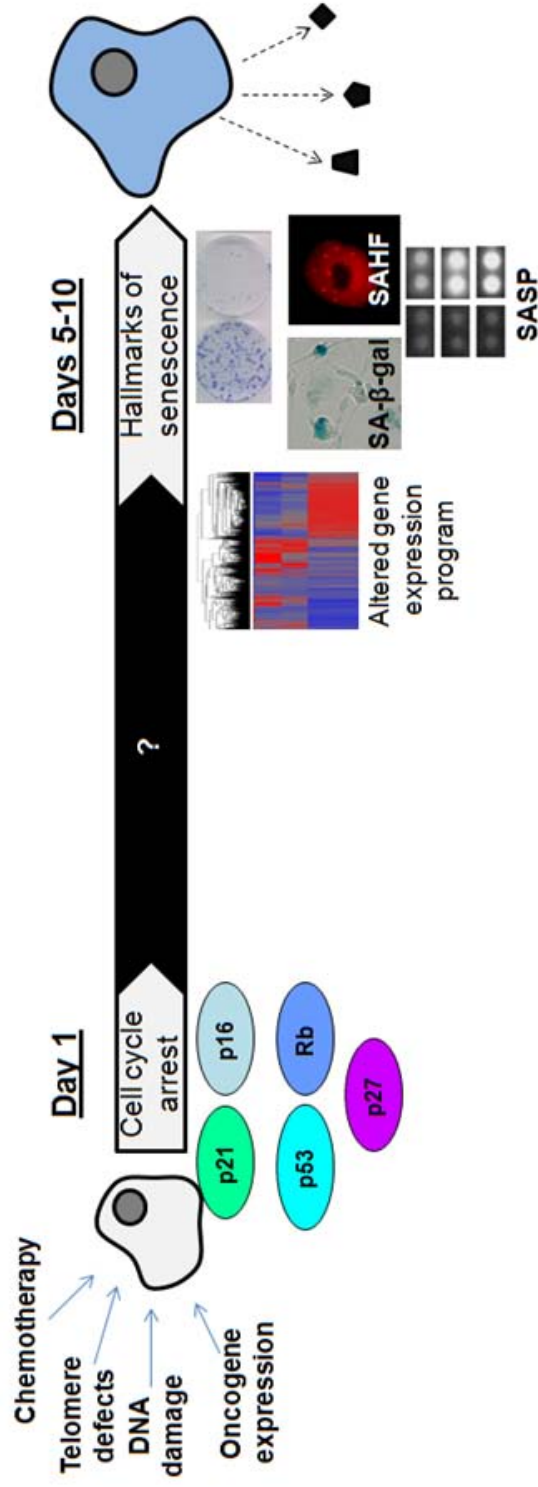


Figure 1.01 The molecular and temporal evolution of senescence.

Many stressors (left) can induce premature senescence. We have a detailed understanding of the molecules that drive cell cycle exit (colored ovals, left); this typically occurs imminently after exposure to the stressor. Many days later, the characteristic hallmarks used to identify senescent cells develop (right). These include accumulation of senescence associated β-galactosidase activity (SA-β-gal), senescence associated heterochromatic foci (SAHF), elaboration of the senescence associated secretory phenotype (SASP; also represented by black shapes secreted by the senescent cell), an enlarged, flattened cell morphology and a failure to return to cell cycle once the stressor is removed (represented by clonogenic outgrowth, blue). However, the molecular requirements for and temporal evolution of senescence remains unclear (black box, ?).

2. MATERIALS AND METHODS

Cell culture

Cell lines were developed from WD/DDLS tumors resected from surgical patients after obtaining informed consent. LS8817 and LS0082 have previously been described using the nomenclature DDLS8817 and WD0082 (Wu et al. 2012). DNA was extracted from cell lines using standard protocols (QIAGEN DNEasy) and lineage confirmed by copy number array to confirm amplification of segment 12q13-15 (Agilent 244K according to manufacturer's specifications).

Cell lines were maintained in DME HG supplemented with 10% heat-inactivated fetal bovine serum and 2mM L-glutamine.

Gene expression analysis

RNA was extracted from cells (RNEasy, QIAGEN) and reverse transcription performed after treatment for 7 days with PD0332991 (Selleckchem).

Gene targeting by shRNA

shRNA were delivered in the pLKO.1 vector (Open Biosystems) and infected cells selected using puromycin (1µg/ml); infection with a virus carrying a scramble control (CAACAAGATGAAGAGCACCAA) was used as a control in all experiments utilizing shRNA. Cell lines were treated with PD0339221 or shRNA directed against CDK4 (GAGATTACTTTGCTGCCTTAA), MDM2 (M376, TTCACTATTCCACTACCAAAG; M380, TACTAGAAGTTGATGGCTGAG), ATRX (588, GCCTGCTAAATTCTCCACATT; 590, CGACAGAACTAACCCTGTAA), HRAS (265, CGGAAGCAGGTGGTCATTGAT;

266, GTGTGTGTTTGCCATCAACAA), ZNF107 (660, CGATTCTCAAACCTAACTATA; 662, CAGGAAACAAACACTTCAAAT) for 48 hours to 7 days.

To rescue the MDM2 knockdown we infected cells with a lentivirus (pLOC, Open Biosystems) encoding either an *MDM2* expression cassette containing a mismatched sequence (ACTATTCTCAACCCTCAACTTCTA) or an *RFP* cassette. 24 hours later after transduction, positive cells were selected in media containing 3µg/ml blasticidin and selection was maintained throughout the experiment. Five days after blasticidin selection began we transduced the cells with a second lentiviral vector encoding either the *shM380* sequence targeting MDM2 or a scrambled sequence (*shSCR*) as described above. 24 hours later these cells were selected in media containing both blasticidin and 3µg/ml puromycin.

Antibodies

Antibodies against CDK4 (3F121), MDM2 (SMP14), total Rb (IF8), cyclin A (H432), p16 (C20), p53 (DO-1 and Bp53-12), tubulin (C20), FLAG (M2), ATRX (H-300) and PML (PG-M3) were obtained from Santa Cruz Biotechnology, phospho-Rb 780 (#9307) from Cell Signalling, Arf (3642), 53BP1 (ab172580) and LC3 (ab48394) from Abcam, HP1γ (05-690) and γH2AX (05-636) from Millipore, GFP (A11122) from Life Technologies and ATRX (A301-045A) from Bethyl Laboratories. Cells were lysed with buffer composed of 50mM Tris-HCl, pH7.4, 250mM NaCl, 5mM EDTA, 0.5% NP40, 2mM PMSF, and supplemented with protease inhibitors. Twenty five to eighty micrograms of protein were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated overnight with antibodies.

Patient Samples

Extracts were prepared from pre-treatment biopsies within two weeks before the first dose of the drug, and post-treatment biopsies were collected within six days of the start of the second cycle. Extracts were prepared in 50mM Tris-HCl, pH7.4, 150mM NaCl, 1mM EDTA, 1% NP40, 0.25% sodium deoxycholate and supplemented with mini-protease inhibitor cocktail (Roche). Tumor response was assessed by reference radiologist by CT scan every six weeks for 36 weeks, and every 12 weeks thereafter. The clinical trial was approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center and all patients provided written informed consent (NCT01209598).

Senescence analyses

Cells were plated at a concentration of 25,000 per well in a 4-well chamber slides (Millipore) and treated for seven days with drug and stained for senescence-associated β -galactosidase (Cell Signaling kit #9860). Cell number was quantitated by DAPI staining and β -galactosidase staining quantitated as a proportion of total cells.

Senescence associated heterochromatic foci were quantitated after cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton, blocked with 2% FBS, and stained with antibodies against HP1 γ (1:5000 dilution, 2MOD-1G6 Millipore). Senescent cells were identified by immunofluorescence after treatment of slides with anti-mouse secondary antibodies and quantitation of focal SAHF as a percent of total cells (Leica Upright Confocal SP5 confocal microscope).

Clonogenic growth assays

Cells were treated with 1 μ M PD0332991 for seven days, collected and then 1000 cells were plated and cultured for three weeks in drug-free medium before staining with crystal violet.

Generation of ATRX mutants

ATRX was mutated using standard QuikChange procedures and Platinum® Taq DNA Polymerase, High Fidelity (ThermoFisher) according to manufacturer's protocols. PCR products were then digested with DpnI (New England Biolabs) according to manufacturer's protocols before being transfected into ElectroMAX™ Stbl4™ Competent Cells (ThermoFisher) according to manufacturer's protocols.

Mutational analysis in U2OS cell lines

ATRX (AddGene #45444) was delivered using a construct generously provided by David Picketts. U2OS cells were transfected using siLentFect Lipid Reagent (BioRad) according to manufacturer's protocols. 48 hours following transfection, cells were selected using G418 (500 μ g/ml). Selected cells were sorted using fluorescent activated cell sorting on a MoFlow™ sorter (Beckman Coulter). Untransfected U2OS cells were used to mark the GFP-negative population and the GFP-low population was collected.

To verify the integrity of each mutant DNA was extracted from the transfected cells (QIAGEN DNEasy) and specific regions of ATRX were amplified by Platinum® Taq DNA Polymerase, High Fidelity (ThermoFisher) (see Table 1 for primers) and sequenced.

Chromatin immunoprecipitation followed by sequencing (ChIP-seq)

ATRX ChIP was performed as previously described (Law et al. 2010). ChIP-seq data was deposited on the Gene Expression Omnibus (GEO,

<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE74619. Barcoded Illumina libraries were generated using the Kapa Hyper Prep Kit (Kapa Biosystems, Wilmington, Massachusetts) according to the manufacturer's instructions, with 12 cycles of PCR amplification. Libraries were pooled and run on an Illumina HiSeq 2500, v4 chemistry, to obtain 30-40 million single read, 50 nucleotide-long reads passing filter. Reads were adapter and quality trimmed using Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore), aligned with BWA mem v0.7.8 (Li and Durbin 2009), and realigned around indels and base quality score recalibrated with the Genome Analysis Toolkit v3.1.1 (McKenna et al. 2010). This was repeated twice with biologic replicates at different times. MACS v2.0.10 (Zhang et al. 2008) was used to call peaks within the irreproducible discovery rate (IDR) framework (<https://www.encodeproject.org/software/idr/>; <https://sites.google.com/site/anshulkundaje/projects/idr>). Peaks were annotated with the most proximal upstream and downstream genes (hg19 RefSeq genes) filtered for technical false positives and intergenic sequences, and then overlapped with -5kb to +1kb promoters. Known telomeric, centromeric, and repetitive sequences were also annotated within the peaks.

RNA sequencing

RNA was extracted from cells treated as described using standard protocols (RNEasy, QIAGEN). All RNA-seq experiments were performed in triplicate (all from biologic replicates at different times). RNA-seq data was deposited on the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE74620. RNA quality was checked on a BioAnalyzer to ensure a minimum RNA Integrity Value (RIN) of 7. Libraries were generated using 500ng of input RNA per sample according to the manufacturer's instructions for TruSeq mRNA Library Prep Kit

V2 (Illumina) with 8 cycles of PCR. Libraries were pooled and run on an Illumina HiSeq 2500, high output, to obtain 30 million paired end, 50 nucleotide-long reads. The RNA-Seq reads were aligned to the human reference sequence hg19 with the RNASeq aligner STAR (version_2.4.0c). Genes annotated in Gencode version 18 were quantified with featureCounts (subhead package version 1.4.3-p1). The raw counts were then subjected to the Bioconductor package DESeq2 to call for differential expression between the groups of samples. Enrichment of differential expression in sets of genes was determined using Gene Set Enrichment Analysis (GSEA) on gene groupings from MSigDB, as well as custom sets (Subramanian et al. 2005).

RNA-sequencing gene list derivation

RNA sequencing data analysis and comparisons were performed with Partek Software. The gene lists included all genes that showed at least a 1.8-fold change (FDR < 0.05) when comparing control and 7 day PD0332991 treated samples.

GO profiling, transcription factor profiling and GSEA

Gene lists were analyzed using the publicly available Enrichr software (<http://amp.pharm.mssm.edu/Enrichr/>) (Chen et al. 2013) and the top four most significantly enriched groups (based on p-value) were reported. For GSEA, gene lists were derived from the RNA-seq data and were compared against gene lists in the publicly available Molecular Signatures Database (MSigDB) v5.1. The specific gene sets analyzed were KONDO_EZH2_TARGETS (M5301), V\$E2F4DP1_01 (M10526) and DNA_REPAIR (M18229). GSEA statistical analysis was carried out with publicly available software from the Broad Institute (<http://www.broadinstitute.org/gsea/index.jsp>).

Real time quantitative qPCR (RT-qPCR)

cDNA was synthesized from 1µg of each RNA sample (extracted as above) using the One Taq® RT-PCR Kit and oligo-dT primers (New England BioLabs). cDNA was diluted 1:5 and 1µl of reaction was used for qPCR using 400nM of each forward and reverse primers and SYBR® Green PCR Master Mix (Life Technologies) according to manufacturer's protocols. qPCR was performed on ViiATM 7 Real-Time PCR System (Thermo Scientific). All primers are listed in Table 1. All RT-qPCR comparisons are by two-sided t test.

Senescence associated secretory phenotype (SASP) cytokine array

Cells were grown as described and treat with 1 µM PD0332991 for 7 days. 24 hours prior to harvest, the media was changed (with drug maintained). Media was harvested, spun down and filtered through a 0.22 µm filter. The adherent cells were trypsinized, counted, and the amount of media used for the cytokine array panel was normalized according to cell number. Cytokine array panels (R&D Systems, ARY005) were performed according to manufacturer's protocols.

Table 1: List of primers

qPCR Primers	
Name	Sequence (5'-3')
ARHGAP18 Fw	ATCAAGAGGTGGTTGTTGTCAAA
ARHGAP18 Rv	ACAATGCTTTCTGTGGATCTC
ATAD2 Fw	GGAAAAACCTCGTCACCAGAG
ATAD2 Rv	CGCCTGTTTCATTTCGTTTACAGTA
CCNY Fw	CAGACGGACGTGAGAGAAAAA
CCNY Rv	TGGTTGACTGACTGTGCTATCA
DHX9 Fw	GCAGCAGAGTGTAACATCGTAG
DHX9 Rv	ACTCAAATCGAACGCTGTAGC
HRAS Fw	CAGATCAAACGGGTGAAGGAC
HRAS Rv	GCCTGCCGAGATTCCACAG
KIF15 Fw	CTGAAGCCTATCAGGTGTTGTC
KIF15 Rv	AGGGAGGTCCGTATATTCACAAT
PARD3 Fw	GCCTTTCAGCCTTACCAAGCA
PARD3 Rv	GGTCACTACTGCGTCGAACAT
SORCS3 Fw	GAATGTGCCGAGACAACACTAGAA
SORCS3 Rv	AGACACGTAGTAGCTGGCTCT
ZNF107 Fw	TTTTCATTTTGCCCAAGACC
NRAS-Fw	TGATTTGCCAACAAGGACAG
NRAS-Rv	CCCTGAGTCCCATCATCACT
KRAS-Fw	TACAGTGCAATGAGGGACCA
KRAS-Rv	TCCTGAGCCTGTTTTGTGTCT
CTNND2-Fw	GCCGGAAGTGATTGAGATGT
CTNND2-Rv	CTACGGTGGACTTCGGTCAT
HCN1-Fw	CCAGCTGATATGCGTCAGAA
HCN1-Rv	GGCATTGTAGCCACCAGTTT
PCLB1-Fw	CCTCGTGAACATCTCCCATT
PLCB1-Rv	GCTTCCGATCTGCTGAAAAC
RNF128-Fw	ATGATCGGCAATCTGAAAGG
RNF128-Rv	GCTTTGAGCTCTTGCAATTCC
ATRX Fw	GTAGACAAGCCAGCCAGGAG
ATRX Rv	CATGAGGTGACCCAGTGTTG
C20orf195 Fw	CCCTGCGTGAGTTCCTGAC
C20orf195 Rv	CCTCGATGTAGTAGGAGCACTTA
COL26A1 Fw	GGCACTCTGTCTTCACAGCA
COL26A1 Rv	TGGGGAGAGCACCTTTATTG
DGKB Fw	GTTGCAGAATACCTTGAGTGGG
DGKB Rv	CCAGAGACACGGTTCATCAT
GAS6-AS1 Fw	CGTCGATGTGGTCATAGCAG

GAS6-AS1 Rv	CTGAGGAGGTCAGGTTCTGG
HIBADH Fw	TGCTGCCACCAGTATCAATG
HIBADH Rv	GCAGGATCAATAGTGCTGGAATC
HRH1 Fw	CTGAGCACTATCTGCTTGGTC
HRH1 Rv	AGGATGTTTCATAGGCATGACGA
SIN3B Fw	AAGACGCCCTCACCTATCTG
SIN3B Rv	GGGAAGAAAAGCGTTGAATCCAA
TRPS1 Fw	GACTGCATAACCGCACCCAG
TRPS1 Rv	CCTGCACATCATAGGTCCCATT
ATRX sequencing primers	
Name/region	Sequence (5'-3')
ADD Fw	CAGAGCTTGCCAAAAGATGG
ADD Rv	CCAATGCAAGATGAGCCTTC
HP1 Fw	GCAGCAGTGGAACTGAACAA
HP1 Rv	TTTCTGTCGGTCGCCTCA
Hel1 Fw	AAGATGCTTCACCCACCAAG
Hel1 Rv	GCTGCAAACACCAAGGATGA
Hel2 Fw	CAGATGCTGATGCTGAGGTT
Hel2 Rv	TGGTCCCAGTTGGTATGTTG

3. MDM2 REGULATES THE SENESCENCE RESPONSE TO CDK4 INHIBITION

3.1 Introduction: CDK4 inhibition as a targeted cancer therapy

Commitment to the mitotic cell cycle occurs in G1 phase is a tightly regulated process in which the cell surveys both environmental cues and intracellular status, ultimately to decide whether it can and should proliferate. Fundamentally, the different stages of the cell cycle are controlled by cyclin dependent kinases (CDKs) that are activated by proteins called cyclins, whose expression is regulated in a cell cycle-dependent manner (Malumbres 2014). The dysregulation of the cell cycle is a hallmark of cancer cells; specifically, oncogenes that drive cell cycle are amplified or activated, whereas tumor suppressor genes that inhibit the cell cycle are deleted or inactivated (Hanahan and Weinberg 2011, 2000). Together, these observations have illuminated that inhibiting G1 CDKs is an attractive therapeutic approach to cancer, ultimately restricting the proliferative potential of these otherwise unrestrained, transformed cells (Malumbres and Barbacid 2006; Malumbres et al. 2008).

Early small molecule inhibitors were broadly promiscuous, targeting multiple CDKs or even other kinases and were either toxic or ineffective in the clinic (Asghar et al. 2015). More recently, molecules that specifically target CDK4 (and its structurally related kinase CDK6) have been developed. CDK4 is a particularly attractive target. It is activated in G1 and its activation is required to push cells through the restriction point. The restriction point is when cells become irreversibly committed to the cell cycle, and will duplicate their cellular content regardless of changes in the extracellular signaling environment (Yao et al. 2008). In most normal, untransformed mesenchymal and epithelial cells, CDK4 integrates stimulatory signals from various growth factor- and

mitogen-activated receptors to initiate the phosphorylation on the tumor suppressor retinoblastoma (Rb). Rb is normally tightly bound to the E2F family of transcription factors (TFs), thus sequestering them from activity. However, upon sequential phosphorylation first by CDK4/6 (bound and activated by the D-type cyclins) and then by CDK2 (bound and activated by cyclin E and cyclin A), Rb becomes hyper-phosphorylated and releases the E2F TFs. E2F release stimulates the synthesis of genes required for S phase, DNA synthesis and mitosis. Over-expression of cyclin E, which is a target of cyclin D-CDK4/6 activity, is sufficient to overcome growth arrest induced by a phosphorylation-resistant Rb mutant; thus, the consequences of sequential Rb phosphorylation manifest in the activation of other critical target(s) (Lukas et al. 1997; Leng et al. 1997). In normal cells, CDK4/6 activity is negatively regulated by tumor suppressor genes; notably, the $p16^{ink4}$ gene product directly inhibits CDK4/6 activity and limits proliferation (Roussel 1999).

The CDK4-Rb signaling axis is critical in cancer, as evidenced by the dysregulation of this pathway in the majority of human tumors (Baker and Reddy 2012). Inhibiting CDK4/6 should theoretically induce growth arrest in all cells that have functional Rb, regardless of tissue type or other oncogenic alterations. Whether cancer cells harbor mutations in hormonal receptors, mitogen receptors, etc. is irrelevant, as these all impinge on CDK4. Notably, many cancer cells inactivate Rb itself. Such mutations bypass the requirement for CDK4 in driving proliferation and their growth is not suppressed by $p16^{ink4}$ over-expression (Medema et al. 1995); thus, the expectation would be that such cells are also refractory to CDK4/6 inhibition. *In vitro* and xenograft studies indicate that Rb-negative cells are indeed resistant to CDK inhibition (Lehn et al. 2011; Dean et al. 2010; Fry et al. 2004; Bosco et al. 2007). CDK4 inhibition should not affect non-proliferating tissue as it is specifically required to drive cell cycle. Proliferating

normal cells do become reversibly growth arrested with CDK4 inhibition and this effect is readily reversible with a “rest” period taken off such inhibitor drugs (Johnson et al. 2010).

Indeed, one such CDK4i, PD0332991 or Palbociclib, has had marked success in clinical trials for hormone receptor-positive breast cancer, which is characterized by cyclin D1 amplification. Palbociclib earned breakthrough therapy designation in combination with a hormone inhibitor and was recently approved by the FDA. Palbociclib exhibited mild side effects largely limited to neutropenia; this was likely due to the quiescence CDK4i induces in normal hematopoietic and stem cells (Sherr et al. 2016; Finn et al. 2016a; Finn et al. 2016b; O’Leary et al. 2016). Despite the overall clinical success of the drug, patient response was not uniform. A subset of patients was consistently failing to respond altogether to CDK4i therapy, despite their Rb-positive status (Finn et al. 2015). Thus, we began to wonder whether a difference in cellular outcome upon exit from the cell cycle might underpin patient outcome.

At Memorial Sloan Kettering Cancer Center, a Phase II clinical trial using Palbociclib as a single agent to treat well differentiated/dedifferentiated liposarcoma (WD/DDLS) was conducted by our colleagues Mark Dickson, William Tap and Gary Schwartz (NCT01209598). WD/DDLS is characterized by genomic amplification of *CDK4* and *MDM2*. Rb remains intact in this disease, thus making it an attractive candidate for CDK4i therapy. Similar to the results in breast cancer, the trial was largely successful on the basis of the extension of progression-free survival over the historically defined benchmark but there was a considerable subset of patients – approximately one third of all patients – whose disease progressed almost immediately whilst on the drug (Dickson et al. 2013). In both the Pfizer breast cancer study (NCT00721409) and the WD/DDLS study, approximately 25% of patients had extraordinary outcomes, with dramatically extended progression-free survival. To investigate the molecular nature of these

differences in patient outcome, the Koff lab employed a panel of seven genetically heterogeneous, patient-derived WD/DDLS cell lines, all harboring both *MDM2* and *CDK4* amplifications and functional Rb.

Not surprisingly, all of the cell lines underwent a rapid and potent G1-phase cell cycle arrest when treated with CDK4i, as measured by flow cytometry to assess DNA content, BrdU incorporation and phospho-Rb and cyclin A levels (an S-phase cyclin whose expression is driven by E2F). Four of these cell lines rapidly returned to cell cycle when the drug was removed after 7 days of continuous treatment. On the other hand, three of the cell lines elaborated a senescent phenotype, characterized by accumulation of SA- β -gal and SAHF (as measured by HP1 γ), elaboration of a SASP, and an inability to return to the cell cycle following drug withdrawal after 7 days of continuous treatment. Senescence was associated with a decrease in the level of MDM2 protein, whereas MDM2 levels were similar in cycling and quiescent cells (Kovatcheva et al. 2015).

MDM2 is a RING domain E3 ubiquitin ligase, capable of conjugating ubiquitin moieties to both itself and an array of substrates (Shi and Gu 2012; Moll and Petrenko 2003). In order for an E3 to ubiquitylate cognate substrates, ubiquitin must first be activated in ATP-dependent reaction by an E1 enzyme. The activated ubiquitin is then transferred to a conjugating E2 enzyme. Finally, the E3 ligase physically interacts with the E2 and the substrate to provide substrate specificity and conjugate the ubiquitin molecule onto a target lysine residue (Pickart 2001). The C-terminal RING domain, which is comprised of a series of cysteine and histidine residues coordinating two zinc ions, is required to bring together the E2 active site with the target lysine, and mutation of such critical residues ablates enzymatic function of MDM2 (Deshaies and Joazeiro 2009), as does disruption of the E2-E3 binding interface (Linke et al. 2008). MDM2 is well known for ubiquitylating itself and its best-characterized substrate, the tumor

suppressor gene p53; in both of these cases, ubiquitylation results in targeting of the substrate for proteasomal degradation (Shi and Gu 2012). p53 is indeed implicated in senescence (Chen et al. 2005; Fuster et al. 2007; Lin et al. 1998; Lujambio et al. 2013; Purvis et al. 2012; Rodier et al. 2007; Rufini et al. 2013; Schmitt et al. 2002); however, MDM2 does have substrates in addition to p53. Moreover, we had observed reductions in p53 levels concomitant with MDM2 decreases (Kovatcheva et al. 2015), suggesting that p53 might not be associated with MDM2 and senescence in these cells.

Thus, I set out to determine whether the change in MDM2 was required for senescence, whether this was related to its role in regulate p53 and whether this was associated with patient response to Palbociclib.

3.2 Results

3.2.1 Reducing MDM2 is sufficient to drive senescence in WD/DDLS

As mentioned above, a previous student in the lab, David Liu had already demonstrated that CKD4i-induced senescence in WD/DDLS cell lines was consistently associated with a decrease in MDM2 protein, whereas there was no reduction of MDM2 protein in cells that were quiescent. Although intriguing, correlation is not causality; thus, I asked whether reducing MDM2 levels would induce senescence. To accomplish this I generated two independent targeting vectors and transduced cells that normally undergo senescence (LS8817 and LS0082) and cells that normally undergo quiescence (LS8107 and LS7785-1) when treated with CDK4i with these hairpins or with a scrambled control. Rb phosphorylation and the amount of cyclin A were reduced in all four cell lines in which I knocked down MDM2, indicative of growth arrest, even in the absence of PD0332991 (Figure 3.01). In all of the cell lines tested, reducing MDM2 by knockdown induced the accumulation of SA- β -gal positive cells (Figure 3.02). SAHF also increased

in all cells except for LS7785-1 (Figure 3.02), which is consistent with the reports that not all senescent cells form such foci (Di Micco et al. 2011; Kosar et al. 2011). The specificity of the shRNAs was confirmed because expression of a mismatched “wobble” *MDM2* allele prevented MDM2 knockdown induced growth arrest and senescence. Thus, although not all cells undergo senescence in response to CDK4i, all cell lines did have the capacity to undergo senescence and this could be induced by reducing MDM2.

Since all cells could undergo senescence but CDK4 inhibition could only trigger it in a subset of them, we wanted to ask whether quiescence could evolve into senescence. Another graduate student in the lab, Mary E. Klein, first treated LS8107 cells with PD0332991 to induce cell cycle exit; as we had previously seen, the amount of p53 was reduced upon CDK4i treatment (Kovatcheva et al. 2015). We subsequently infected these cells with a lentiviral vector expressing an shRNA targeting MDM2. In one experiment we removed PD0332991 two days after infection, and in the other we left the cells in PD0332991 for the duration of the experiment. The results were similar: SA- β -gal positive cells accumulated only after MDM2 knockdown (Figure 3.03). Thus, quiescence can be converted into senescence by means of reducing MDM2. The transition from quiescence to senescence is known as geroconversion.

3.2.2 MDM2 is reduced in a post-translational manner following CDK4 inhibition

We next wanted to gain some insight into the regulation of MDM2. Aimee Crago showed that *MDM2* transcripts were modestly reduced in most cell lines by PD0332991, possibly reflecting the CDK4 inhibitor induced loss of p53 (Figure 3.04 and (Kovatcheva et al. 2015)). In contrast, I found that MDM2 turnover was accelerated in LS8817, LS141 and LS0082 cells that undergo senescence, but not in the LS8107, LS7785-1 and

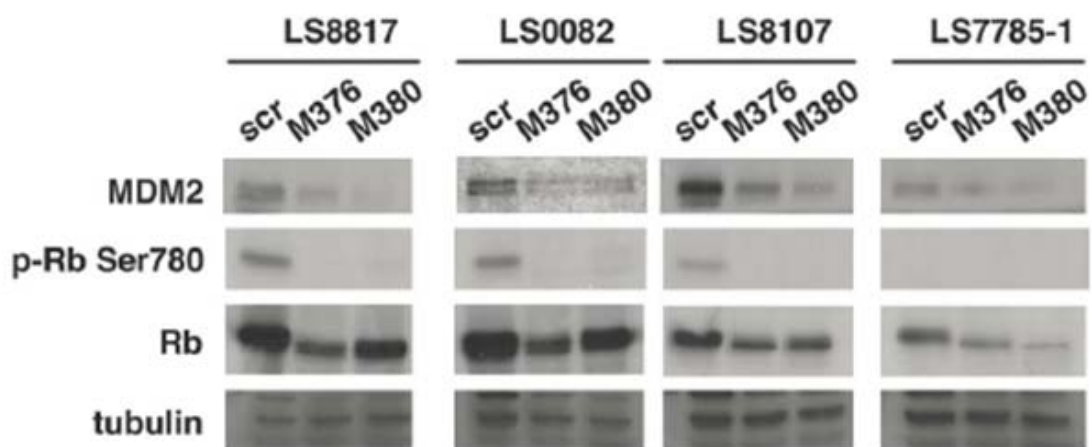


Figure 3.01 Transducing WD/DDLS cell lines with shRNA targeting MDM2 reduces MDM2 protein levels.

The indicated cells were transduced with two different MDM2 knockdown lentiviral vectors (M376 or M380) or a scrambled non-specific vector (scr) and selected in puromycin for five days prior to extraction of proteins for immunoblotting.

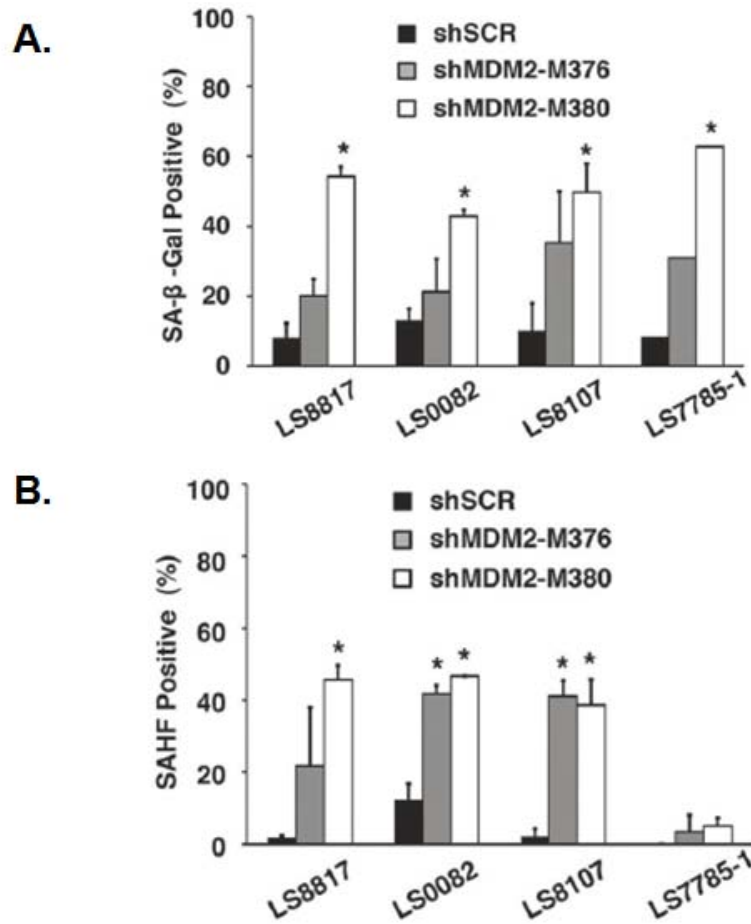


Figure 3.02 Loss of MDM2 can trigger senescence in WD/DDLS.

The indicated cells were transduced with two different MDM2 knockdown lentiviral vectors (M376 or M380) or a scrambled non-specific vector (scr) and selected in puromycin for five days. The percentage of cells staining positive for SA-β-gal (A) and HP1γ foci (B) 10 days after knockdown were quantitated (* $p < 0.05$).

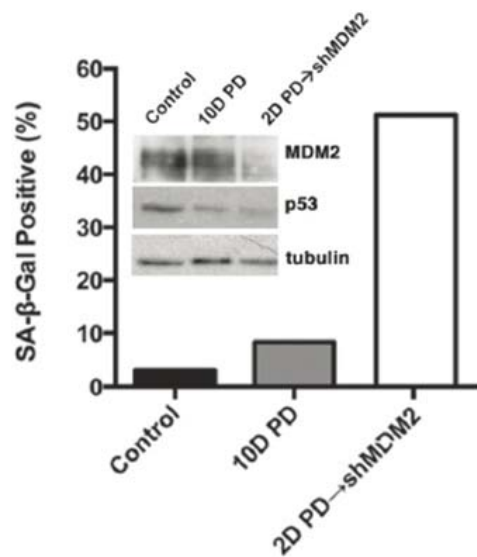


Figure 3.03 Reducing MDM2 can convert quiescence into senescence.

LS8107 cells were first arrested in PD0332991 for 2 days and MDM2 was subsequently knocked down with shM380 and cells were selected in puromycin for 8 days. The effect on the accumulation of SA-β-gal positive cells and expression of MDM2 and p53 is shown. Tubulin is a loading control. Data courtesy of Mary E. Klein

LS7785-10 cells that undergo quiescence following CDK4i (Figure 3.05 and (Kovatcheva et al. 2015). Adding the proteasome inhibitor MG132 to PD0332991-treated LS8817 or LS141 cells prevented the decrease in MDM2 (Figure 3.06). Thus, the PD0332991-triggered loss of MDM2 was at least partially due to increased post-translational, proteasome-dependent turnover.

MDM2 turnover can be regulated by either autoubiquitination or by trans-ubiquitination by multiple E3 ligases. To determine if auto-ubiquitination contributed to MDM2 turnover after CDK4 inhibition I measured the turnover of *MDM2*^{C464A} in LS8817 cells. This mutant bears a C→A substitution in the RING finger domain of MDM2, which is essential for its E3 ubiquitin ligase activity; thus, it can be ubiquitinated in trans but cannot be auto-ubiquitinated (Inuzuka et al. 2010; Linares et al. 2007). As a control I expressed a wild type FLAG-tagged allele of *MDM2*. PD0332991 affected the turnover of wild type FLAG-tagged MDM2 but not the FLAG-tagged C464A mutant (Figure 3.07). Thus, PD0332991-enhanced turnover of MDM2 was dependent on auto-ubiquitination.

3.2.3 Senescence is dependent on functional E3 ligase activity of MDM2 but independent of p53 regulation

To assess whether continued expression of MDM2 would prevent senescence induced by PD0332991 treatment, I transduced the LS8817 cells with lentiviral vectors expressing a FLAG-tagged MDM2 (F-MDM2) and treated them with PD0332991 after selection. As a negative control RFP was introduced. It is known that enforced expression of MDM2 does not necessarily increase its abundance in cycling cells (Calabro et al. 2002); however, enforced expression did prevent the PD0332991-induced reduction in MDM2 (Figure 3.08). Accumulation of phospho-Rb and cyclin A were also reduced (Figure 3.08). Enforcing MDM2 did not prevent growth arrest as evidenced by reduced BrdU incorporation (Figure 3.08). However, it did prevent the

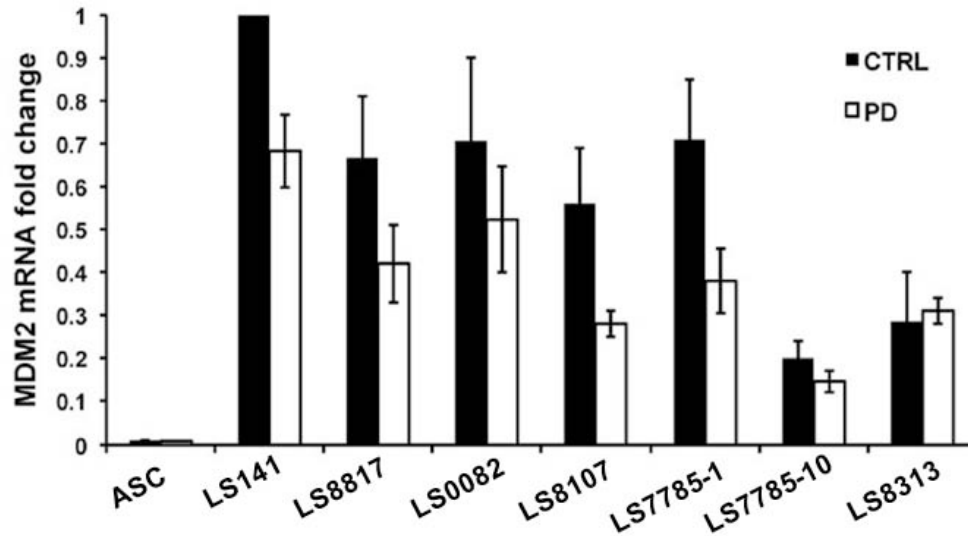


Figure 3.04 MDM2 transcripts are similarly reduced in all the WD/DDLS cells after treatment with PD0332991 regardless of the outcome of this arrest.

The cells were treated with 1 μ M PD0332991 for two days and the effect on MDM2 transcript levels determined by qPCR. This experiment was repeated at least three times on different biologic replicates. All the amounts of mRNA detected were normalized to the amount observed in control untreated LS141 samples, which was set to 1. The level of transcript in a primary adipocyte stem cell (ASC) is shown for comparison. Data courtesy of Aimee Crago.

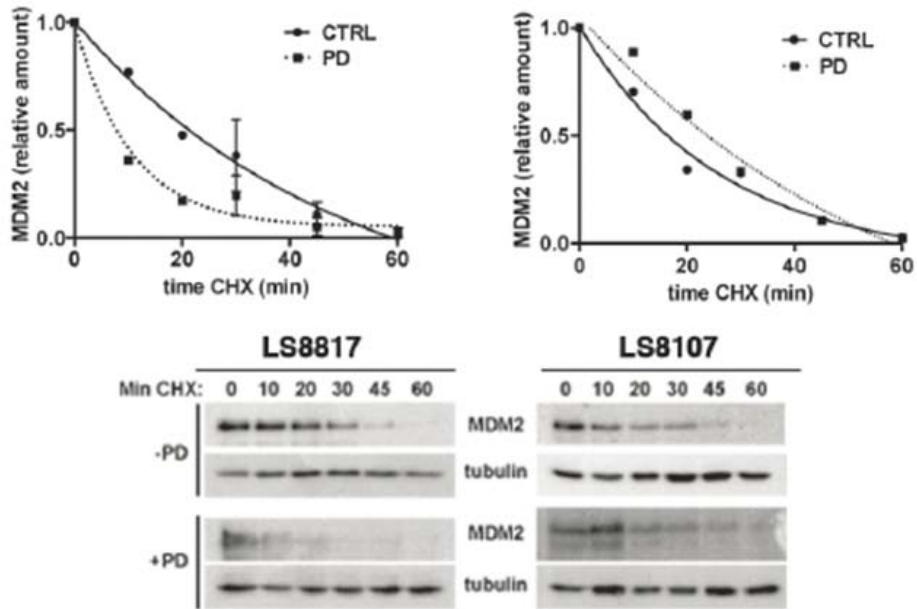


Figure 3.05 MDM2 is regulated post-translationally.

LS8817 (left) and LS8107 (right) cells were treated with 1 μ M PD0332991 for forty eight hours, and 75 μ g/ml cycloheximide was added for the indicated times before proteins were extracted and amount of MDM2 measured by immunoblot. Tubulin is a loading control. Representative autoradiograms are shown below the graphs.

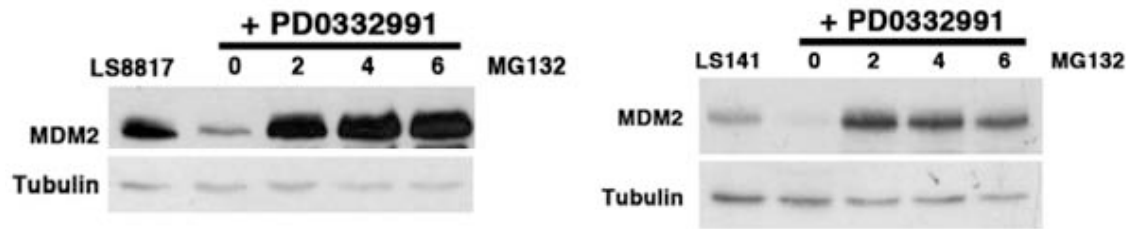


Figure 3.06 MDM turnover following CDK4 inhibition is proteasome-dependent.

The cell lines indicated were treated with PD0332991 for two days after which 10 μ M of the proteasome inhibitor MG132 was added for the indicated times (hours). Tubulin was a loading control.

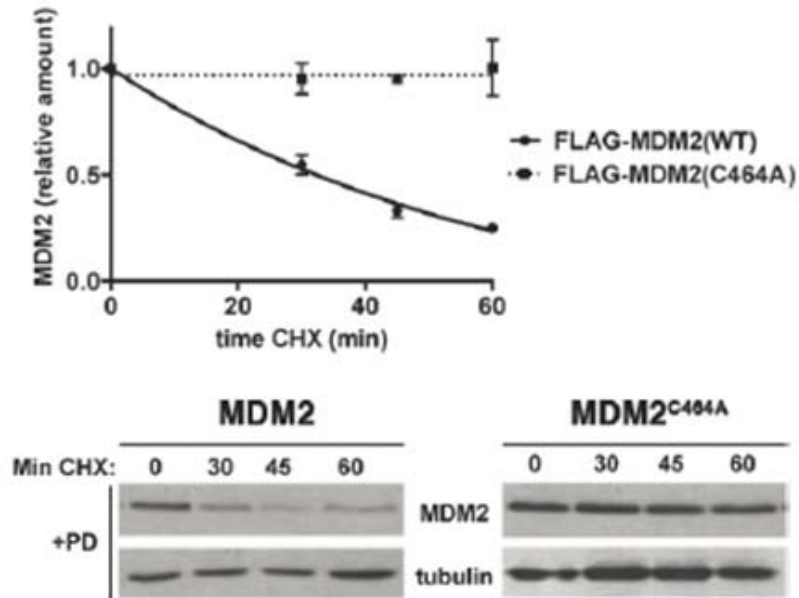


Figure 3.07 MDM turnover following CDK inhibition is dependent on a functional E3 ligase domain.

LS8817 cells were transduced with either a FLAG-tagged MDM2 or a FLAG-tagged C464A mutant of MDM2 as indicated and selected for five days. PD0332991 and cycloheximide were added as described in the legend to Figure 3.05. A representative immunoblot is shown and the graph was compiled from two independent experiments (mean + S.E.M.).

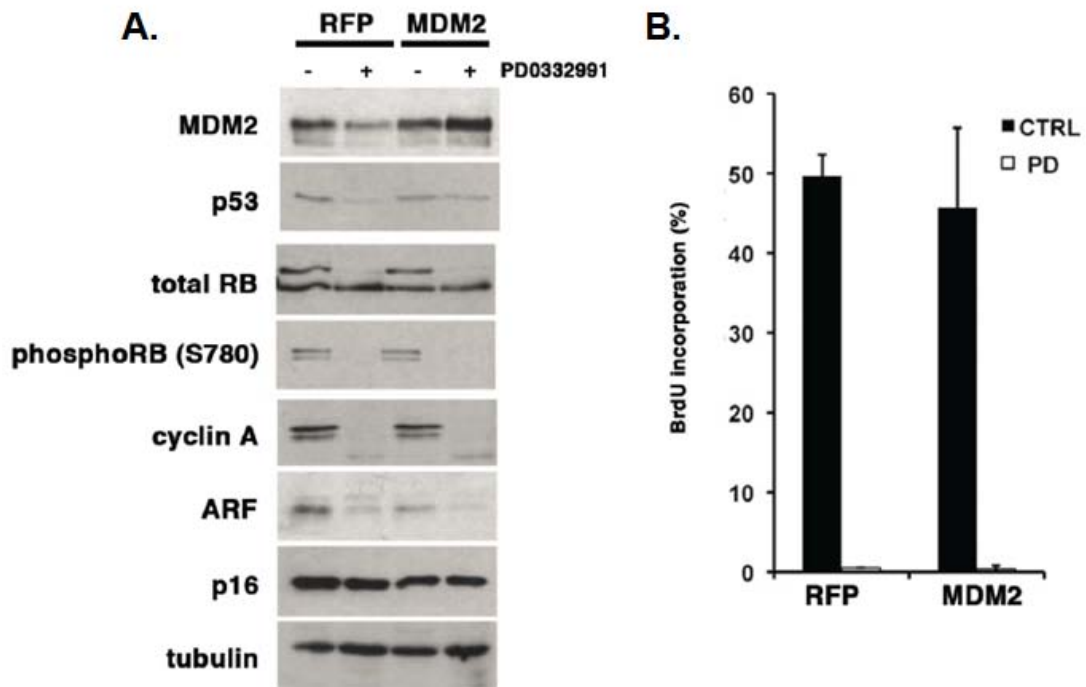


Figure 3.08 Enforcing MDM2 expression does not prevent growth arrest in WD/DDLS cells treated with PD0332991.

(A) LS8817 cells were transduced with a lentivirus engineered to express MDM2 and selected for five days. Cells transduced with a lentivirus expressing RFP were used as a control. After five days of selection the remaining cells were treated with PD0332991 for two days and protein accumulation measured by immunoblot. (B) Cells were treated as described in the legend to panel A; the cells were labeled during the last two hours with 10 μ M BrdU prior to fixation. BrdU-positive cells were quantitated as a fraction of all cells.

CDK4i-induced accumulation of SA- β -gal positive cells (Figure 3.09). Thus, continued MDM2 expression was sufficient to prevent PD0332991 induced senescence. Nevertheless, the cells still exited the cell cycle; thus MDM2 regulates the transition from quiescence into senescence.

To identify which domains of MDM2 were necessary to suppress senescence I expressed different mutant alleles of *MDM2* and scored their effect in PD0332991 treated LS8817 cells. The V75A mutant disrupts binding to p53 (Moll and Petrenko 2003). The C464A mutant eliminates E3 ligase activity by disrupting the cross brace structure of the RING (Foo et al. 2007). The I440A, L468A, or P476A mutants also eliminate E3 ligase activity but do so by selectively disrupting E2 binding (Linke et al. 2008). The Δ 254-264 mutant allele in the acidic domain disrupts multiple MDM2 protein interactions (Sdek et al. 2004). Of these only the V75A mutant was capable of suppressing the PD0332991-induced accumulation of SA- β -gal positive cells (Figure 3.09). Another mutant that affects nucleolar localization (Δ 464 -471) could not suppress accumulation of SA- β -gal positive cells; however, this also disrupts the RING domain, thus precluding a separation of these two functions (Figure 3.09). This indicated that MDM2's ability to suppress PD0332991-induced senescence required its E3 ligase activity and the acidic domain, but was independent of its ability to bind to p53.

Further evidence that the role of MDM2 in senescence following CDK4i is independent of its role in regulating p53, knocking down p53 in LS8817 cells did not disrupt either CDK4 inhibitor induced down-regulation of MDM2 or accumulation of SA- β -gal (or SAHF, not shown), nor did it prevent MDM2 knockdown induced senescence (Figure 3.10). David Liu had shown that p53 levels were in fact decreased following CDK4i treatment in all WD/DDLS cell lines (Kovatcheva et al. 2015). Nevertheless, p53

is wild type and functional in these cell lines, as evidenced by the fact that adding nutlin-3, which inhibits the MDM2-p53 interaction and stabilizes p53, triggers apoptosis (Singer et al. 2007). Typically, p53 mediates senescence induced by DNA damage (d'Adda di Fagagna 2008). Consistent with the observation that CDK4i TIS is p53-independent, we did not observe an increase in the number of 53BP1 or γ H2Ax foci – two markers of double stranded breaks – in drug treated cells (Figure 3.11). Thus, MDM2 mediates senescence in response to CDK4i in a manner that is independent of its regulation of p53.

3.2.4 Amplification of MDM2 is not required for CDK4 inhibitor induced senescence

Senescence caused by CDK4 inhibition has been reported by others in a number of cell lines derived from glioma, breast cancer and non-small cell lung cancer (Hashizume et al. 2016; Baughn et al. 2006; Sawai et al. 2012). These diseases normally are not characterized by MDM2 amplification. Thus, I wanted to examine whether CDK4i-induced downregulation of MDM2 and senescence was dependent on MDM2 amplification, or whether this was a more general result of CDK4i. SNB19, a glioma cell line, is not amplified for MDM2 and expresses a transcriptionally inactive mutant of p53 (R273H) that does not bind to DNA. MDM2 levels decreased in these cells within 48 hours of drug treatment (Figure 3.12) and SA- β -gal and SAHF accumulated in seven days after treatment (Figure 3.12). Similar to what we saw in WD/DDLS cells, enforced MDM2 expression did not affect PD0332991-induced cell cycle exit, but did prevent the accumulation of SA- β -gal (Figure 3.13). Again, mutation of the RING domain abrogated MDM2's ability to suppress senescence and mutation of the p53 binding domain did not affect this (Figure 3.13). Finally, knocking down MDM2 was able to

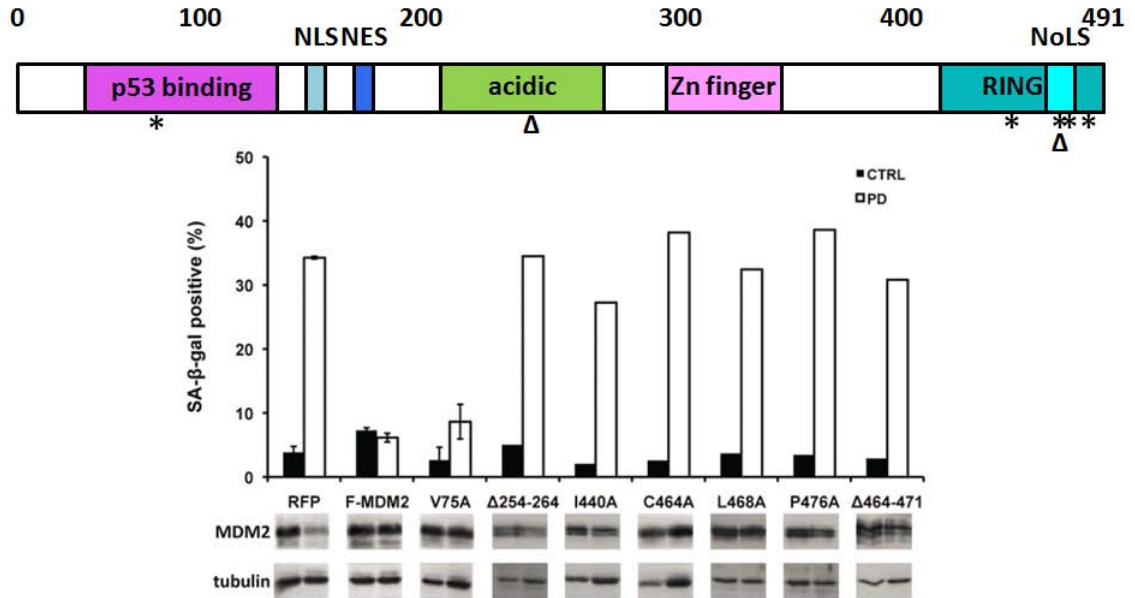


Figure 3.09 Enforcing MDM2 expression prevents senescence in WD/DDLS cells treated with PD0332991 in an E3 ligase-dependent manner.

LS8817 cells were transduced with lentiviruses expressing the indicated mutants as described in the legend to Figure 3.08. Top, diagram representing the domains of MDM2 and corresponding point mutations (*) or deletions (Δ) are indicated. Bottom, The accumulation of SA-β-gal was determined seven days after drug treatment. The expression of MDM2 was measured by immunoblot after 48 hours. Tubulin serves as a loading control.

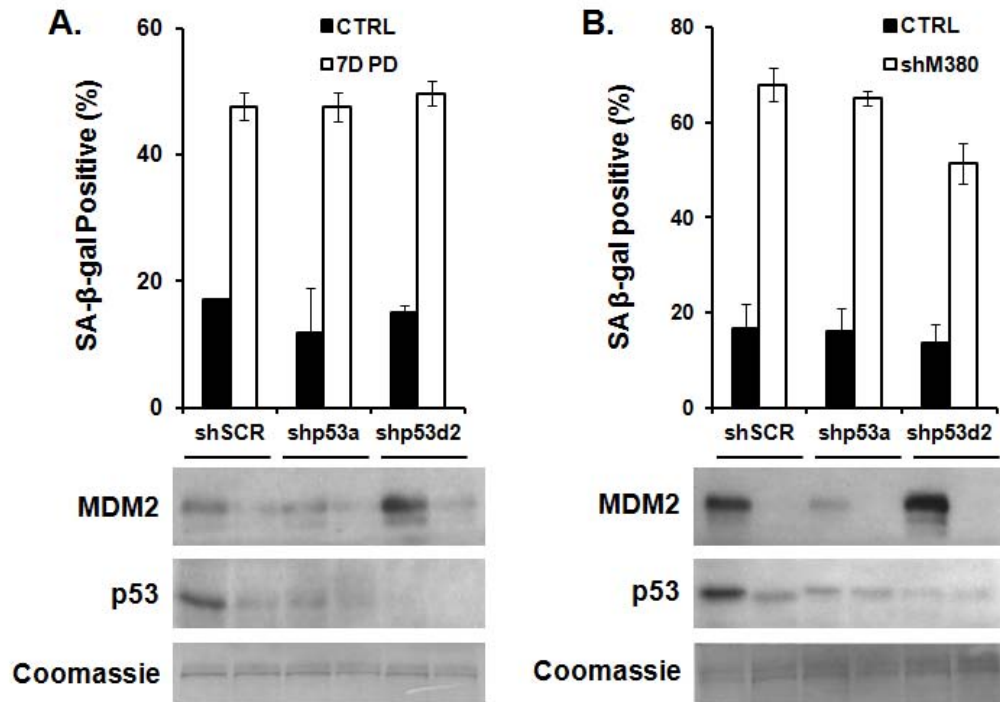


Figure 3.10 Reducing p53 does not affect PD0332991 or MDM2 knockdown induced senescence in LS8817 cells.

LS8817 cells were infected with lentiviruses expressing either a scrambled (shSCR) or two independent p53 targeting (shp53a and shp53d2) shRNAs. After selection these cells were either treated with PD0332991 (A) or MDM2 was reduced by sequential infection with a lentivirus expressing shM380 (B). The accumulation of senescence associated β -galactosidase (top), and the expression of MDM2 and p53 were determined by immunoblot (bottom). Coomassie staining of the gel serves as a loading control.

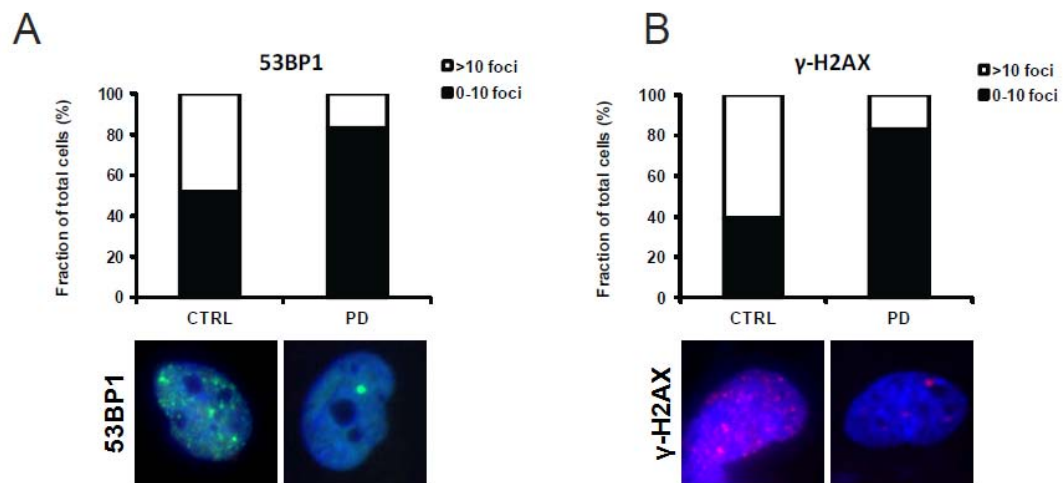


Figure 3.11 CDK4 inhibitor induced senescence causes reduction in the DNA damage markers 53BP1 and γ H2AX in the liposarcoma cell line LS8817.

LS8817 cells were treated with PD0332991 (PD) for 7 days and DNA damage foci detected by 53BP1 (A) γ H2AX (B) immunofluorescence. Graphs are compiled from 200 cells in two independent experiments. Representative images are shown below.

promote accumulation of SA- β -gal positive cells (Figure 3.14). Consequently, MDM2 loss can trigger a p53-independent senescence program even in cancer cells where MDM2 is not amplified.

3.2.5 MDM2 turnover is correlated with a clinically favorable response to PD0332991 in WD/DDLS patients

How to define a senescent cell is problematic because not all hallmarks of senescence are seen with all inducers and many hallmarks are not unique for senescence. Nevertheless, we had shown that changes in MDM2 levels could drive senescence *in vitro*, so I wanted to determine if MDM2 loss associated with CDK4i might correlate with clinical outcome in patients. I was able to look at MDM2 levels in seven paired pre- and post-treatment biopsies from individual WD/DDLS patients enrolled in our colleagues' phase II clinical trial with PD0332991 (NCT01209598) (Dickson et al. 2013).

The specifics of the trial, treatment regimen, criteria for patient outcome are all described (Dickson et al. 2013). Timing of the biopsy is important. Because of neutropenia, patients were given Palbociclib for 3 weeks, and then were taken off the drug for 1 week prior to beginning another 4-week cycle. Post-treatment biopsies were collected during the week off of the drug, between cycle 1 and cycle 2. Three of these patients had stable disease for more than 1 year, one had a complete remission by RECIST 1.1 criteria, and three did poorly on the drug. To measure MDM2 I extracted protein from pre- and post-treatment biopsies and performed immunoblots. I also blotted GAPDH as a loading control and phosphorylated Rb to confirm that the drug had hit the target. MDM2 was reduced in all four post-treatment samples from patients who performed well, and was not reduced in patients who performed poorly (Figure 3.15).

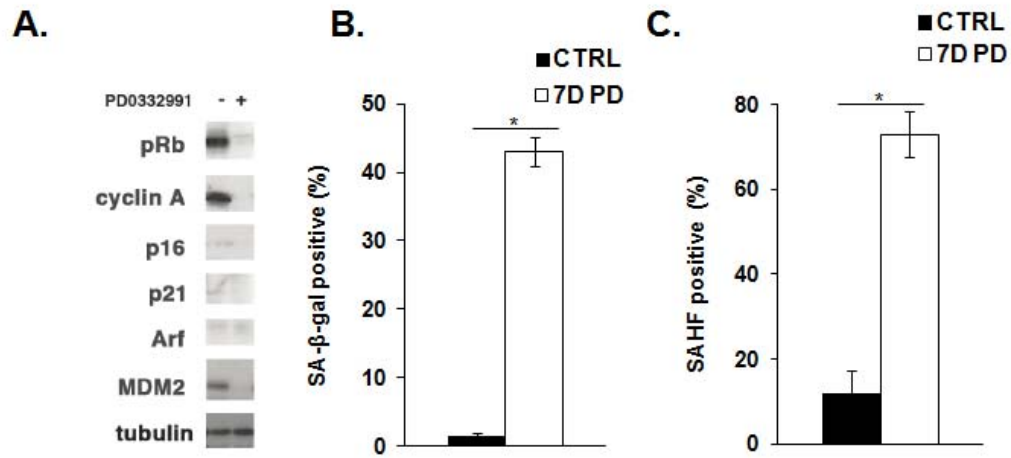


Figure 3.12 PD0332991 induces senescence in the glioma-derived SNB19 cell line.

SNB19 glioma cell lines were treated with 1 μ M PD0332991 and protein expression measured by immunoblot (A) two days later. Tubulin is a loading control. The accumulation of SA- β -gal (B) and HP1 γ foci (C) was measured seven days after drug treatment. * $p < 0.05$.

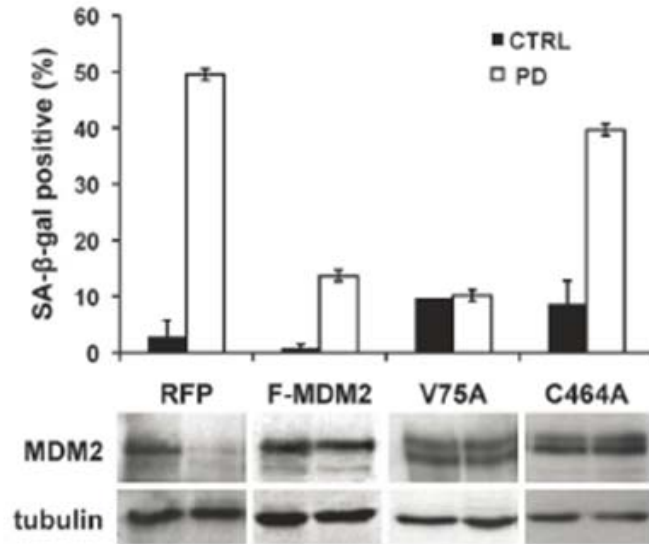


Figure 3.13 Enforced MDM2 expression can bypass PD032991-induced senescence in an E3 ligase-dependent manner in SNB19 cells.

Wild type and mutant MDM2 proteins were expressed in SNB19 glioma cells and analyzed as described in the legend to Figure 3.09. SA-β-gal was plotted (upper) and MDM2 expression levels were measured by immunoblot (lower). Tubulin is a loading control.

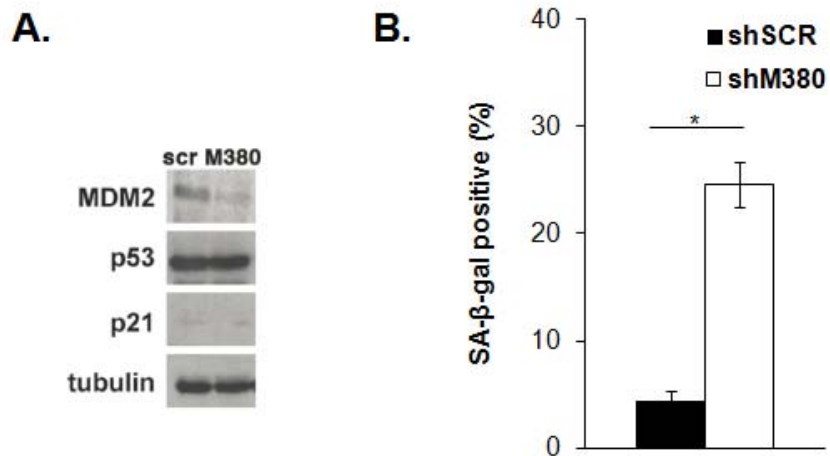


Figure 3.14 MDM2 knockdown can induce senescence in SNB19 cells.

MDM2 was knocked down in SNB19 cells with shM380 as described in the legend to Figure 3.01 and the effect on accumulation of p53 and p21 protein levels (A) and SA-β-gal positive cells (B) are shown. * $p < 0.05$.

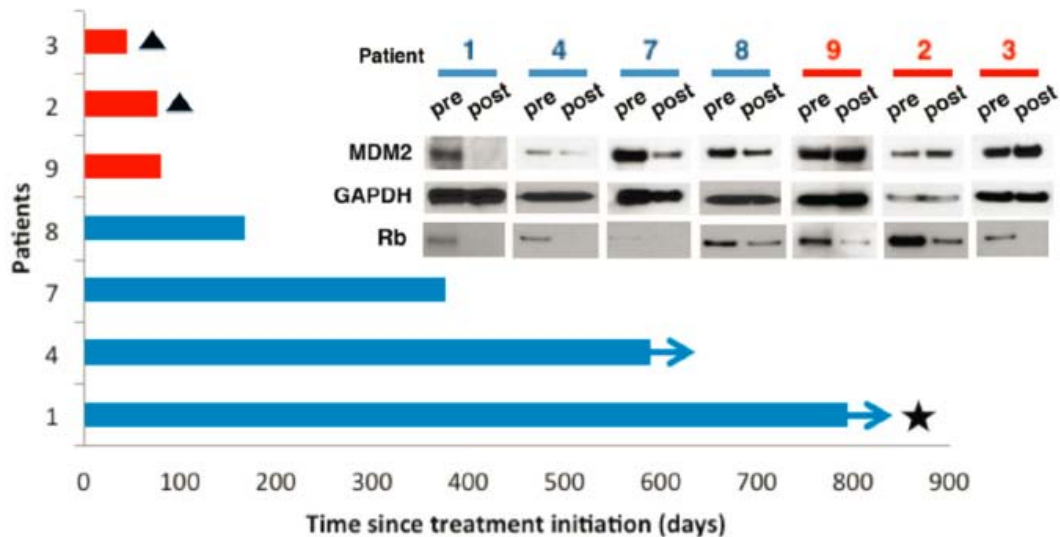


Figure 3.15 MDM2 loss is associated with patient response to Palbociclib.

Seven patients with measurable advanced WD/DDLS confirmed positive for CDK4 amplification and Rb protein expression, and whom had progressed on at least one systemic therapy prior to enrollment (clinicaltrials.gov identifier NCT01209598), consented to pre-treatment biopsies taken prior to therapy and post-treatment biopsies taken after receiving one dose of oral PD0332991 (125mg daily for 21 days). Tumor response was assessed with CT scan by a reference radiologist every 6 weeks for 36 weeks, and every 12 weeks thereafter. Patients were followed up until March 2014. Three patients (red bars) did not derive clinical benefit and stopped treatment within 84 days. Two of these patients (#2 and #3) died on study and are indicated with a triangle. The third patient, #9, came off study as their disease progressed. Four patients (blue bars) had demonstrable clinical benefit, remaining on treatment for more than 84 days. Two of these progressed after 168 (#8) and 376 (#7) days respectively, while two remained on treatment with ongoing benefit (arrows), one achieving a complete response (star). (Inset) Extracts were prepared from pre- and post-treatment biopsies of protein expression measured by immunoblot. GAPDH was a loading control.

Phosphorylated Rb was reduced in all the PD0332991-treated tissues relative to the pre-treatment biopsy, suggesting the drug was hitting the target in all patients, but the cells in some patients were returning to cycle. Thus, in a small pilot study of WD/DDLS patients, PD0332991-induced down regulation of MDM2 associated with a positive response to therapy.

3.3 Discussion

The marked success of CDK4 inhibitor drugs in clinical trials has been partially shadowed by their inability to offer any benefit to a sizeable proportion of patients. The efficacy of CDK4 inhibitor drugs is limited by the lack of appropriate preclinical biomarkers to identify patients as candidates who would derive benefit from CDK4i therapy. To date, only Rb status, p16 expression levels, and *CDK4* amplification status have been screened as qualifiers for CDK4i administration in patients (Konecny et al. 2011; Dickson et al. 2013; Finn et al. 2015). However, none of these biomarkers have proven successful as upwards of 30% treated patients, regardless of their cancer type, exhibit intrinsic resistance to CDK4i drugs.

In the MSKCC WD/DDLS Palbociclib Phase II trial (NCT01209598), patients were also screened for genomic *CDK4* levels via fluorescence *in situ* hybridization; all patients enrolled in the trial were required to exhibit *CDK4* amplification. *CDK4* status did not affect patient response in this case, as nearly one third of patients failed to derive any benefit from the therapy despite amplification (Dickson et al. 2013). It is difficult to test whether WD/DDLS patients without *CDK4* amplification would benefit from Palbociclib, as such tumors are quite rare (less than 10% of all WD/DDLS cases).

High levels of p16 expression are often associated with Rb loss and thus could predict failure to CDK4i therapy (Konecny et al. 2011; Romagosa et al. 2011; Ertel et al.

2010). However, high p16 does not absolutely correlate with Rb loss; in many tumor cell lines the level of p16 is high despite an intact Rb (Romagosa et al. 2011). Thus, while pre-screening both Rb and p16 could be useful, there is concern regarding the utility of p16 as a biomarker.

Another potential biomarker of interest is cyclin D, the cognate binding and activating partner(s) of CDK4/6. Many studies have considered cyclin D1 amplification – which is common in cancer – as a potential biomarker for CDK4i therapy (Musgrove et al. 2011). There is work from breast cancer and pancreatic cancer models to suggest that cyclin D1 upregulation in response to CDK4i treatment may drive resistance, but molecularly how this resistance might occur is unclear (Dean et al. 2010; Franco et al. 2014). Moreover, a Phase II study in estrogen-driven breast cancer that separated patients into two cohorts, one of which had a requirement for cyclin D1 amplification, challenged this hypothesis. There was no measurable difference between the Palbociclib imparted benefit on progression free survival across these two cohorts, suggesting that cyclin D1 levels do not associate with patient response (Finn et al. 2015). A related biomarker to consider may be cyclin E; CDK4/6 is dispensable for driving cell cycle when cyclin E is over-expressed (Lukas et al. 1997). While a direct clinical correlation remains to be established, cyclin E is upregulated in the basal subtype of breast cancer (Finn et al. 2016a) and cell lines derived from basal breast cancers used in an *in vitro* study were found to be most resistant to growth arrest by PD0332991 (Finn et al. 2009).

The work I have presented here suggests that whether a cell undergoes quiescence or senescence in response to CDK4i may underlie the clinical efficacy of this class of drugs. Importantly, we repeated our work with two other CDK4i drugs, Abemaciclib and Ribociclib, as well as with shRNA targeting CDK4 and similar results were obtained,

regardless of the mode of CDK4 inhibition ((Kovatcheva et al. 2015) and data not shown). As discussed in section **1.3 Assaying senescence: a collection of associated hallmarks, but no *bona fide* markers**, measuring senescence – particularly in *in vivo* specimens – is problematic (Sharpless and Sherr 2015). However, we were able to correlate the reduction of MDM2 following CDK4i therapy with senescence *in vitro*; moreover, a reduction of MDM2 also correlated with favorable outcome following CDK4i therapy *in vivo* in a small pilot of seven WD/DDLS patients. Although MDM2 cannot serve as a prognostic biomarker to predict which patients will benefit from CDK4i, the turnover of MDM2 following treatment may predict a patient's trajectory and thus whether they should to continue to receive CDK4i. An understanding of how this MDM2 change is achieved may lead to the development of pre-treatment biomarkers. For a further discussion on this, see section **6.2.1 A prognostic biomarker for CDK4i**.

As discussed in section **1.4 Physiologic consequences of senescence**, there is ample evidence to suggest that senescence may underlie a favorable response to cancer therapy. In our study, one WD/DDLS patient had complete remission of their disease by RECIST 1.1 criteria. It is intriguing that a cytostatic therapy like Palbociclib could result in remission. While we do not have direct evidence of senescence in the tumor cells, nor of infiltration by immune cells, it is tantalizing to speculate, based on the *in vitro* data and MDM2 correlate, that this may have been mediated by SASP-induced tumor clearance. It will be illuminating to test whether CDK4i can stimulate immune clearance of tumor cells in animal models of diseases that are known to undergo senescence in response to therapy, such as lung cancer and breast cancer (Puyol et al. 2010; Choi et al. 2012).

Currently, an additional Phase II clinical trial in WD/DDLS is underway at Memorial Sloan Kettering Cancer Center using the CDK4i drug Abemaciclib (NCT02846987). It will be valuable to assess whether MDM2 levels correlate with outcome in a larger patient population, whether other markers of senescence like SA- β -gal can be detected in post-treatment tissue and how these correlate with MDM2 turnover *in vivo*, and perhaps whether there is an enrichment of infiltrating immune cells in samples from the patients with favorable outcomes. Such observations would strengthen the interpretation that senescence is associated with a positive outcome in response to CDK4i, and future studies could focus on combinatorial therapies that would promote senescence; for a more detailed discussion on this, see **6.3: Clinical Implications**.

Additionally, we have shown that the loss of MDM2 drives the CDK4i-induced transition from quiescence to senescence in a p53-independent manner and that the E3 ubiquitin ligase activity of MDM2 is required to suppress senescence. Collectively, these data suggest that there is an alternative substrate of MDM2 that regulates senescence. In light of these findings, one obvious therapeutic approach would be to inhibit the E3 ligase activity of MDM2 to promote senescence. Unfortunately, while there are ongoing efforts to develop such drugs and there are already existing compounds such as HLI-373, none have been tested in the clinic (Kitagaki et al. 2008). Given the important general role of MDM2 in regulating p53 and the tendency of high p53 levels to induce apoptosis, it is conceivable that such drugs would result in hematologic toxicity and clinical failure, much like the early nutlin compounds (Lane et al. 2011). Thus, it may be more favorable to identify and target (if possible) the relevant substrate(s) of MDM2 in CDK4i induced senescence; for a further discussion on this, see **6.2.2 The MDM2 substrate**

Collectively, I have shown that the downregulation of MDM2 drives the transition from quiescence to senescence (gerconversion), is associated with senescence in response to CDK4i therapy and underlies a favorable clinical outcome. Through a combination of molecular and cellular approaches, using material from *in vitro* cell lines, animal models and human patient samples, we seek to address the outstanding questions regarding the clinical efficacy of CDK4i drugs and ultimately stratify patients for treatment on the basis of prognostic biomarkers and/or rational combinatorial therapies.

4. ATRX IS A REGULATOR OF THERAPY INDUCED SENESENCE REQUIRED FOR FORMATION AND MAINTENANCE OF THE SAHF

4.1 Introduction

CDK4 inhibition allows us to probe the requirements for senescence in a fashion that is different from previous studies. Typically the gene products associated with senescence have been identified by their accumulation or loss comparing cycling cell populations with senescent cell populations (Schwarze et al. 2005; Lackner et al. 2014; Fridman and Tainsky 2008; Narita et al. 2006). Generally, the causal relationship of these observations to senescence are established when the investigator removes the identified gene product and the cell no longer undergoes senescence when challenged with the same senescence inducer, but rather remains in the proliferative cell cycle. Consequently many of these so-called senescence regulators are proteins that govern cell cycle exit, such as p16, proteins that regulate p16 expression, p53, p21, and so on (Lundberg et al. 2000; Kuilman et al. 2010). Cell cycle exit – although a prerequisite for senescence – is certainly not exclusive to senescent cells.

CDK4 inhibitors provide a new experimental system from which to gain insight into senescence. As discussed in **Chapter 3**, CDK4 inhibition causes cell cycle exit in Rb positive cells, but only some cells progress further into senescence (i.e. geroconversion). Geroconversion is probably related to the stress associated with ongoing receptor tyrosine kinase and integrin signaling in a cell in which the activity of cyclin D-CDK4 is inhibited (Blagosklonny 2014). Regardless, this distinction between quiescence and senescence allows one to identify gene products that are directly involved in geroconversion, independent of their role(s) in the cell cycle.

Having established that the change in MDM2 level was associated with whether cells underwent geroconversion in response to CDK4 inhibition, I was interested in identifying other gene products that might regulate this transition. While we know a great deal about the molecular events that govern cell cycle exit (Giacinti and Giordano 2006), we have substantially less knowledge about the events that occur following growth arrest and before the acquisition of the senescent state (Figure 1.01). I set out to fill this gap with the hope that it might provide insight into common events and core machinery that drive cells into the irreversible senescent state, regardless of the nature of the signal or type of the cell.

In this chapter I discuss how I mined genomic information from TCGA to identify ATRX, a chromatin remodeling enzyme, as a regulator of senescence. In **Chapter 6: Discussion**, I elaborate on other experimental approaches that might be useful to identify novel senescence regulators in the future.

4.1.1 ATRX is commonly mutated in soft-tissue sarcoma.

The third most commonly dysregulated gene in WD/DDLS (after MDM2 and CDK4) is ATRX (alpha-thalassemia mental retardation, X-linked) (Lee et al. 2015). ATRX is a chromatin remodeling protein that belongs to the SWI/SNF family of helicases. ATRX bears an atypical PHD-finger domain that facilitates chromatin binding by way of direct interactions with H3K9me3/H3K4me0 modified histone monomers. ATRX can also bind to chromatin via an interaction with heterochromatin protein 1 (HP1) (Eustermann et al. 2011). ATRX is involved in replication-independent deposition of the histone variant H3.3 and has been implicated in the maintenance of constitutive heterochromatin at telomeres and pericentric regions (Wong et al. 2010; Goldberg et al. 2010; Lewis et al. 2010), in the regulation of mH2A (Ratnakumar et al. 2012), as well as at heterochromatic sites throughout the genome, like silenced imprinted alleles in embryonic stem cells (Voon et

al. 2015) and in X-chromosome inactivation (Sarma et al. 2014). Loss or mutation of ATRX has been associated with the genetic syndrome from which its name is derived, as well as with the alternative lengthening of telomeres (ALT) in a variety of cancers (Basehore et al. 2015; Stevenson 1993; Lovejoy et al. 2012; Napier et al. 2015). ALT is a homologous recombination-based mechanism employed to maintain telomeres in telomerase-deficient cells (Cesare and Reddel 2010; Dilley et al. 2016).

To identify potential senescence regulators, I used the gene association pathway analysis tool GeneMANIA (Warde-Farley et al. 2010) and queried known components and/or regulators of the senescence associated heterochromatic foci (SAHF) including PML, mH2A, histone H3.3 and the HP1 family of proteins (Zhang et al. 2007). Intriguingly, ATRX was one of the genes that this software associated with the SAHF components (Figure 4.01).

SAHF contain repressive chromatin modifications like trimethylation on histone H3 (H3K9Me3) and aforementioned repressive proteins associated with chromatin compaction and gene silencing including the HP1 family, macro H2A (mH2A) as well as the structural protein HMGA (Adams 2007; Chandra and Narita 2013; Narita et al. 2006; Tu et al. 2013; Rai et al. 2014; Kennedy et al. 2010; Rai et al. 2011; Zhang et al. 2005). Functionally, the SAHF can contribute to the stability of growth arrest as cells become senescent (Narita et al. 2003). It has been reported that these repressive proteins and modifications, along with the tumor suppressor Rb, are recruited to the promoters of E2F target genes in senescent, but not in reversibly arrested quiescent cells. Their recruitment is required for the suppression of E2F target genes that normally drive proliferation.

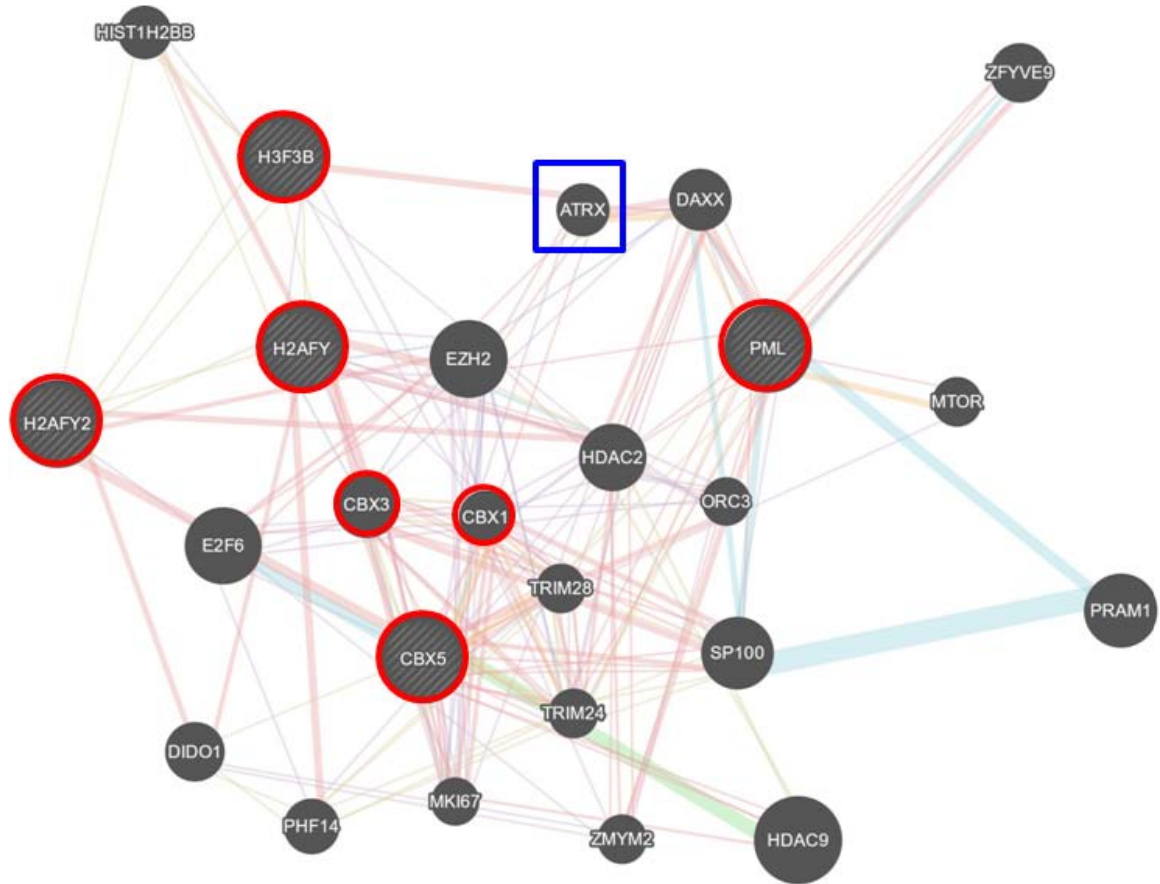


Figure 4.01 SAHF components are functionally associated with ATRX.

The SAHF components mH2A (H2AFY, H2AFY2), HP1 (CBX1, CBX3, CBX5), PML and histone H3.3 (H3F3B) (all circled in red) were entered into the predictor program GeneMANIA (Warde-Farley et al. 2010), which determines genes related to the input genes based on extremely large sets of functional association data. ATRX, which was identified in the network by the program, is boxed in blue.

PML nuclear bodies (PML-NBs) are also important for SAHF formation. PML-NBs range in size from 0.1-1.0 μm and are composed of their key organizing protein, the tumor suppressor promyelocytic leukemia (PML), along with a host of other dynamic and condition-specific proteins (Bernardi and Pandolfi 2007; Vernier et al. 2011; Vernier and Ferbeyre 2014). PML-NBs are present in most cell lines and their numbers increase under a variety of stress conditions; they play roles in apoptosis, the response to viral infection, DNA damage and senescence. In the context of senescence, HP1 γ has been shown to transit through the PML-NBs, where it becomes phosphorylated (Zhang et al. 2007). This modification is essential for the incorporation of HP1 γ into the SAHF. Additionally, the histone chaperone HIRA has been reported to transit through the PML-NBs and is important for senescence (Zhang et al. 2005). HIRA, along with another chromatin regulator ASF1, deposits the histone variant H3.3 in a replication-independent manner, which has important implications for how chromatin structure can be maintained in a non-dividing senescent cell (Corpet et al. 2014). A proteolytically cleaved product of histone H3.3 has been shown to drive senescence (Duarte et al. 2014).

Based on the frequency of ATRX mutation in WD/DDLS, the interaction of ATRX with components of the SAHF, and the importance of SAHF formation to entering senescence, I decided to ask whether ATRX had a role in driving the transition from quiescence to senescence.

4.2 Results

4.2.1 ATRX is required for therapy induced senescence

Approximately one third of soft tissue sarcomas are characterized by the alternative lengthening of telomeres (ALT) (Lee et al. 2015; Lee et al. 2012). Thus, we looked at

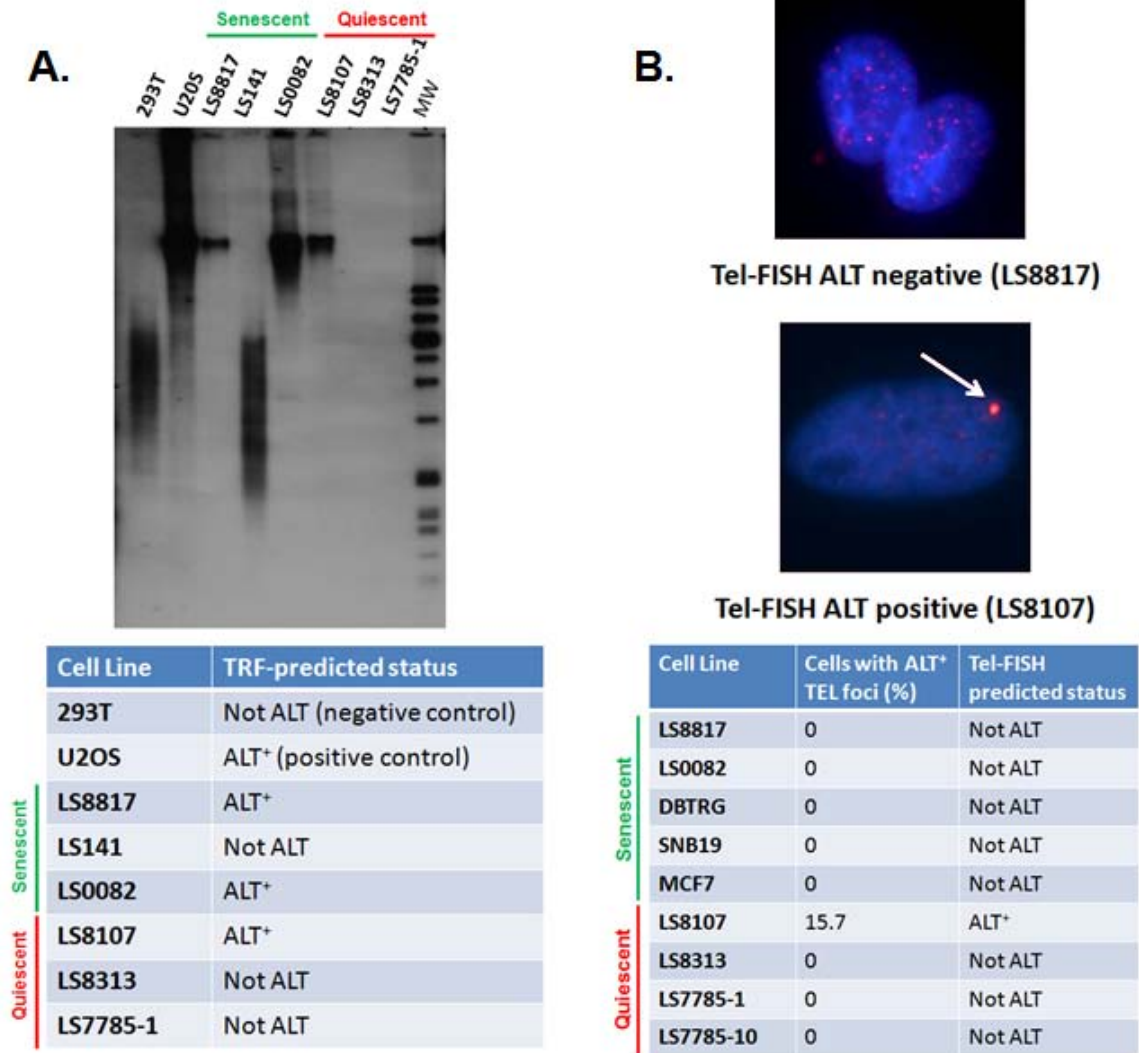


Figure 4.02 Senescence response to CDK4 inhibition does not associate with the ALT status of the cell lines.

(A) The telomere restriction fragment length assay (TRF) and (B) the telomere-FISH assay (tel-FISH) were used to assess ALT status in the WD/DDLS cell lines. Representative examples are shown (upper) and the outcome tabulated (lower). In the tel-FISH assay the arrow points to the large “dot” indicative of ALT whereas the smaller distributed dots are indicative of a non-ALT cell. “Senescent” and “Quiescent” refers to the cell line’s response to CDK4 inhibition. Data courtesy of Beth Ashbridge (TRF) and Akiko Inagaki (Tel-FISH).

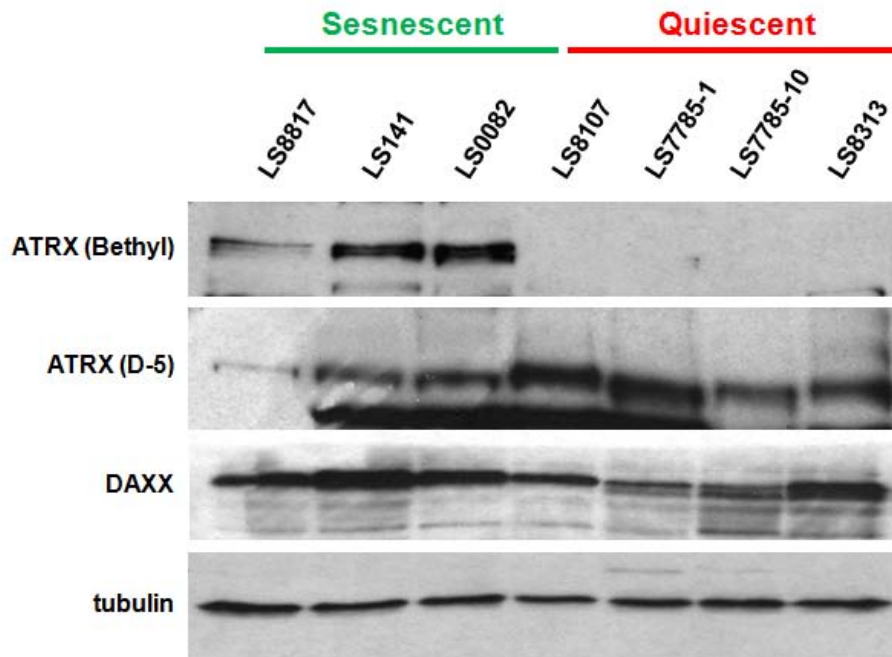


Figure 4.03 Senescence response to CKD4 inhibition correlates with ATRX reactivity to a Bethyl antibody.

Extracts were prepared from asynchronously growing cells and immunoblotted with the indicated antibodies. Tubulin was a loading control. “Senescent” and “Quiescent” refers to the cell line’s response to CDK4 inhibition.

whether ALT was associated with the response of cells to CDK4i. With the help of Akiko Inagaki and Beth Ashbridge, we measured ALT by both telomere-FISH and telomere restriction fragment assays (Engelhardt et al. 1998; Lovejoy et al. 2012) (Figure 4.02). I also looked at the expression of ATRX and DAXX as these proteins are commonly not expressed in ALT-positive cells (Heaphy et al. 2011). Decreased ATRX levels have also been reported to associate with melanoma progression (Qadeer et al. 2014). All seven of the WD/DDLS cell lines expressed both ATRX and DAXX (Figure 4.03) and ALT did not associate with response. However, I noted that the ATRX antibody from Bethyl Laboratories did not detect ATRX in all four of the quiescent cell lines. Thus, expression of this isoform of ATRX was associated with response to CDK4i (Figure 4.03).

Consequently, I asked whether ATRX was needed for CDK4i to induce senescence. To explore this, I acutely knocked down ATRX in LS8817 cells with two different shRNA vectors (Figure 4.04) and measured the effect on accumulation of SA- β -gal positive cells and SASP. In these ATRX-deficient cells, the drug still induced cell cycle exit, but MDM2 did not decrease, nor was its turnover accelerated (Figure 4.04). PD0332991 treatment did not induce significant accumulation of SA- β -gal positive cells or secretion of SASP factors as measured by cytokine array panels (Figure 4.05). This indicated that ATRX was required for the regulation of MDM2 in cells that undergo senescence when treated with CDK4i. However, this experiment did not assess the role of ATRX directly in senescence, as I had already shown that stable MDM2 levels suppress senescence.

To examine whether this was the only role that ATRX had in senescence I knocked down MDM2 and ATRX in the LS8817 cell line. As expected, knocking down MDM2 induced the accumulation of HP1 γ foci and SA- β -gal (Figure 4.06). However, the accumulation of HP1 γ foci and SA- β -gal was significantly reduced when knocking down

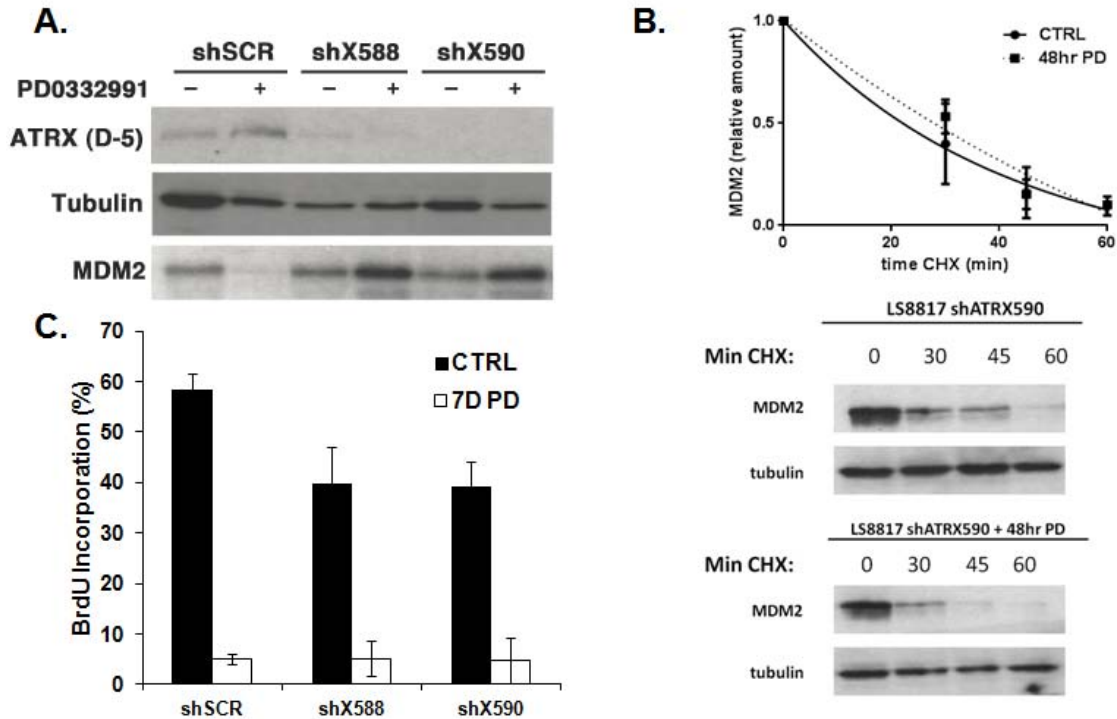


Figure 4.04 ATRX is required for PD0332991 induced down-regulation of MDM2.

(A) Protein expression was measured by immunoblot 48 hours after drug treatment in cells transduced with lentiviral vectors targeting ATRX (shX588, shX590) or a scramble control (shSCR). (B) MDM2 turnover rates were measured in LS8817 shX590 expressing cells 48 hours after treatment with PD0332991 and compared to turnover in untreated cells as described in the legend to Figure 3.05. This experiment was repeated twice and the mean and SEM were plotted (upper). Representative immunoblots are shown (lower). (C) Loss of ATRX does not affect proliferative arrest induced by PD0332991. The same cells as in panel (A) were treated with PD0332991 for 48 hours and pulsed with BrdU. The fraction of cells incorporating BrdU were plotted.

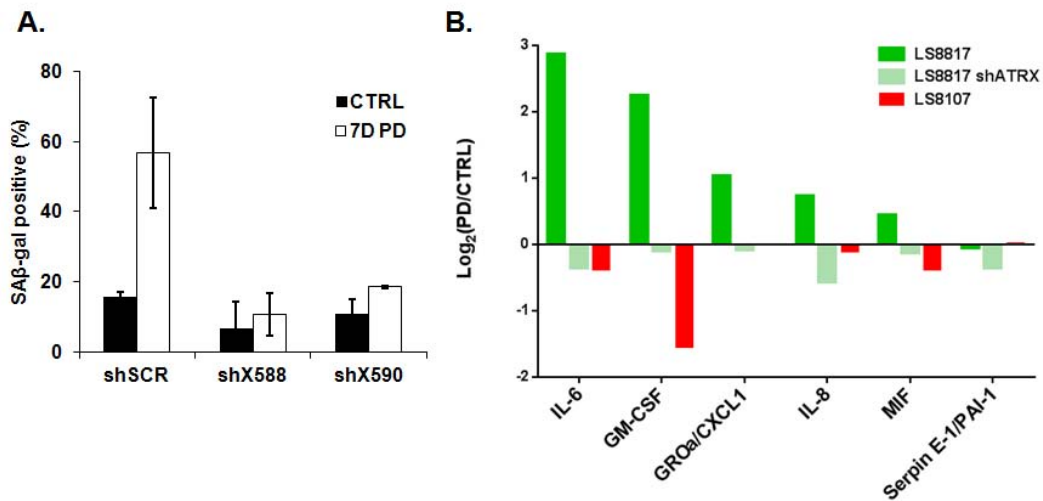


Figure 4.05 ATRX is required for SA-β-gal induction and elaboration of the SASP in response to CDK4 inhibition in WD/DLS cells.

The cells described in Figure 4.04 were treated with 1 μM PD0339221 for seven days. (A) SA-β-gal accumulation was measured. (B) 24 hours prior to harvest, media was changed (with drug treatment maintained). Media was harvested, filtered through 0.22 μm filters and volume was normalized according to cell number. Cytokine arrays (R&D Biosystems ARY005) were performed according to manufacturer's instructions. Autoradiograms were quantified and the log₂ fold change of SASP factors in the drug treated versus control conditioned media was plotted. LS8017 cells, which undergo quiescence in response to CDK4 inhibition, were included as a negative control.

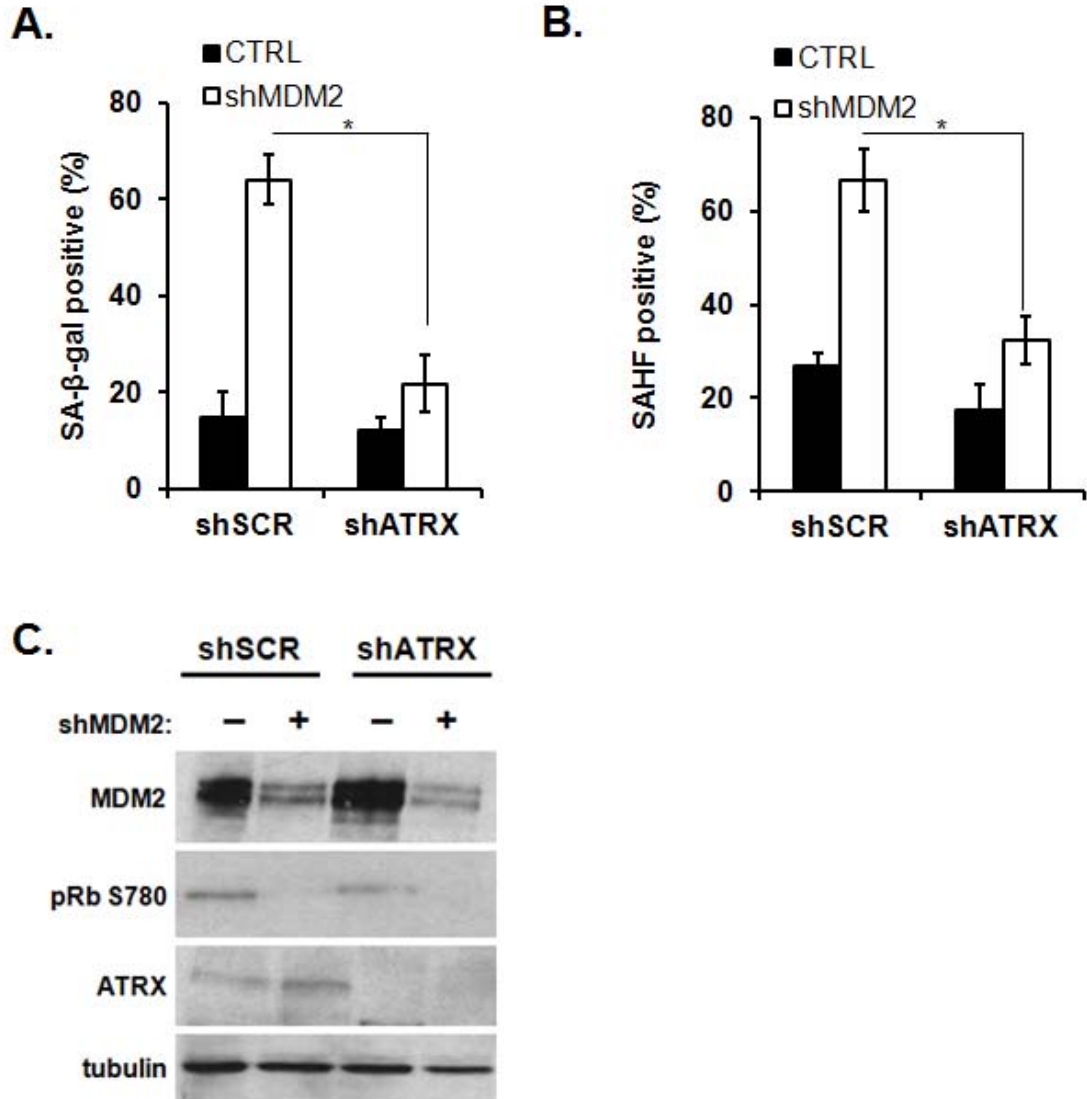


Figure 4.06 ATRX is required for MDM2 knockdown induced senescence in WD/DDLS cells.

(A-C) The LS8817^{scr} and LS8817^{shATR^X} cells described in Figure 4.04 were transduced with an shRNA vector targeting MDM2 and the effect on accumulation of SA-β-gal (A), SAHF formation (B) and protein abundance (C) was determined. * p < 0.05.

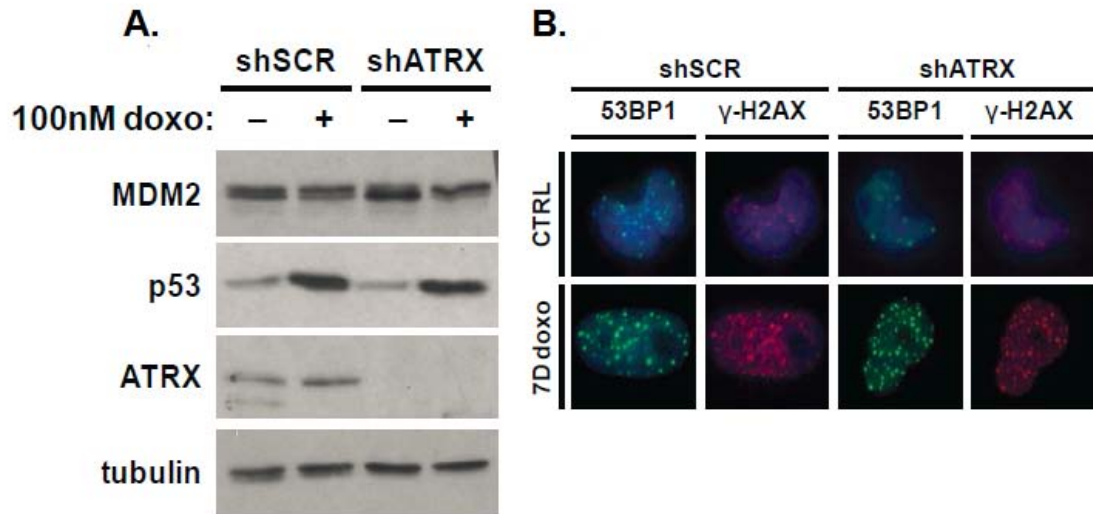


Figure 4.07 : Loss of ATRX does not affect the DNA damage response to doxorubicin.

LS8817 cells were transduced with either a scrambled (shSCR) or ATRX specific (shATRAX) lentiviral knockdown vector and subsequently treated with 100nM doxorubicin for 7 days. (A) Extracts were made from the cells indicated above each lane and the expression of proteins determined by immunoblot as indicated on the left of each panel. (B) Cells were stained with the antibodies indicated on top of each panel.

both ATRX and MDM2, albeit the cells still exited the cell cycle as determined by the decrease in the amount of phosphorylated Rb (Figure 4.06). Thus, ATRX does have a direct role in driving the transition from quiescence to senescence, at least in the context of CDK4i, independent of its role in regulating MDM2 turnover.

As previously described, there are many different stressors that can induce senescence. CDK4 inhibition is an example of therapy induced senescence (TIS) that is induced independently of p53; however, many other forms of TIS require functional p53. To determine if ATRX played a more general role in other forms of TIS I treated the ATRX deficient LS8817 cells with doxorubicin. Doxorubicin causes DNA damage in the control LS8817 cells as judged by the accumulation of p53 and an increase in the number of 53BP1 and γ H2Ax foci (Figure 4.07). Doxorubicin also induced the accumulation of SA- β -gal and HP1 γ foci, as well as increased the expression of CXCL1, IL-6, and IL-8 mRNAs, markers of the SASP (Figure 4.08). At this dose the cells exit the cell cycle and there was no increase in cell death (Figure 4.08). ATRX deficiency prevented neither the accumulation of p53 nor the increase in the number of 53BP1 and γ H2Ax foci (Figure 4.07) and the cells still exited the cell cycle (Figure 4.09). However, knocking down ATRX attenuated the accumulation of SA- β -gal and HP1 γ foci, and the increase in the SASP cytokines (Figure 4.08). Thus ATRX is a broader regulator of TIS, acting both upstream of MDM2 following CDK4 inhibition, and downstream of this in both p53-independent CDK4 inhibitor TIS and DNA damage p53-dependent TIS.

4.2.2 The number of ATRX foci increases in senescent cells

ATRX is a chromatin remodeling enzyme and its expression is largely unchanged in cells treated with CDK4 inhibitors (Figure 4.04) or following MDM2 knockdown (Figure 4.06), or after exposure to doxorubicin (Figure 4.07). I looked to see if there was a

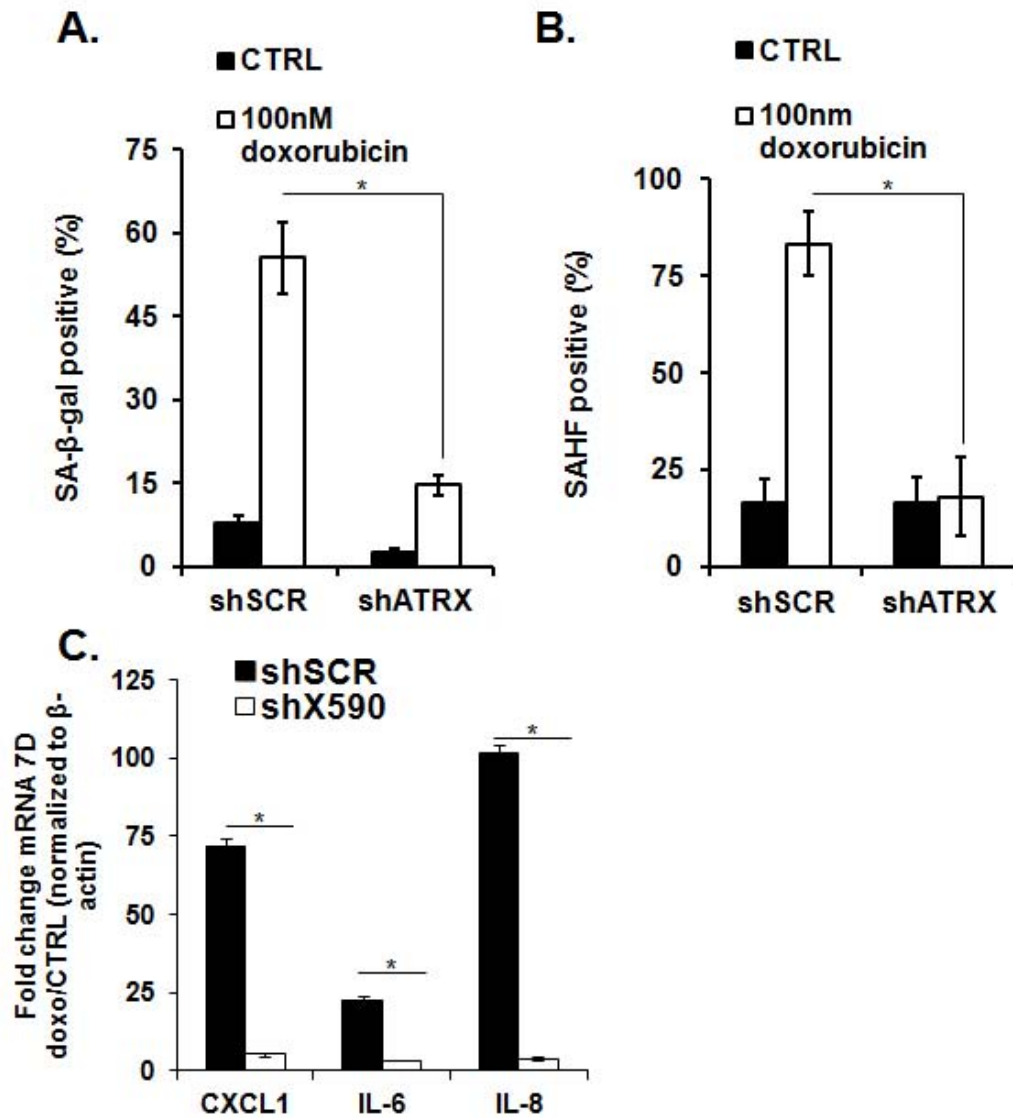


Figure 4.08 ATRX is required for doxorubicin-induced senescence in WD/DDLS cells.

Cells were treated as described in Figure 4.06 The accumulation of SA-β-gal positive cells (A) or SAHF-positive cells (B) for each individual treatment condition was measured. (C) The accumulation of three of the liposarcoma SASP transcripts was measured by qPCR under each indicated condition and their induction in treated cells relative to that in untreated cells is plotted and compared. * $p < 0.05$.

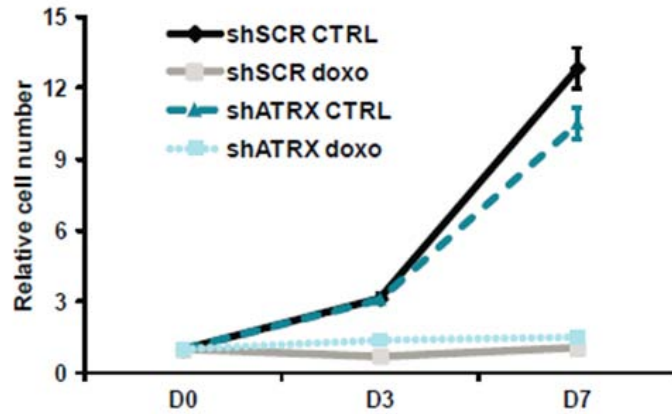


Figure 4.09 Loss of ATRX does not affect cell cycle growth kinetics of LS8817 cells.

LS8817 cells with or without ATRX as indicated were plated on day 0 and either treated with 100nM doxorubicin (doxo) or left untreated (CTRL). The number of cells was counted at the indicated days and was plotted.

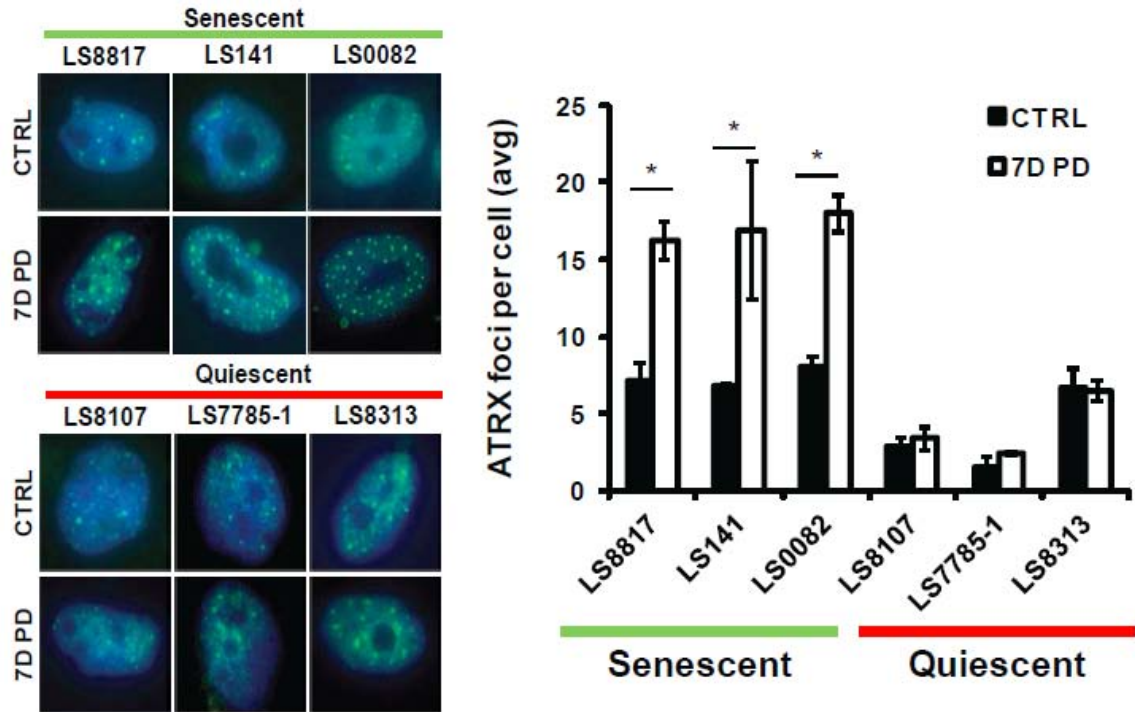


Figure 4.10 ATRX accumulates in nuclear foci in senescent but not quiescent WD/DDLS cells.

The indicated liposarcoma cell lines were treated with PD0332991 for 7 days as previously described (Kovatcheva et al., 2015). The outcome of this treatment vis a vis quiescence or senescence is indicated. ATRX foci were detected by immunofluorescence and the average number of foci per cell was plotted (right). Representative images are shown (left). * $p < 0.05$.

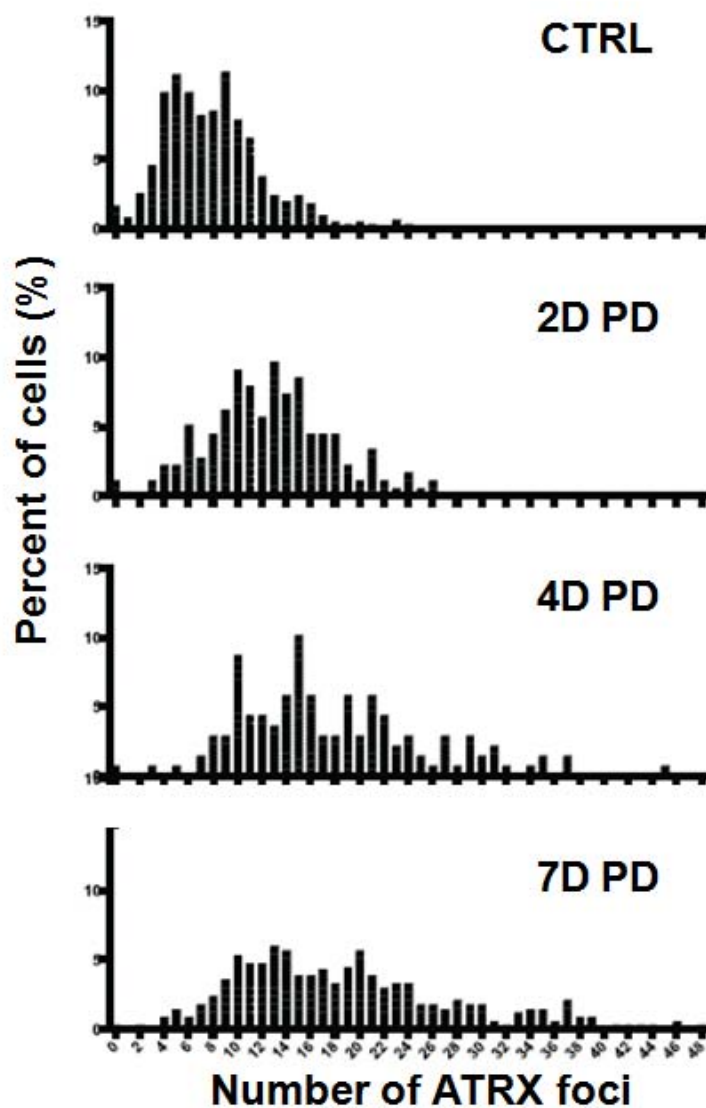


Figure 4.11 Kinetics of ATRX foci formation following PD0332991 treatment in WD/DDLS cells that undergo senescence.

LS8817 cells were treated with PD0332991 for 2, 4, or 7 days, fixed and stained with ATRX antibodies. The numbers of ATRX foci were counted in these and in control untreated cycling cells. The percentage of total cells with the number of foci indicated on the x-axis is plotted. At least 150 cells were counted on each day.

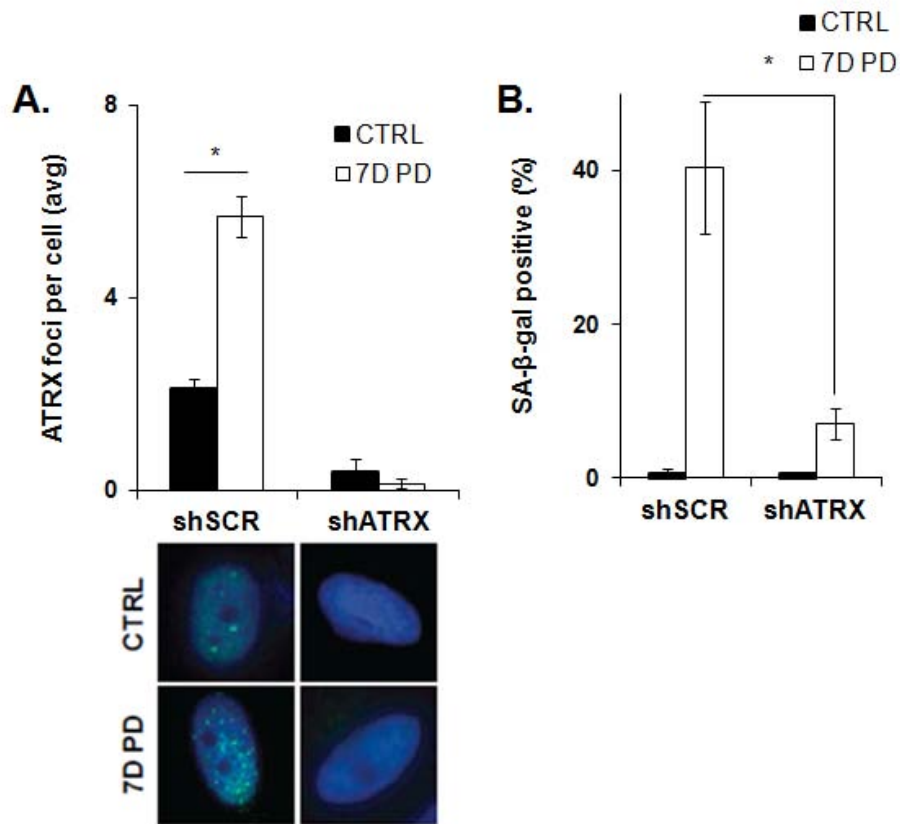


Figure 4.12 ATRX foci accumulate in glioma-derived SNB19 cells that undergo CDK4i induced senescence.

SNB19 glioma cells were transduced with targeting vectors and subsequently treated with PD0332991 and the accumulation of ATRX foci (A) and SA-β-gal (B) positive cells was measured. Representative images of ATRX foci are shown (bottom). * p < 0.05.

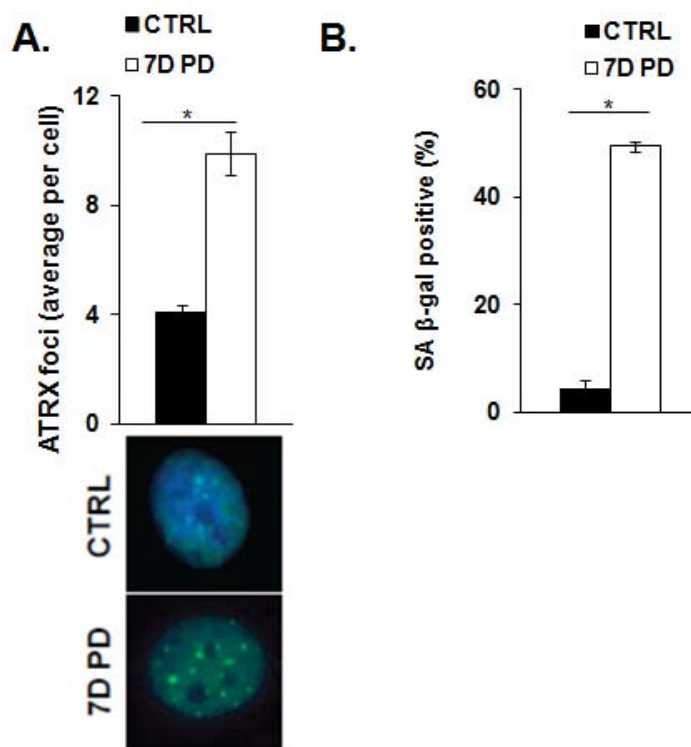


Figure 4.13 ATRX foci accumulate in breast cancer-derived MCF7 cells that undergo CDK4i induced senescence.

MCF7 breast cancer cells were transduced with targeting vectors and subsequently treated with PD0332991 and the accumulation of ATRX foci (A) and SA- β -gal (B) positive cells was measured. Representative images of ATRX foci are shown (bottom). * $p < 0.05$.

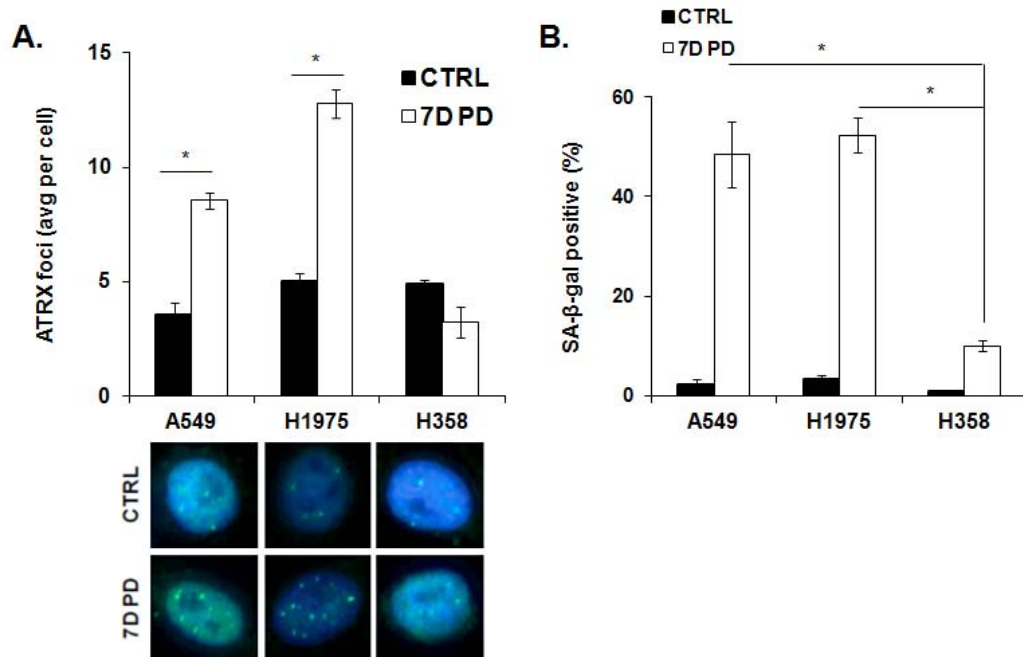


Figure 4.14 ATRX foci accumulate in transformed lung cancer cells that undergo CDK4i induced senescence but not during quiescence.

Three lung cancer derived cell lines were treated with PD0332991 for 7 days and the accumulation of ATRX foci (A) and SA-β-gal positive cells (B) was determined. Representative images of ATRX foci are shown (bottom). * p < 0.05.

cytologic change in ATRX localization as cells underwent senescence. First, in our panel of WD/DDLS cell lines in which CDK4 inhibition can induce either quiescence or senescence, I noted a significant increase in the number of ATRX foci in those that undergo senescence after CDK4 inhibition compared to those that undergo quiescence, which remained unchanged (Figure 4.10). In a more detailed time course, I saw that the number of foci began to increase as early as two days after addition of the CDK4i drug and continued to increase until day 7 (Figure 4.11).

ATRX foci also increased in PD0332991 treated SNB19 glioma cells that underwent senescence (Figure 4.12). Similarly, CDK4i induced accumulation of ATRX foci in the MCF7 breast cancer epithelial cell line (Figure 4.13), and in two lung cancer cell lines (A549 and H1975) (Figure 4.14) that accumulated SA- β -gal and underwent senescence as previously described (Kovatcheva et al. 2015). The number of ATRX foci also increased in the LS8817 cells following MDM2 knockdown (Figure 4.15) and after treatment of LS8817 cells with doxorubicin (Figure 4.16). Thus, the increase in ATRX foci was not cell type or inducer specific during TIS.

No accumulation of ATRX foci was observed in the three WD/DDLS cell lines in which PD0332991 induced quiescence (Figure 4.10), or in the lung cancer cell line (H358) in which the CDK4i-induced quiescence (Figure 4.14). Additionally, no increase in ATRX foci was detected when LS8817 cells were serum starved and quiescent (Figure 4.17), nor when MCF7 (Figure 4.18) became autophagic after serum starvation, or when LS8107 cells were differentiated into adipocytes (Figure 4.19). Based on these data, the increase in ATRX foci occurs soon after cells exit the cell cycle and is specific for cells on a path destined to senesce. Thus, ATRX may represent a novel, early stage marker of such cells.

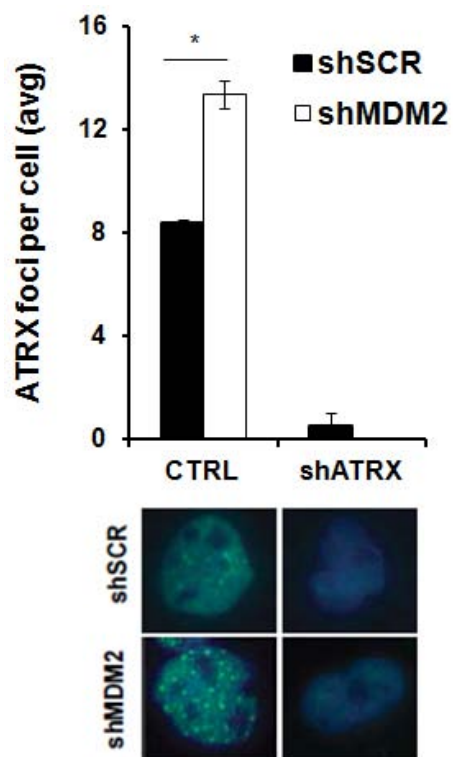


Figure 4.15 ATRX foci accumulate in WD/DDLS cells induced to senesce via MDM2 knockdown.

ATRX immunofluorescence was carried out in LS8817 cells in which senescence was induced by MDM2 knockdown as described in the legend to Figure 4.05. * $p < 0.05$.

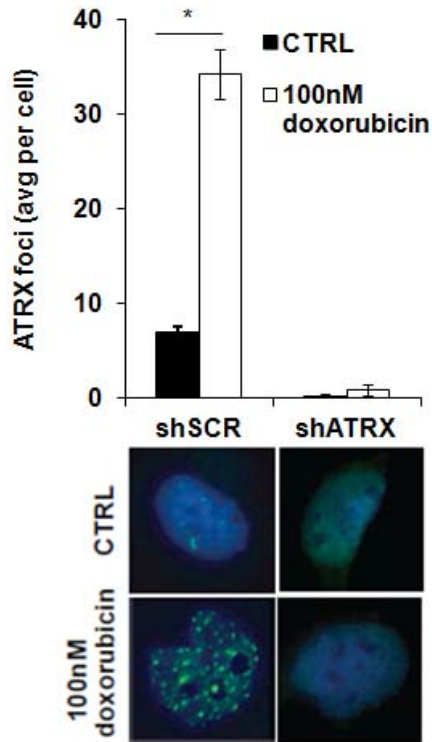


Figure 4.16 ATRX foci accumulate in WD/DDLS cells induced to senescence via DNA damage.

LS8817^{scr} and LS8817^{shATRX} were treated with doxorubicin as described in the legend to Figure 4.06 and ATRX foci detected by immunofluorescence, counted, and average number plotted. * $p < 0.05$.

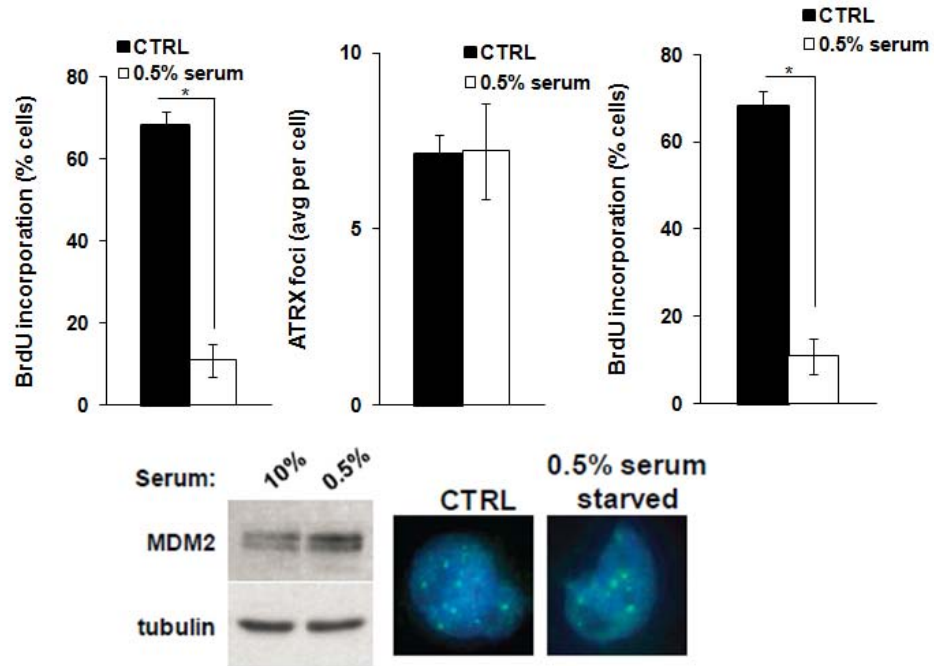


Figure 4.17 ATRX foci do not accumulate in quiescent LS8817 cells.

LS8817 cells were serum starved (0.5%) for 5 days. The proliferation of these cells was assessed by BrdU incorporation. Senescence was assessed by the expression of MDM2 and accumulation of SA- β -gal positive cells. The number of ATRX foci was measured. * $p < 0.05$.

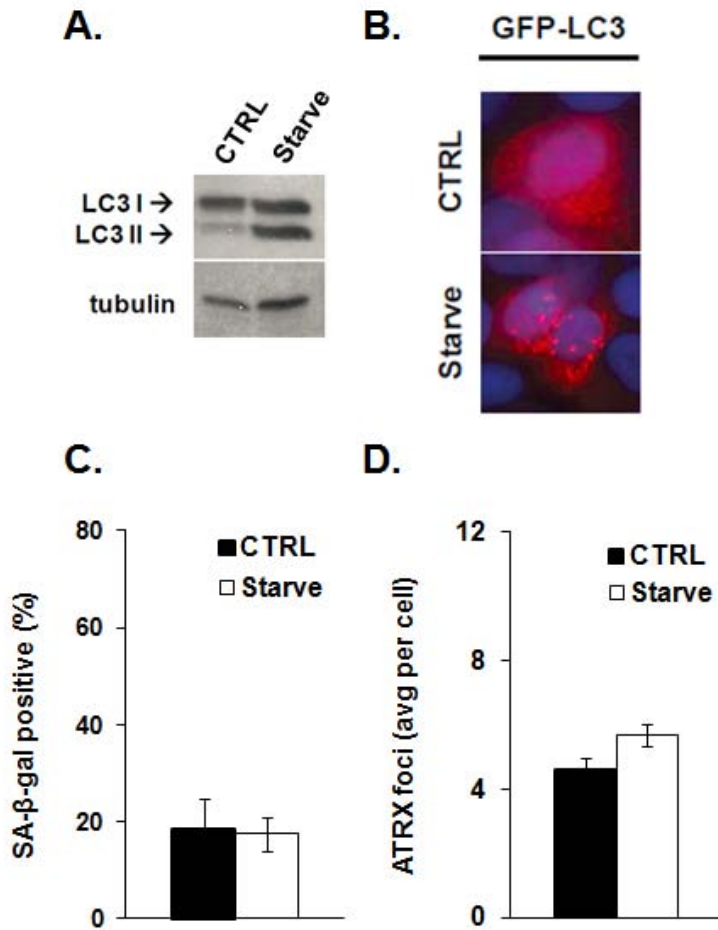


Figure 4.18 ATRX foci do not accumulate in MCF7 cells undergoing autophagy.

MCF7 cells were serum starved and underwent autophagy as described (Donohue et al., 2011). Autophagy was monitored by conversion of LC3-I to LC3-II by immunoblot (A) and by the puncta staining pattern of GFP-LC3 (B). The accumulation of SA-β-gal (C) and the number of ATRX Foci (D) was also measured.

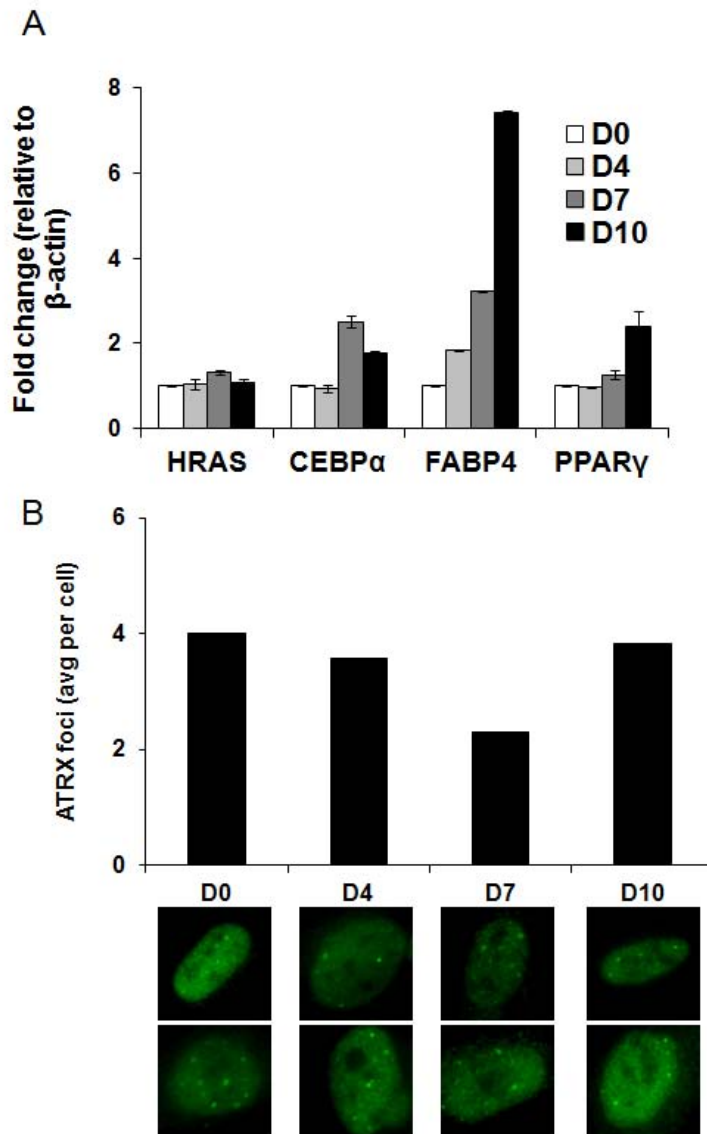


Figure 4.19 ATRX foci do not accumulate in WD/DDLS cells induced to differentiate.

LS8107 cells were induced to differentiate into adipocytes. 10 days later the expression of differentiation genes was assessed by RT-qPCR (A) and ATRX foci were measured by immunofluorescence (B). Foci were quantified (upper) and representative images are shown (lower)

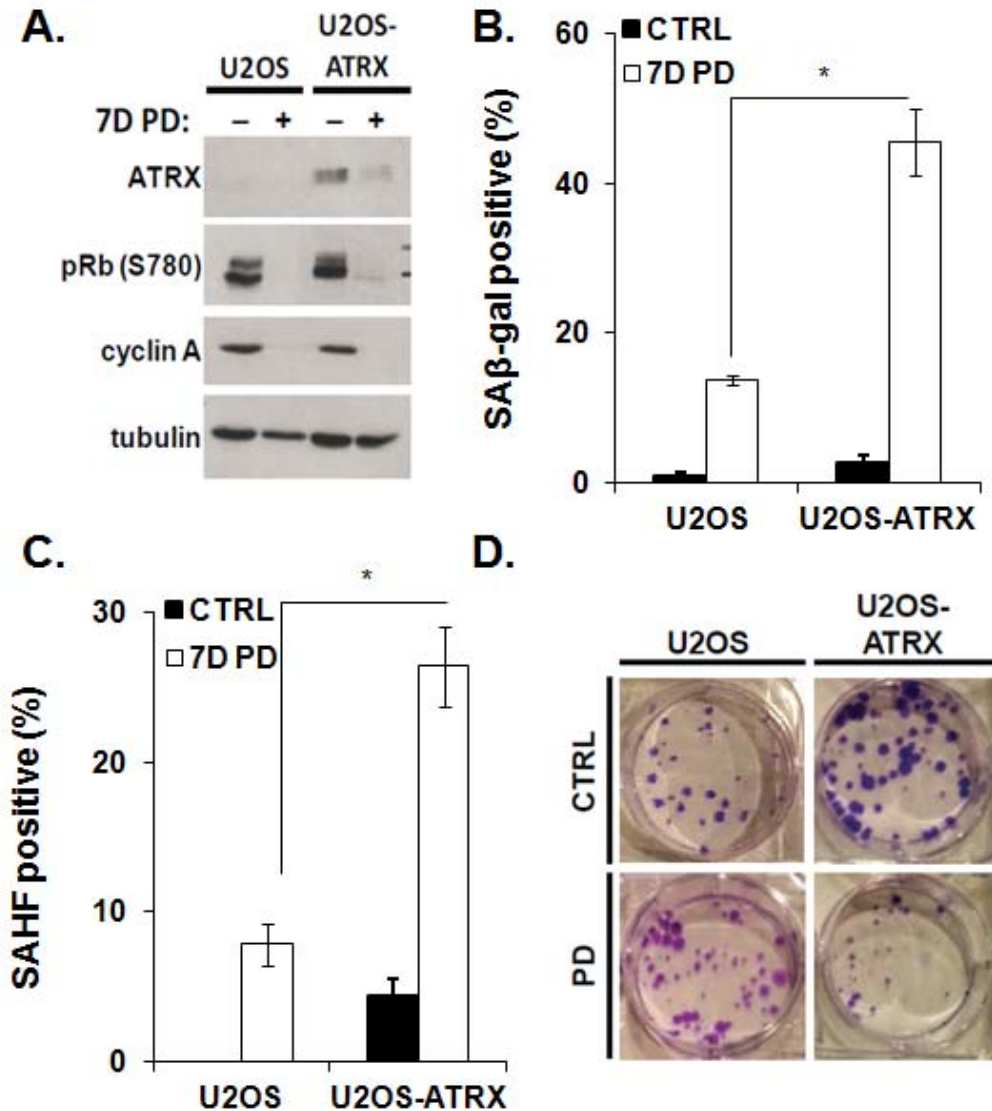


Figure 4.20 ATRX facilitates senescence in response to CDK4 inhibition in the ATRX-deficient cell line U2OS.

U2OS cells were stably transfected with wild type or mutant ATRX as described in the legend to Figure 3. (A) The expression of ATRX, cyclin A and phosphorylated Rb before and after drug treatment in parental U2OS cells and U2OS cells expressing ATRX was determined by immunoblot before and after drug treatment. (B and C) The accumulation of SA-β-gal (B) and SAHF (C) were measured in each condition. (D) Clonogenic growth was measured by crystal violet staining three weeks after the removal of the drug and replating the cells at low density in the absence of drug. * $p < 0.05$.

4.2.3 The ability of ATRX to support senescence requires HP1 and H3K9me3 binding sites.

U2OS cells are Rb positive and have a homozygous deletion encompassing exons 2-19 at the *ATRX* loci (Lovejoy et al. 2012). Treating these with PD0332991 induced cell cycle exit as evidenced by the decrease in cyclin A and phosphorylated Rb (Figure 4.20). There was a modest increase in the number of SA- β -gal and HP1 γ foci as well (Figures 4.20), however, these cells were overall not senescent as they resumed proliferation when CDK4 inhibitors were removed (Figure 4.20). Enforced expression of ATRX increased the number of these cells that accumulated SA- β -gal and HP1 γ foci after CDK4i were added, and the ability of these cells to re-enter the cell cycle after removing the CDK4i was compromised (Figure 4.20). Expressing ATRX did not significantly alter the proliferation of these cells during unperturbed culture, nor affect the viability of the cells after CDK4i-induced cell cycle exit, at least over the time course of my experiments which are carried out typically within one month.

This allowed me to ask what domains of ATRX were important for senescence. Using a panel of previously well characterized mutants (a schematic of ATRX is shown in Figure 4.21) I made U2OS lines in which ATRX mutants in the H3K4me0 binding (E218A), H3K9me3 binding (C240G), and HP1 binding (V588E) sites were expressed (Eustermann et al. 2011). I also included two mutations in the helicase domain: L1612V and K1650N. L1612V was identified in a surgical patient sample by Samuel Singer and was expected to not affect the function of ATRX based on sequence conservation. K1650N has hypomorphic helicase activity (Mitson et al. 2011). The C240G, V588E, and K1650N mutations were compromised in their ability to enhance the accumulation of SA- β -gal positive cells when CDK4 inhibitors were added (Figure 4.21). However, Rb phosphorylation was reduced (Figure 4.22), indicating that these cells still exited the cell

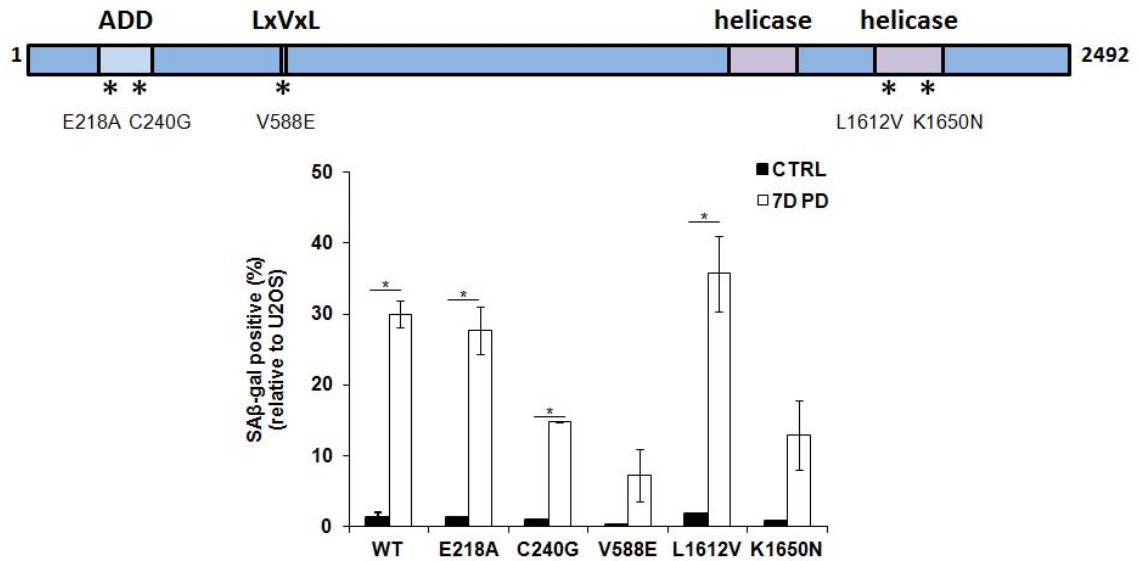


Figure 4.21 The ability of ATRX to support senescence requires HP1 and H3K9me3 binding sites.

A schematic of ATRX indicating the domains and amino acid residues numbered as annotated on UniProt is shown (upper). U2OS cells were transfected with a vector expressing wild type or mutant ATRX as indicated and stable transformants were selected with G418 and sorted using a flow cytometer to recover a GFP-low population as described in the methods. The accumulation of SA-β-gal positive cells was scored 7 days after PD0332991 (PD) treatment (lower). * p < 0.05.

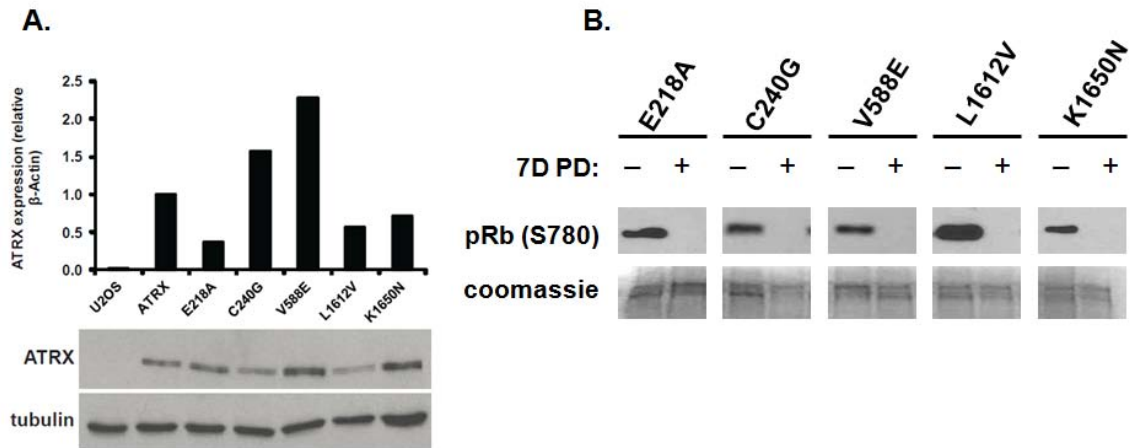


Figure 4.22 ATRX expression and capacity of PD0332991 to induce accumulation of unphosphorylated Rb is not affected by ATRX mutation status.

U2OS cells were stably transfected with ATRX constructs as described in Figure 4.23 (A) The amount of ATRX mRNA was measured by qPCR (upper) using a probe that binds within the middle of the transcript (sequence in Table 1) and ATRX protein was measured by immunoblot (lower). (B) Rb phosphorylation, a measure of cell proliferation, was measured in the U2OS cells expressing different mutant ATRX constructs before and after treatment with PD0332991 (PD) for seven days.

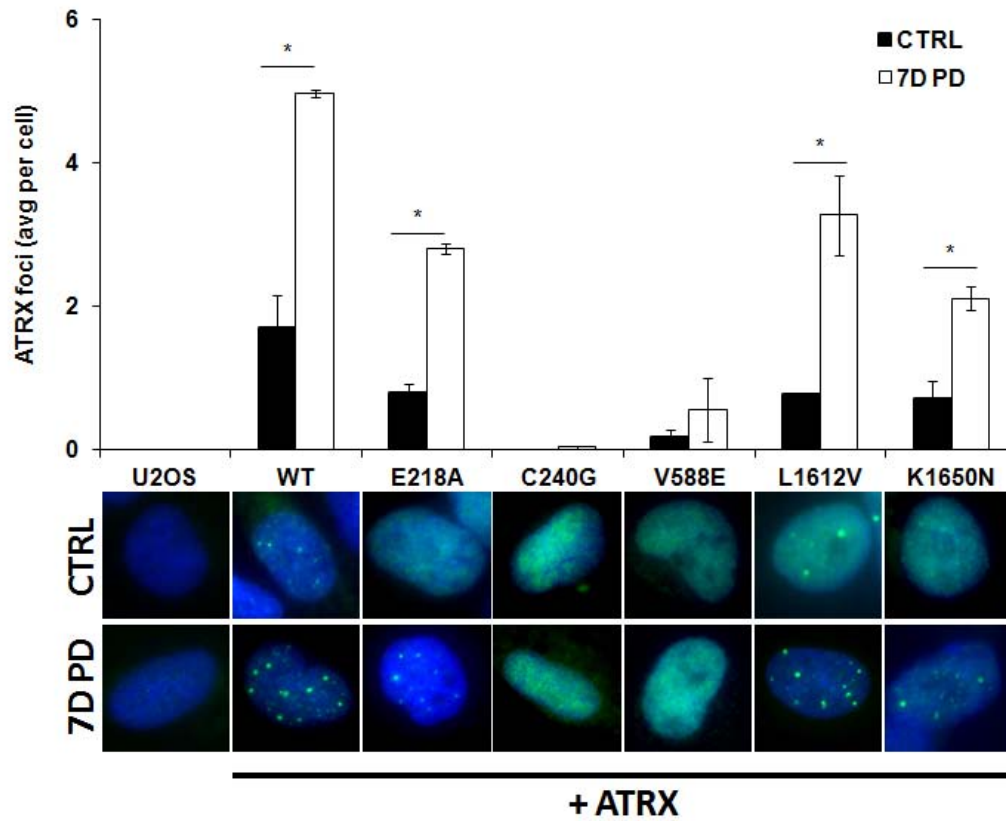


Figure 4.23 ATRX foci accumulation depends on H3K9Me3 and HP1 interactions.

U2OS cells were stably transfected with ATRX constructs as described in Figure 4.23. ATRX immunofluorescence was carried out. The average number of ATRX foci per cell is plotted (upper) and representative images are shown (lower) for each mutant. * $p < 0.05$.

cycle. In contrast, the E218A mutation and the L1612V mutation did not affect the ability of ATRX to support CDK4i induced accumulation of SA- β -gal (Figure 4.21). The expression of the mutants, at the level of mRNA or protein, did not correlate with their ability to promote the accumulation of SA- β -gal (Figure 4.22). There was, however, a correlation between the ability of the mutant to form ATRX foci upon drug treatment and its ability to promote senescence, with the exception of the K1650N mutant (Figure 4.23). This mutant formed foci but did not support senescence to the full capacity of wild type ATRX, perhaps reflecting the importance of chromatin remodeling to the role of ATRX in senescence. Thus, the interaction of ATRX with H3K9me3 histones and the HP1 family of proteins as well as the helicase activity were necessary for ATRX to promote senescence following inhibition of CDK4

4.2.4 ATRX is involved in the establishment and maintenance of SAHF

I next wanted to determine how ATRX foci formation was related to other hallmarks of a senescent cell. In LS8817 cells treated with PD0332991 the PML nuclear bodies accumulated within one day, contemporaneously with cell cycle exit (Figure 4.24). HP1 γ foci positive cells began to accumulate sometime around day 3 and continually increased with time. SA- β -gal positive cells began to accumulate shortly after that, rising between days 4 and 5. As mentioned previously, ATRX foci began to increase in number around day 2 and continued to increase over the next few days. These kinetics are summarized graphically in Figure 4.25. In the senescent LS8817 cells, ATRX co-localized with both HP1 γ and with PML (Figure 4.26). LS8817^{shATRAX} cells failed to form HP1 γ foci, but PML nuclear bodies still accumulated (Figure 4.27). This suggested that the formation of ATRX foci is probably downstream or independent of the accumulation of PML foci, but it might be involved in the formation or maintenance of the SAHF.

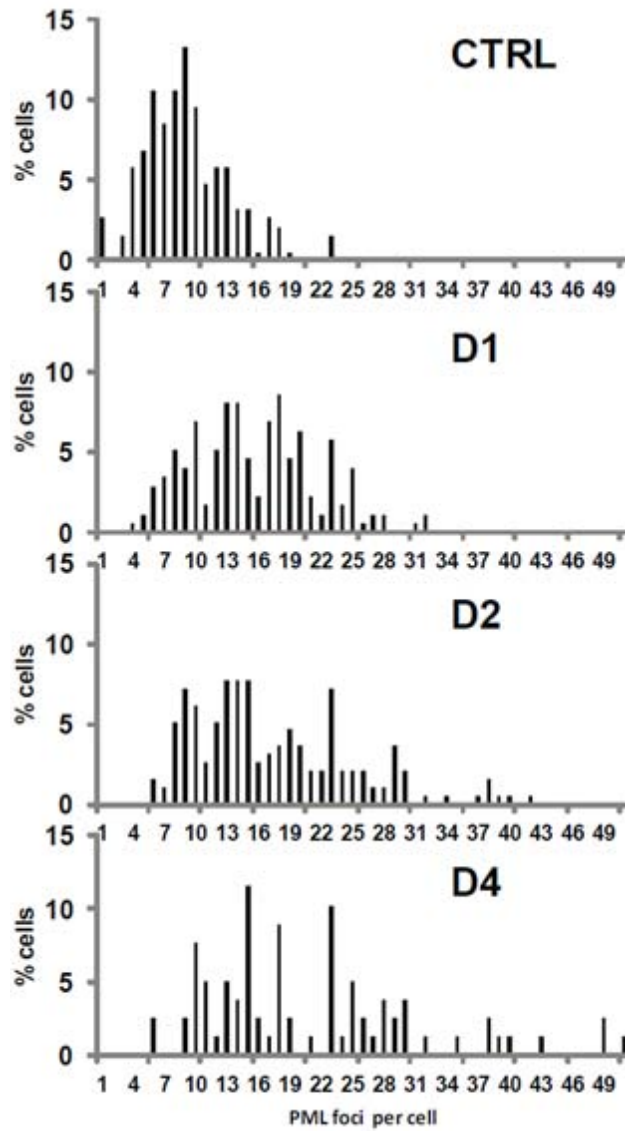


Figure 4.24 PML foci begin to accumulate within one day of PD0332991 treatment.

PML foci were stained in LS8817 cells treated with CDK4 inhibitor and the number of foci were plotted as described in the legend to Figure 4.10.

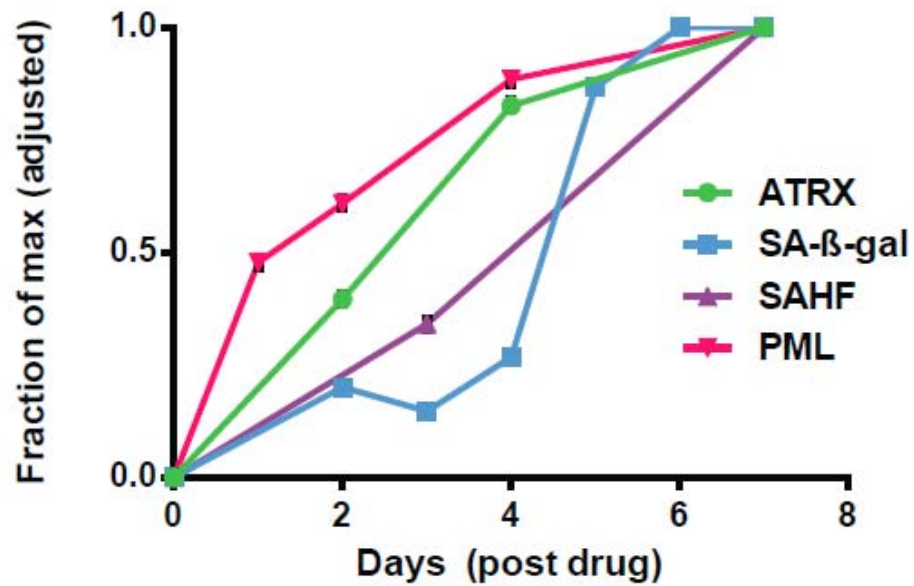


Figure 4.25 Temporal accumulation of various senescence hallmarks.

The appearance of SA-β-gal positive cells or HP1γ positive cells and the average number of ATRX foci and PML foci were plotted in a single experiment in which LS8817 cells were treated with PD0332991 for the indicated length of time. Values were plotted as a percent of the maximum observed at day seven and normalized by setting the number at day zero to zero.

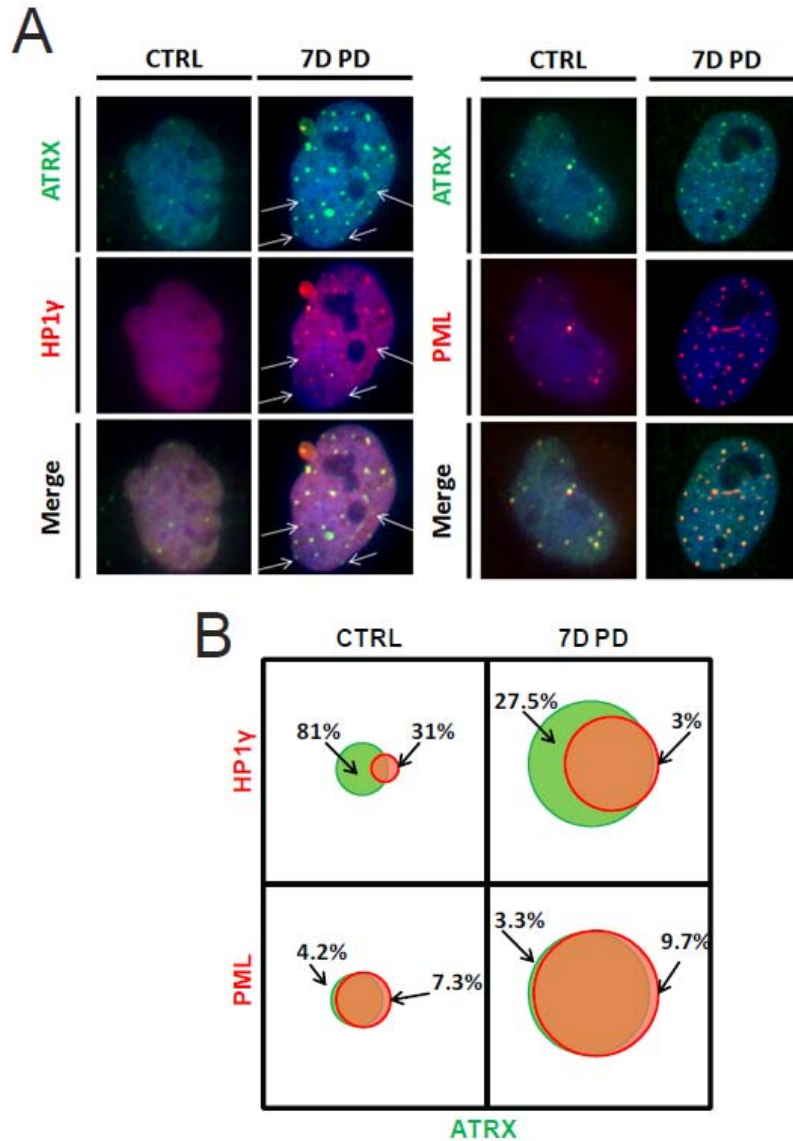


Figure 4.26 ATRX co-localizes with SAHF and PML in senescent cells.

(A) LS8817 cells were treated with PD0332991 for seven days and the co-localization of HP1 γ (left) and PML foci (right) with ATRX foci determined by immunofluorescence. Representative images are shown. White arrows indicate ATRX foci that do not co-localize with HP1 γ . (B) The fraction of ATRX foci co-localized with either HP1 γ or PML foci was quantified in control and treated cells. Circles in the Venn diagrams are drawn to scale relative to the number of the indicated foci in each condition.

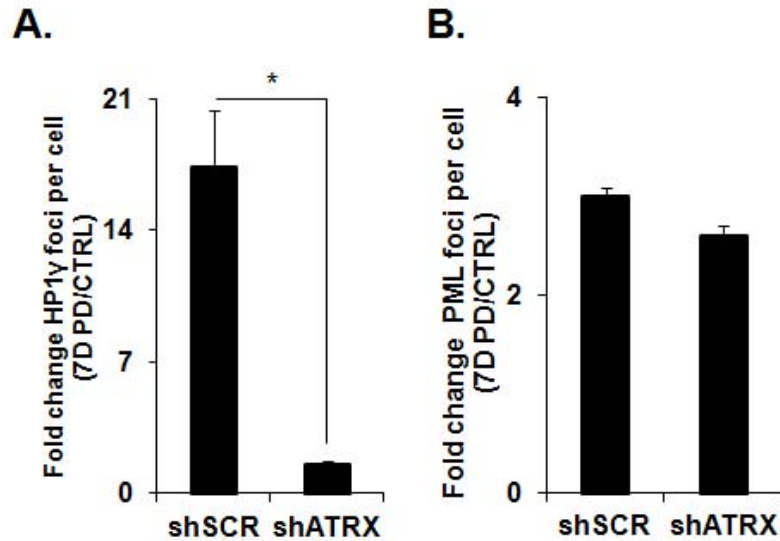


Figure 4.27 ATRX is required for establishment of HP1 γ SAHF but not PML foci in senescent cells.

The presence of HP1 γ foci (A) and PML foci (B) was assessed in LS8817^{scr} and LS8817^{shATR γ} cells after treatment with PD0332991 for seven days. Graphs represent the fold-change in the number of foci per cell in PD0332991 treated cells vs. untreated controls. * $p < 0.05$.

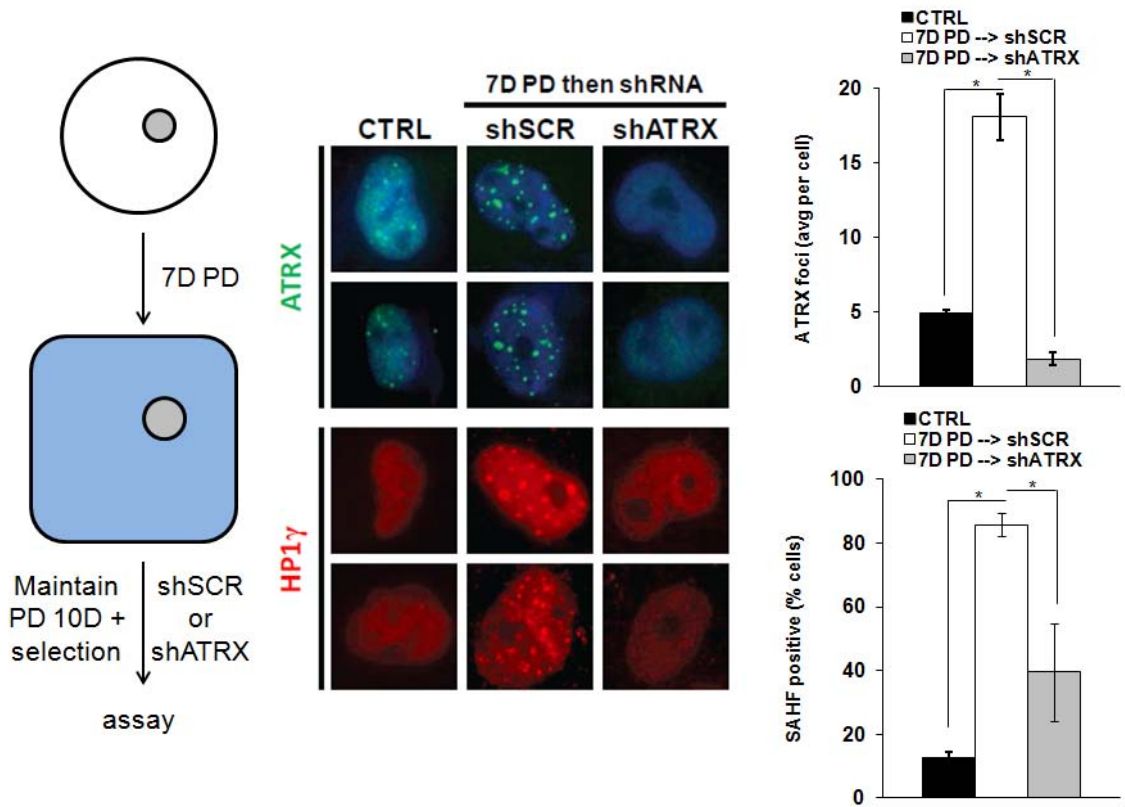


Figure 4.28 ATRX is required for maintenance of HP1γ SAHF in senescent cells.

LS8817 cells were treated as described in the schematic (left) and then fixed and processed for immunofluorescence. Representative images are shown (middle). The number of ATRX foci and the accumulation of HP1γ-positive cells (SAHF) was plotted (right). * $p < 0.05$.

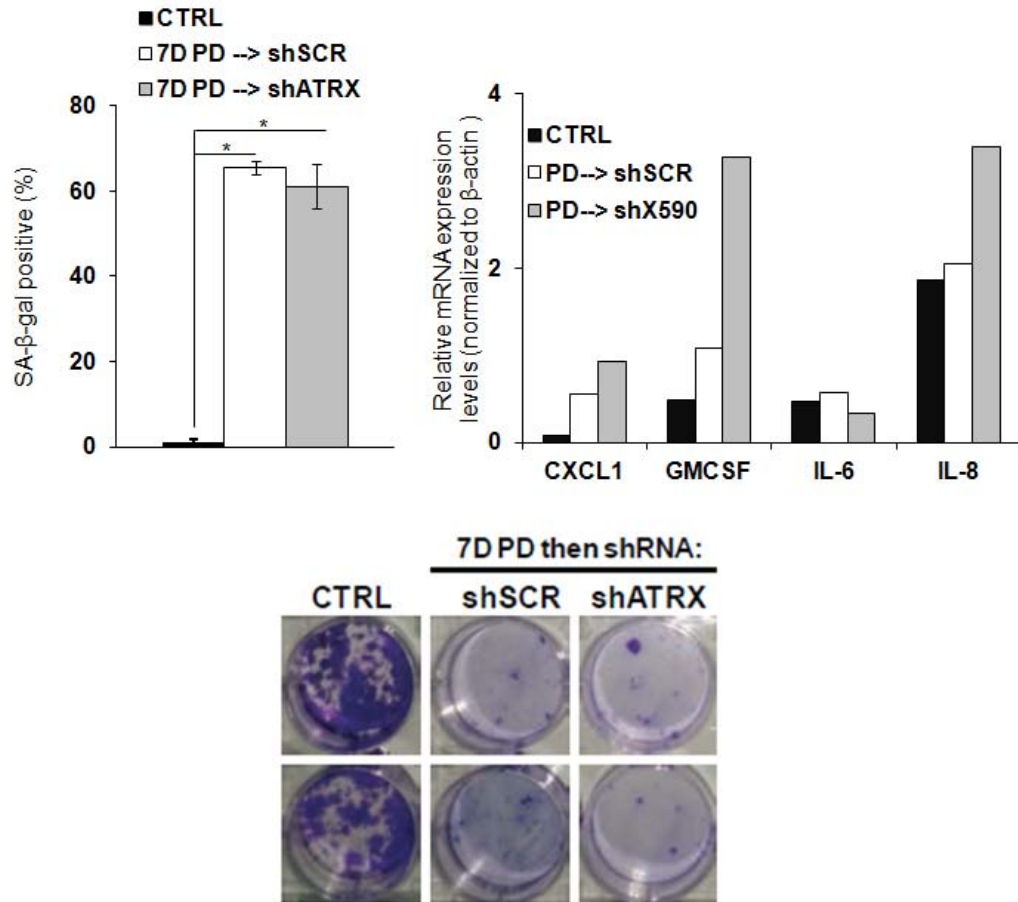


Figure 4.29 ATRX foci are dispensable for senescence maintenance.

LS8817 cells were treated as described in the schematic to Figure 4.28. The accumulation of SA-β-gal positive cells, the accumulation of four mRNAs of the SASP cytokine program and clonogenic outgrowth after cells were replated at low density the absence of drug and were allowed to grow for three weeks were assessed. * $p < 0.05$.

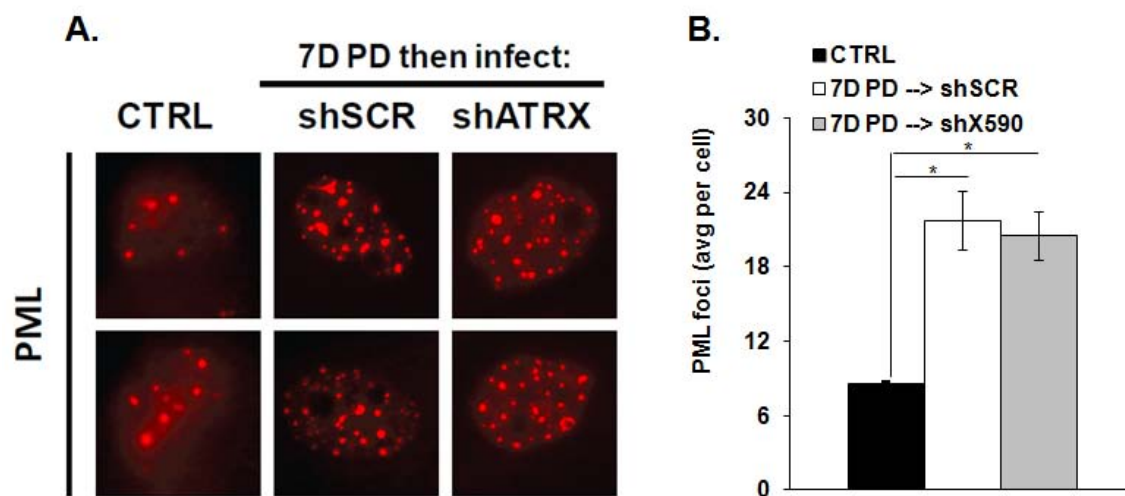


Figure 4.30 Loss of ATRX does not affect PML foci.

Cells were treated as described in the schematic to figure 4.28 and PML foci were detected and quantitated as previously described. Representative images are shown (A) and the number of PML foci per cell was quantified (B). * $p < 0.05$.

To determine if ATRX was directly required for the maintenance of SAHF I transduced senescent LS8817 cells with a lentivirus expressing the ATRX hairpin and after selection for 10 days in the presence of CDK4 inhibitor (Figure 4.28) I measured accumulation of ATRX foci, the accumulation of SA- β -gal positive cells, the expression of the SASP genes, and the accumulation of HP1 γ positive cells, and the irreversibility of arrest after removal of the CDK4 inhibitor, both by BrdU incorporation and clonogenic growth. Loss of ATRX significantly reduced the number of cells with HP1 γ foci (Figure 4.28). However, loss of ATRX did not reduce the accumulation of SA- β -gal positive cells, the expression of the SASP genes (some of which were even modestly enhanced), or the ability of cells to return to cell cycle (Figure 4.29). However, the PML foci were unchanged (Figure 4.30). Thus, ATRX is required for the establishment and maintenance of the SAHF in senescent cells but both SAHF and ATRX became dispensable for the maintenance of growth arrest, SA- β -gal and SASP factors once TIS had been established.

SAHF are known to be important for senescence in normal non-immortalized and non-transformed human diploid fibroblasts (HDFs) (Narita et al. 2003; Tu et al. 2013). Thus, I wondered whether ATRX would be important in various different modes of senescence induction in HDFs. I looked at foci formation in WI38 HDFs following either exposure to doxorubicin or gamma-irradiation (DNA damage induced senescence), or after enforced expression of the KrasV12 oncogene (OIS). As expected, these treatments induced SA- β -gal expression; ATRX foci were also increased (Figure 4.31). During replicative senescence the number of ATRX foci also increased (Figure 4.32). Knocking down ATRX in WI38 cells and IMR90 cells (a similar normal HDF cell line) induced massive amounts of cell death, preventing me from assessing whether ATRX was required for senescence in these cells. Nevertheless, the increase in ATRX foci

was a common event associated with senescence caused by a wide range of inducers, regardless of whether it was occurring in transformed cancer cell lines or in normal HDFs.

4.2.5 ATRX contributes to E2F target repression in senescence

SAHF are known to contribute to E2F target gene repression during senescence induction. Given the role for ATRX in forming SAHF, I wondered whether ATRX would affect the gene regulatory role of the SAHF. I carried out genome wide RNA-sequencing (RNA-seq) to identify genes differentially expressed in ATRX deficient and wild type LS8817 cells before and after the addition of PD0332991.

As expected the changes in gene expression associated with CDK4 inhibition were dramatically different in these cells (Figure 4.33). CDK4i treatment significantly changed the expression 4894 genes in LS8817scr cells and 739 in LS8817^{shATR_X} cells by a factor of at least 1.8 fold. 324 of these overlapped. Consistent with previous reports, the most commonly down-regulated GO terms included those relating to the cell cycle, DNA replication and repair (Table 1). This indicates that ATRX plays an integral role shaping the gene expression program between quiescent and senescent cells induced by CDK4 inhibition.

I then used gene set enrichment analysis (GSEA) to compare the complete transcriptomes of drug treated cells to their asynchronous counterparts in both ATRX proficient and deficient populations (Subramanian et al. 2005). E2F4 normally binds and represses its target genes to promote cell cycle arrest (Lee et al. 2011). As expected in normal LS8817 cells, E2F4 targets were significantly enriched (i.e. activated, $p < 0.01$) in the cycling population as compared to its CDK4i treated, senescent counterpart. In the LS8817^{shATR_X} cells, these E2F targets were no longer significantly enriched in the cycling

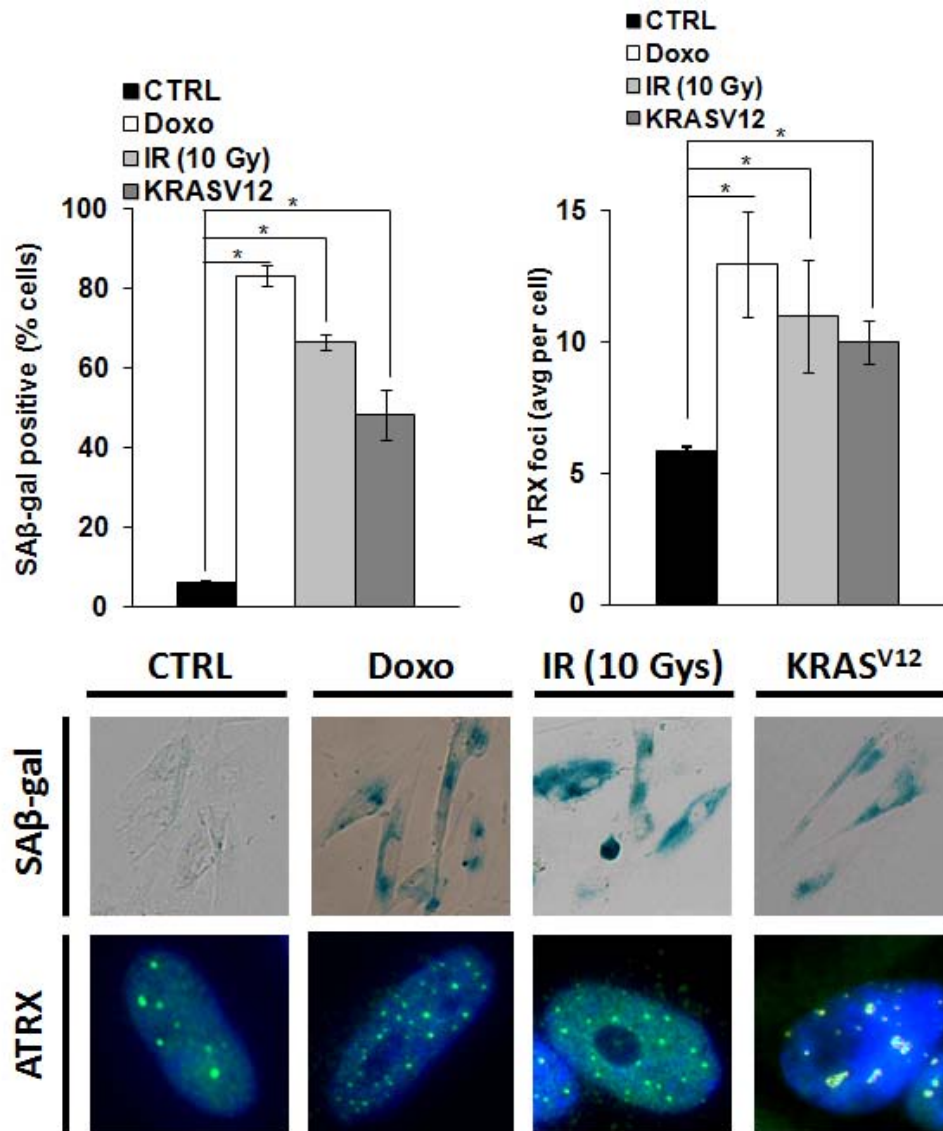


Figure 4.31 ATRX foci accumulate in normal human diploid fibroblasts induced to undergo premature senescence.

WI38 normal human diploid fibroblasts were treated as indicated with either 100nM doxorubicin (doxo), γ -irradiation (IR) or expression of KRAS^{V12}. Control untreated cells are shown as well. SA- β -gal and the number of ATRX foci per cell was assessed seven days later and quantified (upper) and representative images are shown (lower). * $p < 0.05$.

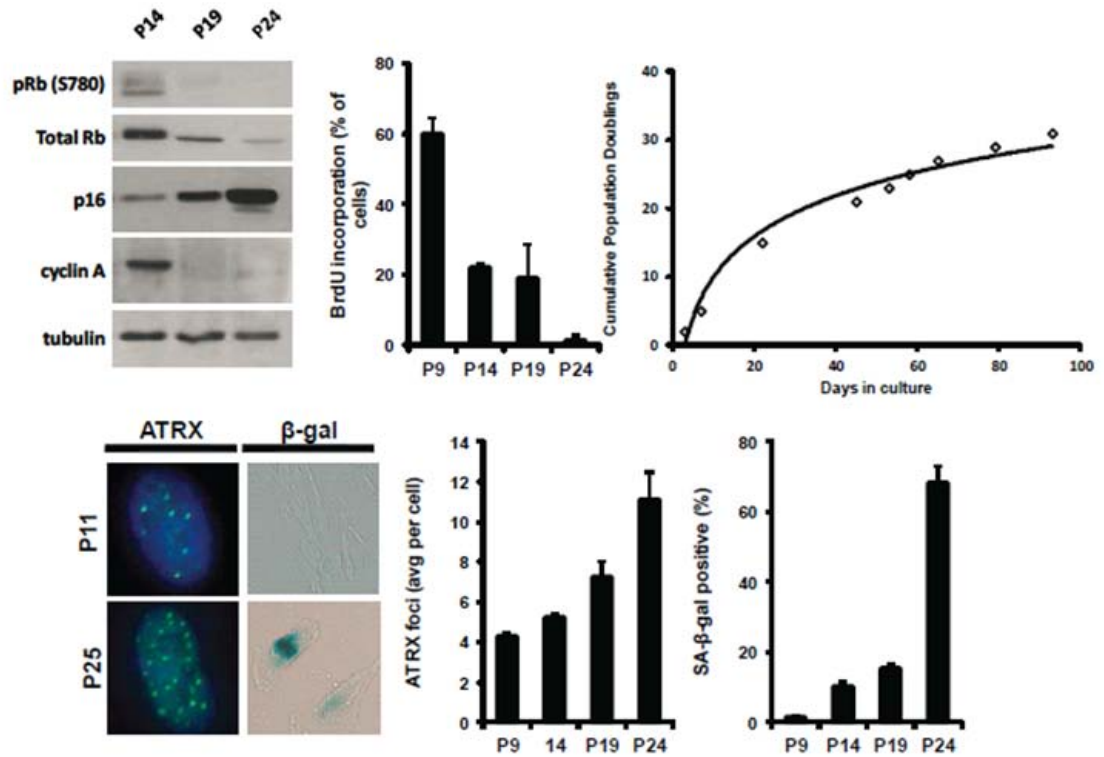


Figure 4.32 ATRX foci accumulate in normal human fibroblasts undergoing replicative senescence.

WI38 cells were cultured to replicative exhaustion with samples removed at the indicated passages for immunoblot, analysis of BrdU incorporation, detection of SA-β-gal positive cells and ATRX foci. Representative images of young (P11) and old (P25) cells stained for ATRX foci and SA-β-gal are shown. The number of population doublings are indicated (upper right).

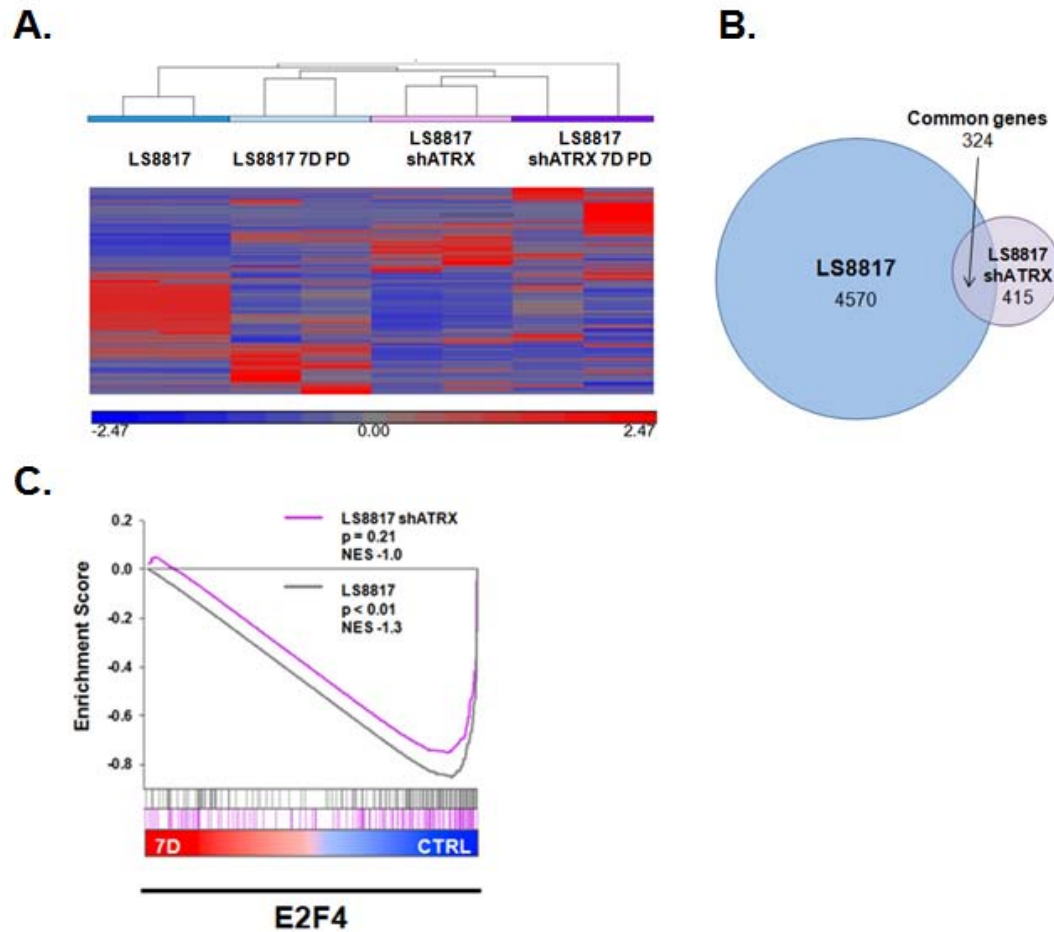


Figure 4.33 ATRX represses E2F4 target genes in senescent cells.

(A) Hierarchical clustering based on expression of all genes sequenced by RNA-seq. Each column represents a single sample, and the conditions are indicated above; RNA-seq was performed in duplicate on each condition. (B) Venn Diagram indicating the number of genes that were differentially expressed with a fold change of at least -1.8 or 1.8 and an FDR < 0.05 across PD-treated vs. control samples in unperturbed and ATRX-deficient LS8817 cells; the genes that were found in common across both cell types are indicated. (C) Gene set enrichment analysis (GSEA) was performed on across PD-treated vs control samples in unperturbed and ATRX-deficient LS8817 cells and E2F4 gene signatures are shown with the corresponding normalized enrichment score (NES) and FWER p-values.

Table 2: Enrichr analysis of GO Biological Processes that are enriched in the differentially expressed genes following PD0332991 treatment

LS8817 only	
GO Biological Process	-log(p value)
extracellular matrix organization (GO:0030198)	7.44
extracellular structure organization (GO:0043062)	7.41
homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156)	6.60
cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742)	5.84
mitotic cell cycle (GO:0000278)	61.53
DNA repair (GO:0006281)	34.49
cell cycle phase transition (GO:0044770)	33.04
mitotic cell cycle phase transition (GO:0044772)	32.55
Common (LS8817 and LS8817^{shATRX})	
GO Biological Process	-log(p value)
regulation of cyclin-dependent protein serine/threonine kinase activity involved in G1/S transition of mitotic cell cycle (GO:0031657)	2.81
positive regulation of neuroblast proliferation (GO:0002052)	2.24
protein carboxylation (GO:0018214)	2.59
peptidyl-glutamic acid carboxylation (GO:0017187)	2.59
RNA splicing (GO:0008380)	16.32
mRNA splicing, via spliceosome (GO:0000398)	16.63
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile (GO:0000377)	16.63
RNA splicing, via transesterification reactions (GO:0000375)	16.30

Legend:
up-regulated in PD0332991-treated cells
down-regulated in PD0332991-treated cells

Note: LS8817^{shATRX} only GO terms were not listed very few (< 10) genes from the indicated list belonged to any gene set; thus statistical power was limited

vs. drug treated population ($p = 0.21$), consistent with a role for ATRX in regulating the SAHF and thus E2F target repression (Figure 4.33).

4.3 Discussion

Here, I have shown that ATRX plays a pleiotropic role in regulating senescence, both in a CDK4i-specific manner (i.e. MDM2 turnover) and more broadly in therapy induced senescence (TIS). ATRX accumulates in nuclear foci soon after cells exit the cell cycle, well before SA- β -gal, SAHF, and SASP accumulate. Moreover, ATRX is required for the elaboration of these aforementioned senescence hallmarks in TIS. Cells without ATRX still undergo growth arrest, indicating that ATRX regulates the transition from quiescence to senescence known as geroconversion. Recruitment of ATRX to foci occurs in WI38 human diploid fibroblasts undergoing DNA damage induced senescence, replicative senescence, or OIS, as well as in transformed cell lines undergoing TIS, by DNA damage in both a p53-dependent manner or CDK4 inhibition in a p53-independent manner.

Specific to CDK4i, ATRX plays a role in the down-regulation of MDM2 in quiescent cells. This is a prerequisite for senescence induced by CDK4i but not other stresses. This is clinically relevant because the dosing schedule of Palbociclib requires a one week interval off the drug between each cycle of 14-21 days (Dickson et al. 2016; Dickson et al. 2013; Turner et al. 2015). Upon drug removal, quiescent cells can return to the cell cycle and imbalances in their metabolism or transcriptional networks may make them prone to error (Menu et al. 2008; Huang et al. 2012; Yang et al. 2015; Niesvizky et al. 2015). Senescence (over quiescence) is the preferred outcome of a cytostatic drug and thus understanding how ATRX contributes to senescence is important. The *in vitro* observation that the Bethyl antibody reacts with an ATRX isoform only in those cell lines that down-regulate MDM2 following CDK4i, as well as the clinical

observation that decreases in MDM2 are associated with a favorable outcome suggest that ATRX may have utility as a prognostic biomarker for CDK4i therapy. Unfortunately, we did not have sufficient clinical material to investigate whether ATRX detection correlates with patient outcome at this time, but we plan to interrogate this using material from future clinical trials.

More broadly, I showed that ATRX is required for the acquisition of senescence phenotypes in both p53-dependent and p53-independent TIS. Without ATRX, cells did not accumulate SA- β -gal activity, did not accumulate SAHF, nor did they elaborate a SASP, and their growth arrest was reversible once the inducer was removed. ATRX was also important for the maintenance of SAHF, indicating that these chromatin domains are dynamic in senescent cells and that the molecular requirements for entry vs. maintenance of senescence are unique. Consistent with ATRX's role in regulating SAHF formation, ATRX deficiency resulted in a de-repression of E2F target genes in quiescent drug treated cells as compared to their ATRX-proficient, senescent counterparts. We were unable to test the requirement for ATRX in senescence in normal human diploid fibroblasts (HDFs), as loss of ATRX resulted in cell death. This is perhaps related to the fact that over-expression of E2F1 in quiescent fibroblasts induces cell death (Kowalik et al. 1995).

Although it has been reported that some cell types and/or inducers can undergo senescence without detectable accumulation of SAHF (Kosar et al. 2011; Di Micco et al. 2011), the functional importance of these structures cannot be overlooked. In cells where SAHF are established during senescence, a loss of the gene products required for SAHF formation inhibits senescence entirely and instead promotes proliferation. For example, knockdown of Asf1 resulted in the failure of WI38 HDFs to form SAHF when triggered by oncogenic Ras^{V12}. Asf1-deficient cells had a much higher rate of

proliferation than control cells infected with Ras^{V12} (Zhang et al. 2005). Knocking down HMGA in IMR90 HDFs also prevented SAHF formation when transduced with oncogenic Ras, once again accompanied by increased BrdU incorporation, improved colony formation, reduction in the accumulation of SA- β -gal and derepression of E2F target genes (Narita et al. 2006). IMR90 cells in which SAHF formation was prevented due to Rb loss also exhibited reduced SA- β -gal, increased BrdU and upregulation of genes with E2F consensus binding sites like MCM2, cyclin A and cyclin E when challenged with oncogenic Ras (Chicas et al. 2010).

SAHF also have important physiologic consequences; a failure to generate SAHF due to inactivation or loss of the assembly components can support oncogenic growth. SuvH1 (required for H3K9me3 accumulation) knockout mice developed invasive T-cell lymphoma when challenged with activated N-Ras and failed to senesce when treated with standard chemotherapy, unlike their SuvH1-competent counterparts (Braig et al. 2005). HIRA-deficient mice rapidly and uniformly developed aggressive papillomas when challenged with activated Braf (Rai et al. 2014). Furthermore, chromatin changes are a hallmark of aging, including events like global loss of DNA methylation, changes in lamin organization and telomeric defects (Reviewed in (Dimauro and David 2009)).

However, unlike these previously identified regulators of SAHF, loss of ATRX does not push cells back into proliferation but rather maintains them in a quiescent state. Thus I have identified, for the first time to our knowledge, a chromatin regulator that distinguishes between quiescence and senescence. Geroconversion has important implications in the efficacy of cancer therapy ((Yoshida et al. 2016; Kovatcheva et al. 2015), tumor dormancy (Bellovin et al. 2013; Aguirre-Ghiso 2007) and the maintenance and functionality of stem cell pools (Krizhanovsky et al. 2008a; Cheung and Rando 2013; Sousa-Victor et al. 2014; Garcia-Prat et al. 2016).

Finally, this work suggests that accumulation of ATRX foci may serve as a novel, specific marker of senescent cells. ATRX foci are observed earlier than the other hallmarks of senescence and ATRX foci occur in many different cell types, both untransformed and transformed, and with a diverse collection of senescence inducers. Foci formation is remarkably specific and was not observed in quiescent, autophagic or differentiated cells. Moreover, the capacity of ATRX foci to distinguish between a cell that has embarked on the path to irreversibility and a reversibly quiescent cell has important implications for how screens to identify novel regulators of senescence, both generic and context-specific, might be performed (see **6.3 ATRX foci as a tool**). Nevertheless, we are currently the only group to have reported senescence-associated changes in ATRX foci. Thus, the breadth and utility of ATRX foci as a marker of senescence will eventually be determined as more laboratories begin to test it in their own systems.

5. ATRX DIRECTLY REGULATES *HRAS* EXPRESSION TO DRIVE GEROCONVERSION IN THERAPY INDUCED SENESENCE

5.1 Introduction: A role for ATRX beyond the SAHF

Despite the fact that ~70% of the ATRX foci co-localized with HP1 γ foci in senescent cells, ~30% of ATRX did not co-localize with the SAHF (Figure 4.26). This raised the possibility that ATRX may have additional, non-SAHF related roles in senescent cells.

Chromatin immunoprecipitation studies have localized ATRX not only to constitutive heterochromatin at telomeric and pericentromeric regions, but also to facultative euchromatic regions featuring tandem repeats (TRs) and CpG islands (Law et al. 2010). ATRX can regulate the expression of specific genes from TRs; mutations identified in patients with ATRX syndrome resulted in dysregulation of genes found within TR loci. Additionally, ATRX can regulate gene expression by inhibiting the enrichment of mH2A at the α -globin gene locus; this may be responsible for the α -thalassemia phenotype that gives the gene product its name (alpha-thalassemia, mental retardation X-linked) (Ratnakumar et al. 2012). Thus, in this chapter I asked whether ATRX might regulate a gene expression program important for driving geroconversion and independent of SAHF-associated E2F regulation.

5.2 Results

5.2.1 ATRX regulates EZH2 targets in CDK4 inhibitor induced senescence

Having generated gene expression profiles using RNA-sequencing for LS8817 cells with and without ATRX treated with PD0332991, I used this data and Enrichr software (Chen et al. 2013) to identify what transcription factor (TF) targets were specifically

affected by the loss of ATRX (Figure 5.01). Myc, Max, and p300 target genes were enriched the untreated populations of both ATRX-proficient and ATRX-deficient cells as compared to their drug-treated counterparts. This was expected, as both quiescent (shATRX) and senescent (ATRX^{WT}) cells have exited the cell cycle. Consistent with the GSEA analysis (see **4.2.5 ATRX contributes to E2F target repression in senescence**), E2F4 target genes were enriched exclusively in the ATRX proficient cells. In the LS8817^{shATRX} cells, the targets of SUZ12 and SMC3 were specifically down-regulated following drug treatment. EZH2 targets were overall up-regulated following drug treatment in the LS8817 (ATRX^{WT}) cells, but this regulation was absent in LS8817^{shATRX} cells: some EZH2 targets were up-regulated and others were down-regulated following CDK4i treatment. EZH2 and SUZ12 are both components of the polycomb repressive complex 2 (PRC2), which has known functions in regulating tumor suppressor genes (Yamaguchi and Hung 2014; Hock 2012). Consistent with the TF Enrichr analysis, EZH2 targets were significantly enriched in the drug-treated population (as compared to untreated cells) in normal cells as assessed by GSEA ($p < 0.01$), but this enrichment was lost in the LS8817^{shATRX} cells ($p = 0.6$) (Figure 5.01). Collectively, this suggests a general perturbation of PRC2 target gene expression as a result of ATRX loss.

5.2.2 ATRX directly binds to and represses HRAS in senescent cells

Overall changes in gene expression program may reflect both the direct and indirect contribution of ATRX. To identify genes that were directly regulated by ATRX in senescent cells I performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) in doxorubicin-treated and CDK4 inhibitor-treated LS8817 cells after seven days of drug treatment. By combining these data sets I focused on identifying common regulatory targets of TIS rather than inducer specific targets. In addition, to ensure that

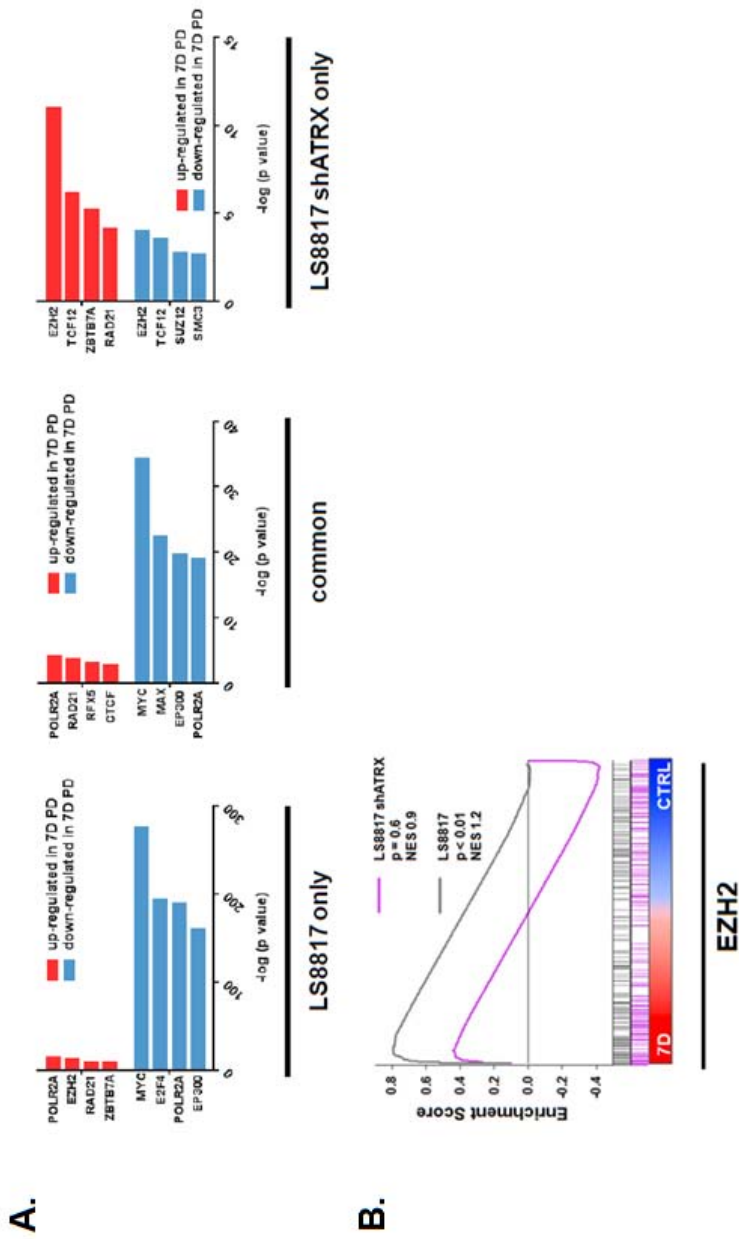


Figure 5.01 ATRX affects EZH2 target gene expression in senescent cells.

RNA-seq from the experiments described in Figure 4.33 was analyzed. (A) Enrichr analysis of the top predicted transcription factors that regulate the up-regulated and down-regulated gene lists from Figure 4.33 B. The negative log of the p-value for the enrichment scores is plotted. A similar analysis of GO categories is provided in Table 2. (B) Gene set enrichment analysis (GSEA) was performed on PD-treated vs control samples in unperturbed and ATRX-deficient LS8817 cells analyzing an EZH2 gene signature. The profiles are shown with their corresponding normalized enrichment score (NES) and FWER p-values.

the binding sites were related to senescence and not just growth arrest, I also carried out CHIP-seq from quiescent serum starved and cycling LS8817 cells. Confirming the specificity of the immunoprecipitation, antibodies to ATRX failed to pull down sufficient DNA from U2OS cells to prepare a library. We filtered out previously reported centromeric and telomeric peaks, as well as other repetitive regions.

There were 166 ATRX binding sites shared in common and not enriched in serum starved or cycling cells (Figure 5.02). One-third of these were in gene bodies or promoters (Figure 5.02) representing 41 unique genes (Table 3). The remaining were in intergenic sequences, including annotated satellite, centromeric and telomeric repeats. This ratio of ATRX binding sites is consistent with that previously reported by others (Law et al. 2010; Sarma et al. 2014). We were unable to discern any relationship between the distribution of the intergenic sites along the chromosomes or relative to centromeres and telomeres. Likewise, we were also unable to discern a common structural or sequence element in these intergenic regions, including G-quadruplexes which are known to be enriched at ATRX binding sites (Law et al. 2010).

I used the Enrichr software to determine what transcription factors regulate this subset of 41 genes. E2F1, MAX and FOXC1 were the most strongly enriched (Figure 5.02 and Table 3). The predominance of E2F binding sites was consistent with the hypothesis that ATRX was involved in the maintenance of the SAHF.

39 of the 41 genes were identified in our RNA-seq data. 18 of these were similarly regulated in both the CDK4i and doxorubicin treated cells compared to cycling or serum starved cells (Table 3). Eight were decreased in at least three of the comparisons (*KIF15*, *ATAD2*, *DHX9*, *ARHGAP18*, *PARD3*, *HRAS*, *ZNF107*, and *SORCS3*), and ten were increased in all four of the comparisons (*RNF128*, *PLCB1*, *HCN1*, *CTNDD2*,

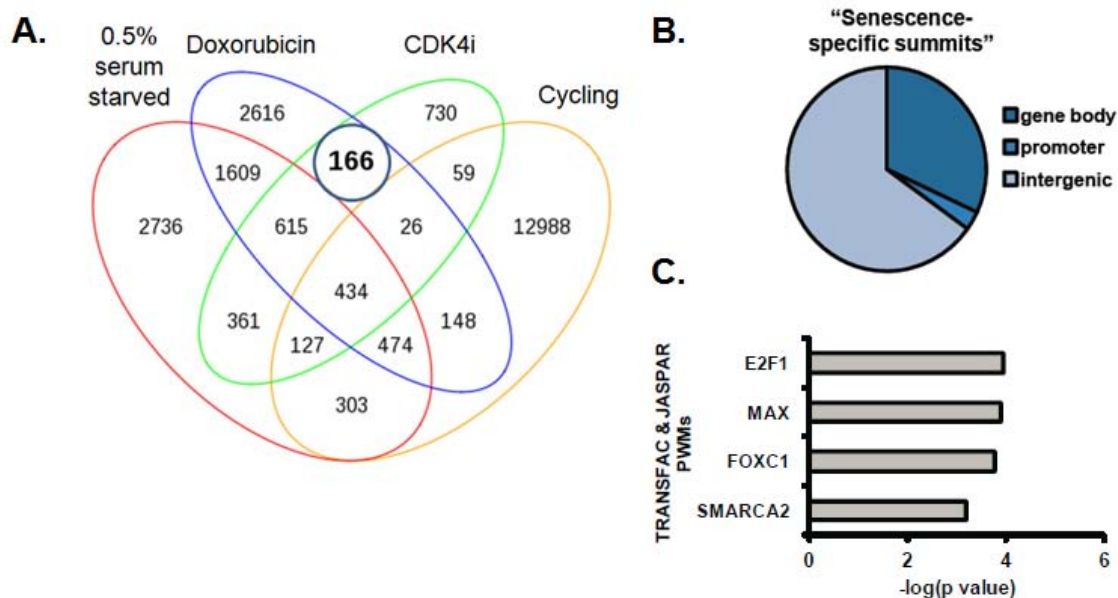


Figure 5.02 ATRX binding sites in senescent cells.

(A) Venn diagram indicating the number of ATRX-specific summits identified by ChIP-seq performed in untreated (cycling) LS8817 cells, senescent LS8817 cells treated with either PD0332991 (CDK4i) or doxorubicin for seven days, and quiescent cells induced by growth in low serum for five days (0.5% serum starved). The senescence-specific summits are circled in blue. (B) Pie chart summarizing the distribution of the 166 senescence-specific summits within gene bodies, associated with promoters or in intergenic regions. (C) Enrichr analysis of the top predicted transcription factors that regulate the “gene body” and “promoter” associated genes from panel B. The negative log of the p-value for the enrichment scores is plotted. The specific genes that comprise each gene set can be found in Table 3.

Table 3: RNA expression of genes in which ATRX binding was enriched (within the promoter or gene body) in senescent cells

Gene locus	Enrichr TRANSFAC/JASPAR PWMs				Fold change by RNA-seq			
	FOXC1 Target	E2F1 Target	Max Target	SMARCA2 Target	PD vs CTRL	Doxo vs CTRL	PD vs 0.5% serum	Doxo vs 0.5% serum
KIF15	X				0.0	0.2	0.1	0.4
ATAD2	X	X		X	0.1	0.3	0.1	0.4
DHX9	X	X			0.3	0.3	0.6	0.6
ARHGAP18	X				0.5	0.4	0.8	0.6
PARD3	X	X	X	X	1.0	0.7	0.7	0.5
HRAS	X	X	X		0.5	0.7	0.8	1.1
ZNF107	X			X	0.7	0.6	1.0	0.8
PGK1		X		X	1.1	0.4	1.1	0.4
CCNY	X				0.8	0.7	1.1	1.0
RGS17		X	X		0.8	1.1	0.7	1.1
SORCS3	X	X	X		0.3	0.2	1.2	0.6
CAMTA1	X	X			1.2	1.0	0.8	0.7
SPATA13	X				1.1	0.6	1.2	0.6
ABCA11P					1.1	0.7	1.7	1.1
C12orf56	X				0.7	2.8	1.0	4.1
TBC1D5	X	X	X		0.8	1.3	0.9	1.5
LOC729444					n.d.	n.d.	n.d.	n.d.
RBAK-LOC389458					n.d.	n.d.	n.d.	n.d.
SMAD2	X	X		X	1.0	1.0	0.9	1.0
IMMP2L	X				1.3	1.0	1.2	0.9
PCGF3	X		X		1.3	1.1	1.2	1.1
ZSCAN32					1.3	1.1	1.3	1.1
PRKAR1B	X		X		1.1	1.7	1.1	1.8
GRIN1	X	X	X		1.0	2.4	1.0	2.4
ATRX				X	1.5	1.3	1.3	1.1
HRH1	X	X			1.7	1.1	1.8	1.2
LHFPL3	X				1.4	1.9	1.0	1.4
CELF5	X				1.6	3.0	1.0	1.9
DMXL2	X	X			3.1	1.3	2.6	1.1
DGKB					0.9	6.7	1.2	9.5
CPQ					5.1	2.5	1.8	0.9
C20orf195	X				1.3	1.2	1.3	1.2
HIBADH					1.5	1.3	1.5	1.3
SIN3B	X				1.4	1.4	1.8	1.7
GAS6-AS1					2.5	2.3	1.5	1.4
COL26A1					1.5	5.7	1.4	5.7
TRPS1	X	X		X	4.0	2.0	2.4	1.2
CTNND2		X	X	X	2.0	11.6	1.6	9.7
HCN1	X		X		1.8	6.8	2.4	9.4
PLCB1	X				2.7	3.0	2.5	2.9
RNF128	X				4.9	2.8	5.3	2.9
RNA expression repressed in senescent cells is indicated in green								
RNA expression induced in senescent cells is indicated in brown								
Shading indicates the degree of repression or activation								
Fold repression/induction are indicated by the numbers								
n.d. not determined								

TRPS1, *COL26A1*, *GAS6-AS1*, *SIN3B*, *HIBADH*, and *C20orf195*). I was most interested in identifying direct targets that might control progression from quiescence into senescence in a cell type-independent manner. Thus, as I progressed to validating these results I also carried out secondary assays to identify and eliminate targets that were cell type dependent.

I first compared the expression of these 18 genes in three cell lines, two of which undergo CDK4i-induced senescence (LS8817 and LS0082) and one which undergoes quiescence upon treatment (LS8107) (Figure 5.03-5.04). Only the expression patterns of *PARD3*, *HRAS*, *ZNF107*, and *SORCS3* made it through this validation screen. These four were reduced in both of the senescent cell lines, and not in the quiescent cell line following treatment with PD0332991.

I then asked if these four were regulated in an ATRX dependent manner using the LS8817 (Figure 5.05) and U2OS (Figure 5.06) isogenic cell pairs in which ATRX expression was manipulated. *PARD3* dropped out since it was not repressed in U2OS cells that expressed ATRX. Changes in *ZNF107* expression were consistent with our expectations, but more modest than with *HRAS* or *SORCS3*.

I then asked if ATRX binding was observed and reduced using ChIP-qPCR at these sites before and after drug treatment. I could not design primers to evaluate binding to the *SORCS3* locus because the sequences identified in chromatin immunoprecipitation were AT-rich. ATRX binding was strongly enriched at *HRAS* locus in both the senescent LS8817 and LS0082 cells and not in the quiescent LS8107 cells. Binding was modestly enriched at the *ZNF107* locus, being stronger in LS0082 than in LS8817 (Figure 5.07).

Finally, I wanted to make sure that this was not a mechanism that was limited to sarcoma cell lines, so I looked at the expression of *HRAS* and *ZNF107* in other cell lines

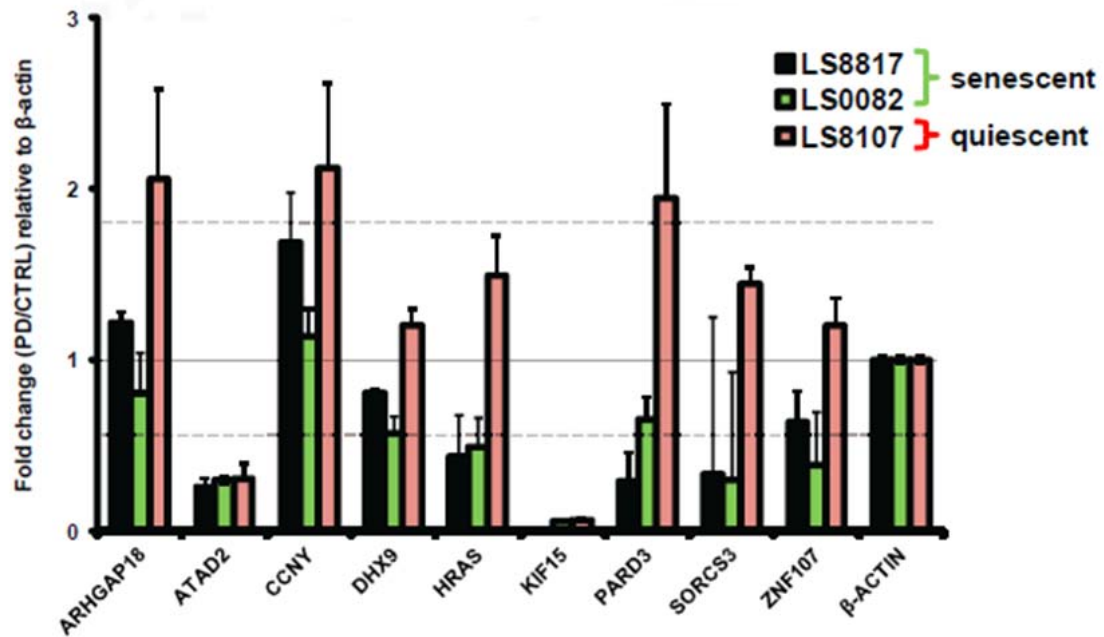


Figure 5.03 Validation of genes potentially bound to and down-regulated by ATRX in senescence.

qPCR analyses of expression of the ATRX bound genes identified to be downregulated in senescence by RNA0seq. Expression was plotted as a fold change in PD0332991 treated (7 days) cells compared to untreated cells, normalized to beta-actin. The solid line indicates no change in expression and dashed lines indicate cutoffs at 1.8-fold change in expression to demonstrate significant repression or activation.

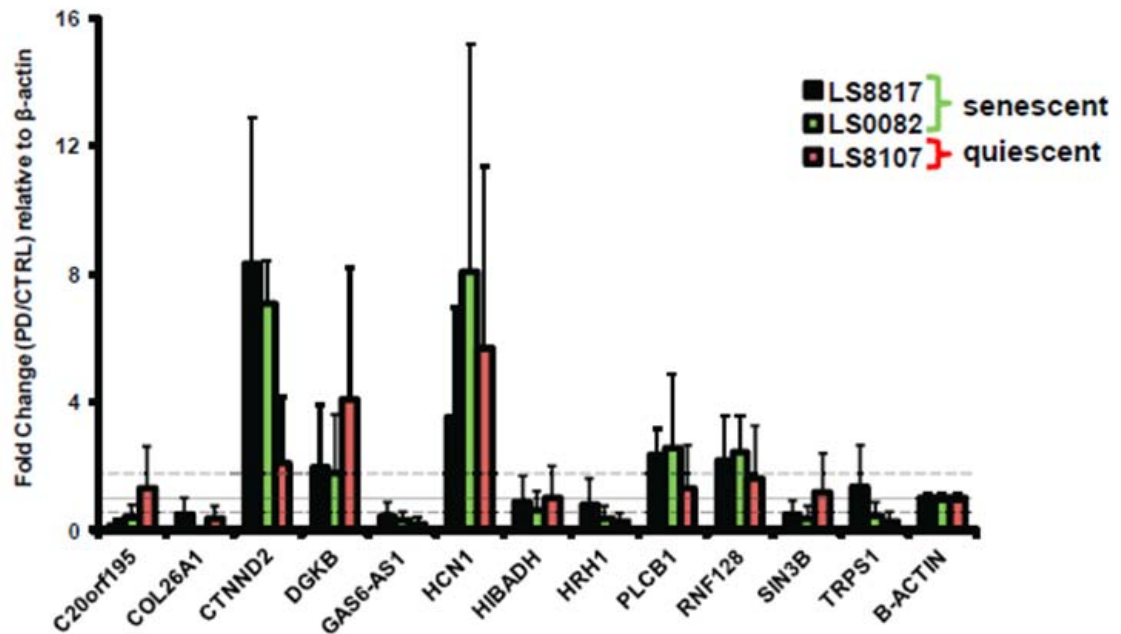


Figure 5.04 Validation of genes potentially bound to and up-regulated by ATRX in senescence.

qPCR analyses of expression of the ATRX bound genes identified to be upregulated in senescence by RNA0seq. Expression was plotted as a fold change in PD0332991 treated (7 days) cells compared to untreated cells, normalized to beta-actin. The solid line indicates no change in expression and dashed lines indicate cutoffs at 1.8-fold change in expression to demonstrate significant repression or activation.

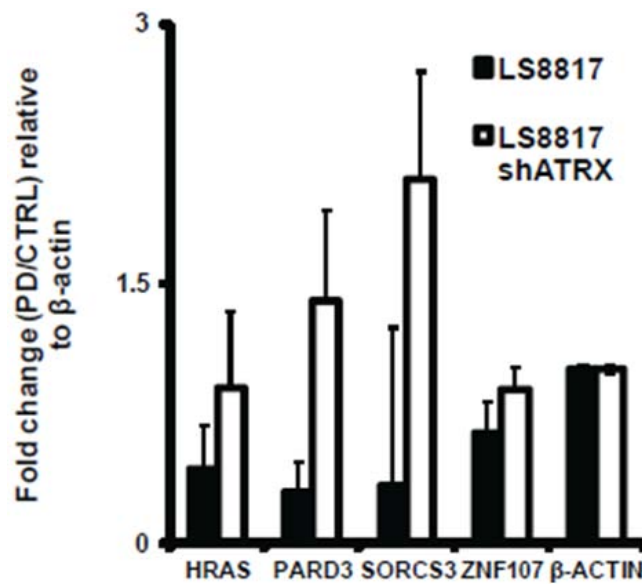


Figure 5.05 ATRX is required for efficient repression of *HRAS* in LS8817 cells.

ATRX was stably reduced in LS8817 cells and the expression of the indicated genes measured as described in Figure 5.03.

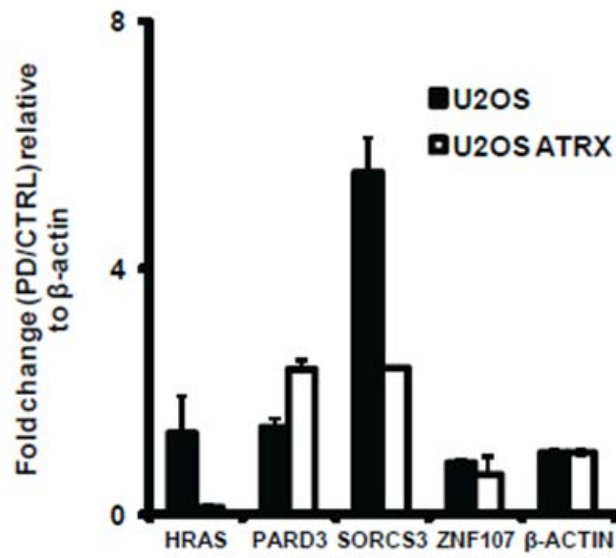


Figure 5.06 ATRX is required for efficient repression of *HRAS* in U2OS cells.

U2OS cells were stably transfected with wild type ATRX and expression of genes measured as described in Figure 5.03.

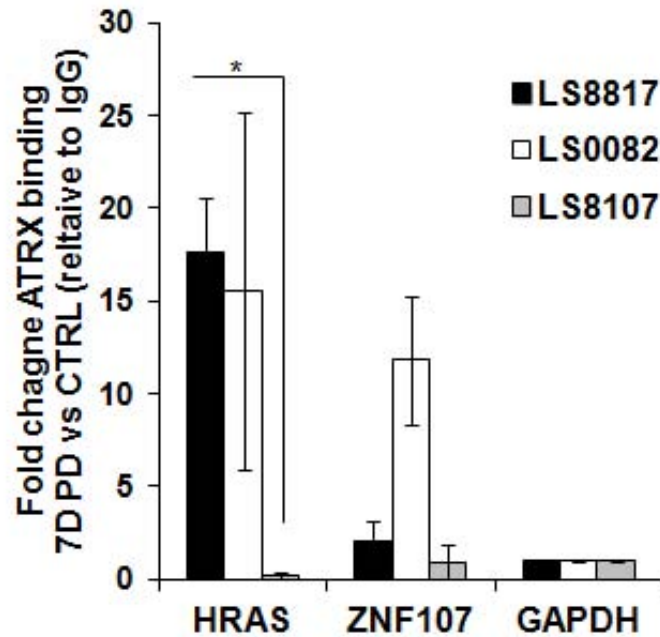


Figure 5.07 ATRX accumulates at genomic DNA of the *HRAS* locus in senescent but not quiescent WD/DDLS cells.

ATRX ChIP experiments were performed in untreated controls and treated cells as indicated. The relative amount of ATRX binding at the indicated loci (as originally identified by ChIP-seq) was analyzed by qPCR. * $p < 0.05$.

treated with CDK4i. *HRAS* (but not *ZNF107*) expression was suppressed in senescent glioma (SNB19) and lung cancer (A549, H1975) cells following CDK4 inhibition, but not in the quiescent lung cancer cell line (H358) (Figure 5.08). Both *HRAS* and *ZNF107* mRNA were suppressed in the LS8817 cells in which MDM2 was knocked down. Collectively, this suggests that the repression of *HRAS* is not cell type specific during CDK4i-induced TIS.

Consistent with this, ATRX ChIP-seq binding tracks around the *HRAS* locus showed clear focal binding enriched in senescent LS8817 cells compared to the serum starved cells (Figure 5.09). This locus also contains a number of G-quadruplex forming sequence elements, which have a propensity for ATRX-mediated regulation of target gene expression (Law et al. 2010).

ATRX binding to *HRAS* was detected most strongly in LS8817 cells late after the addition of CDK4i (Figure 5.10), a time by which these cells have accumulated other markers of senescence (Figure 4.25). In U2OS cells expressing wild type ATRX, ATRX bound to *HRAS* (Figure 5.11). In cells expressing the C240G and V588E mutant proteins, I could not detect *HRAS* binding; this is consistent with the inability of these mutants to repress *HRAS* as strongly as the wild type following CDK4i treatment (Figure 5.12). Thus, I concluded that *HRAS* was a bona fide target of ATRX.

5.2.3 *HRAS* repression drives geroconversion: the transition from quiescence into senescence

The data above indicated a role for ATRX in geroconversion and suggested that some of this activity may relate to its ability to bind to and repress expression from the *HRAS* locus. Thus, I asked whether suppressing *HRAS* in quiescent cells would induce

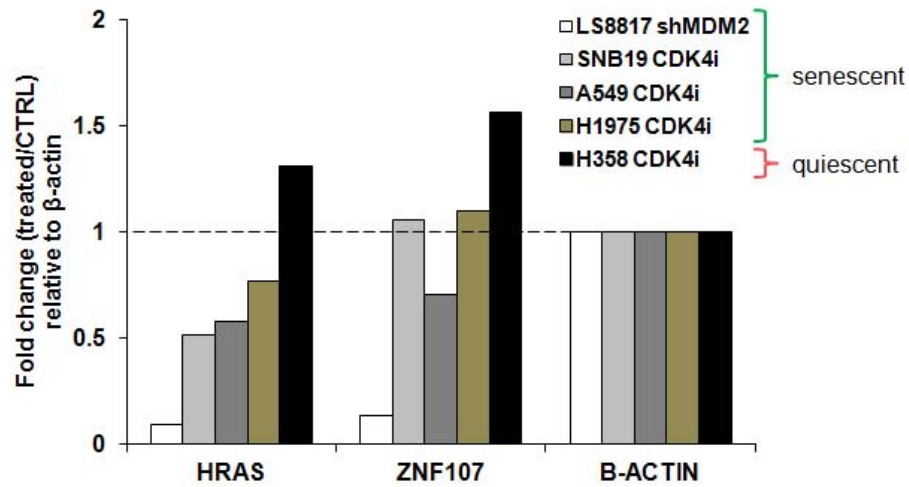


Figure 5.08 *HRAS* is repressed in a variety of different transformed cell lines undergoing senescence.

The cells were treated as indicated and expression of *HRAS*, *ZNF107*, and β -actin were measured as described. The outcome of treatment, vis a vis quiescence or senescence is indicated in the legend of the graph.

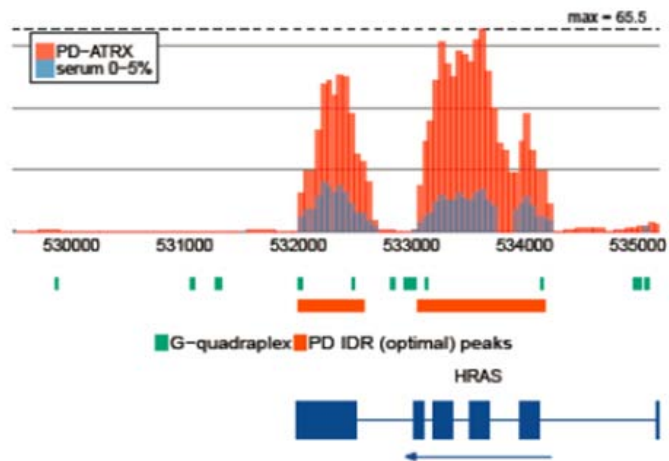


Figure 5.09 ATRX is enriched at the *HRAS* locus in senescent as compared to quiescent LS8817 cells.

Genome browser view of ATRX enrichment at the *HRAS* locus in quiescent (serum 0-5%, blue) and senescent (PD-ATRX, red) LS8817 cells as determined by ChIP-seq. Orange bars indicate the peaks called via the IDR algorithm and green bars indicate DNA sequences predicted to form G-quadruplex structures.

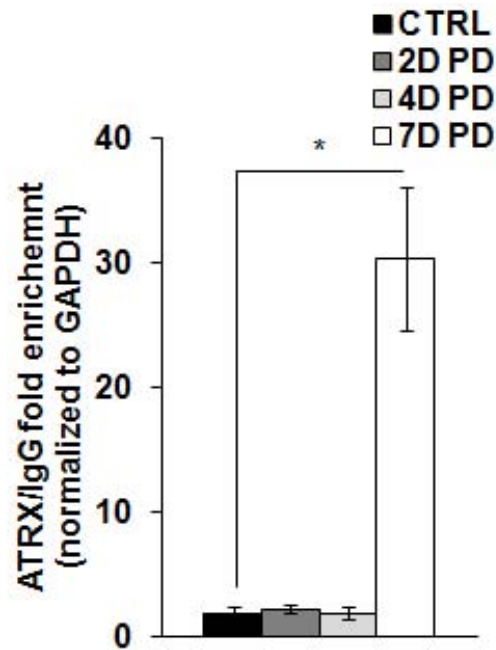


Figure 5.10 ATRX does not accumulate at genomic *HRAS* in LS8817 cells until 7 days following PD0332991 treatment.

ATRX ChIP was performed in LS8817 at the times indicated following treatment with PD0332991 and in untreated cycling cells (CTRL). The level of ATRX enrichment specifically at the *HRAS* locus as determined by qPCR was plotted. * $p < 0.05$.

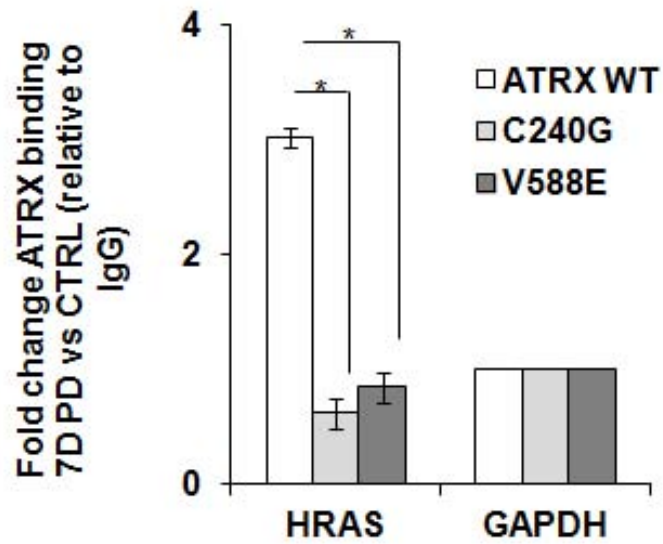


Figure 5.11 ATRX binding to HRAS following PD0332991 treatment in U2OS cells requires H3K9Me3 and HP1 interactions.

ATRX was immunoprecipitated from CDK4 inhibitor treated and untreated U2OS cells in which either wild type (WT) or mutant (C240G, V588E) protein was expressed. ATRX enrichment at the *HRAS* locus as determined by qPCR was quantified. * $p < 0.05$.

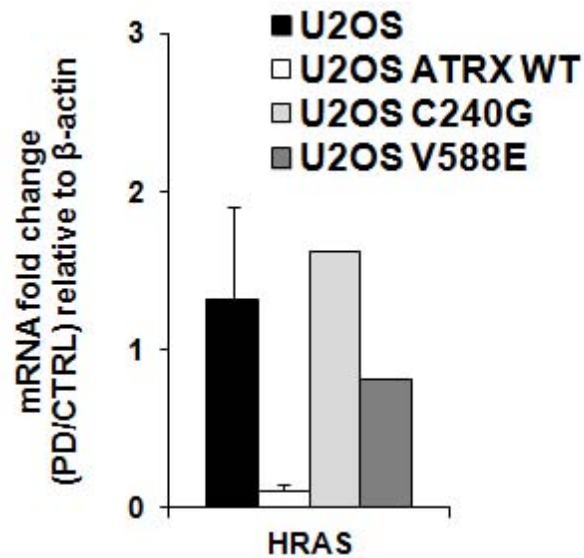


Figure 5.12 ATRX repression of HRAS following PD0332991 treatment in U2OS cells requires H3K9Me3 and HP1 interactions.

HRAS mRNA levels were measured from CDK4 inhibitor treated and untreated U2OS cells in which either wild type (WT) or mutant (C240G, V588E) protein was expressed.

geroconversion. To accomplish this I first identified two independent shRNAs that when introduced into LS8817 cells would specifically repress *HRAS* expression but not *KRAS* or *NRAS* (Figure 5.13). I subsequently used these in two different experimental situations to determine if reducing *HRAS* would promote geroconversion.

First, I serum starved LS8817 cells to induce quiescence and then knocked down *HRAS* and measured the accumulation of SA- β -gal positive cells, HP1 γ foci positive cells, and the ability of the cells to return to the cell cycle and grow when serum was restored. The extent of *HRAS* repression was similar to that seen following treatment with CDK4 inhibitors (Figure 5.13). Following the loss of *HRAS*, but not in cells transduced with a vector expressing a scrambled control shRNA, the number of SA- β -gal positive cells increased (Figure 5.14). The number of HP1 γ positive cells also increased and clonogenic growth was reduced following restoration of serum (Figure 5.14).

Second, I treated LS8107 cells with CDK4 inhibitors to induce quiescence and asked if knocking down *HRAS* would induce geroconversion. Again I validated that *HRAS* was reduced following knockdown in this cell line by qPCR (Figure 5.15). Knocking down *HRAS* also led to the accumulation of SA- β -gal positive cells, HP1 γ foci positive cells. The cells did not incorporate BrdU (Figure 5.15). Collectively then, it appears that the loss of *HRAS* can contribute to geroconversion.

I then asked whether enforcing the expression of *HRAS* would prevent CDK4i-induced senescence. I was able to express *HRAS* at physiologic levels in LS8817 cells using a lentiviral vector. Levels of *HRAS* were maintained during seven days of CDK4i treatment (Figure 5.16 A). Although *HRAS* expression did not significantly reduce the

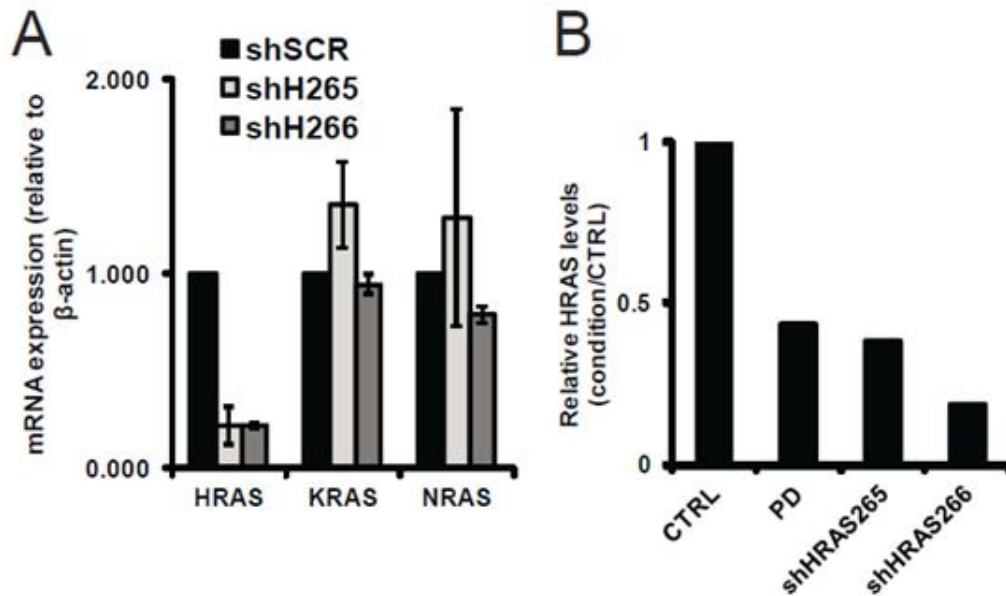


Figure 5.13 shRNA reduces *HRAS* to levels comparable to CD4k inhibition in LS8817 cells.

(A) *HRAS* was stably knocked down with two independent hairpins in cycling LS8817 cells and transcript levels were measured by qPCR. Expression levels of each gene were individually normalized to 1 in the shSCR cells. (B) LS8817 cells were treated with CDK4i for seven days or serum starved (0.5%) for three days and subsequently infected with the indicated shRNA encoding lentiviruses. After an additional five days of selection, the accumulation of *HRAS* mRNA was measured.

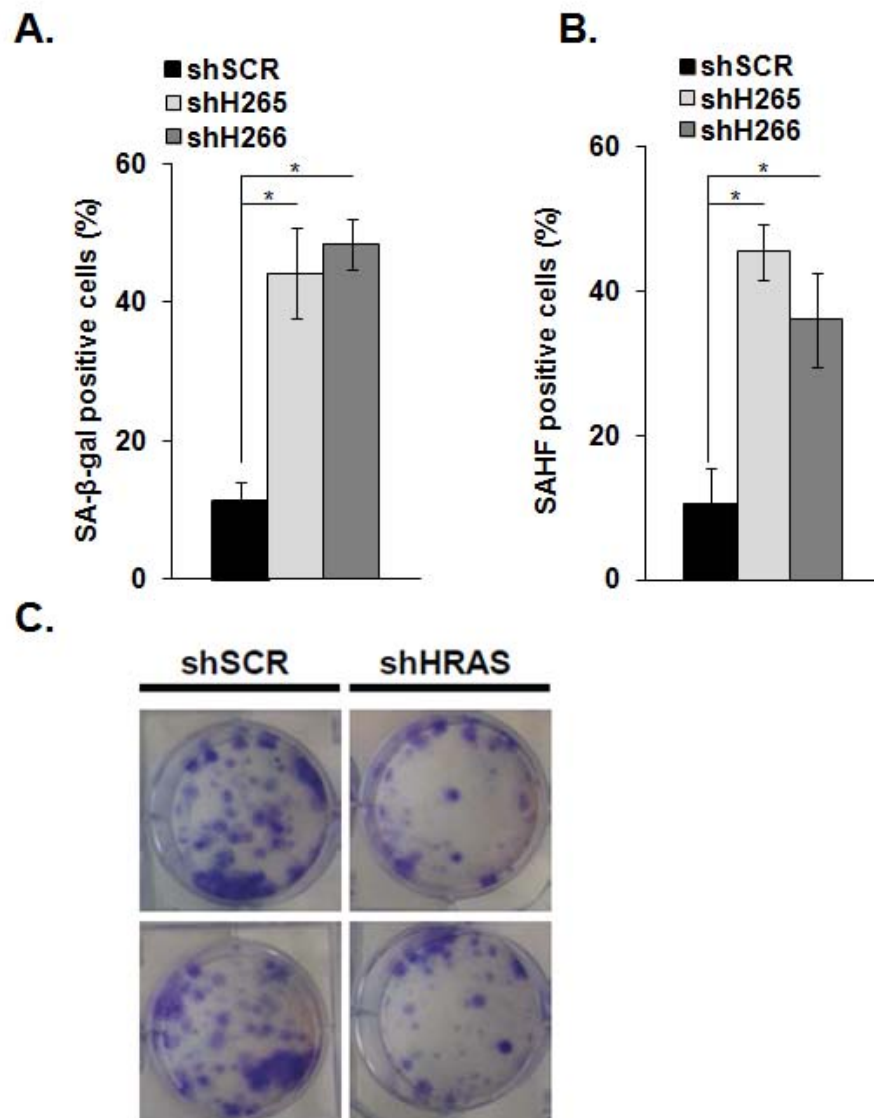


Figure 5.14 Reduction of *HRAS* is sufficient to drive geroconversion: the transition from quiescence into senescence.

LS8817 cells were serum starved (0.5%) for three days and subsequently infected with the indicated shRNA encoding lentiviruses. After an additional five days of selection, the accumulation of SA-β-gal (A) and SAHF (B) were measured. Long term clonogenicity was measured by crystal violet staining of colonies three weeks after the cells were replated in complete medium (C). * $p < 0.05$.

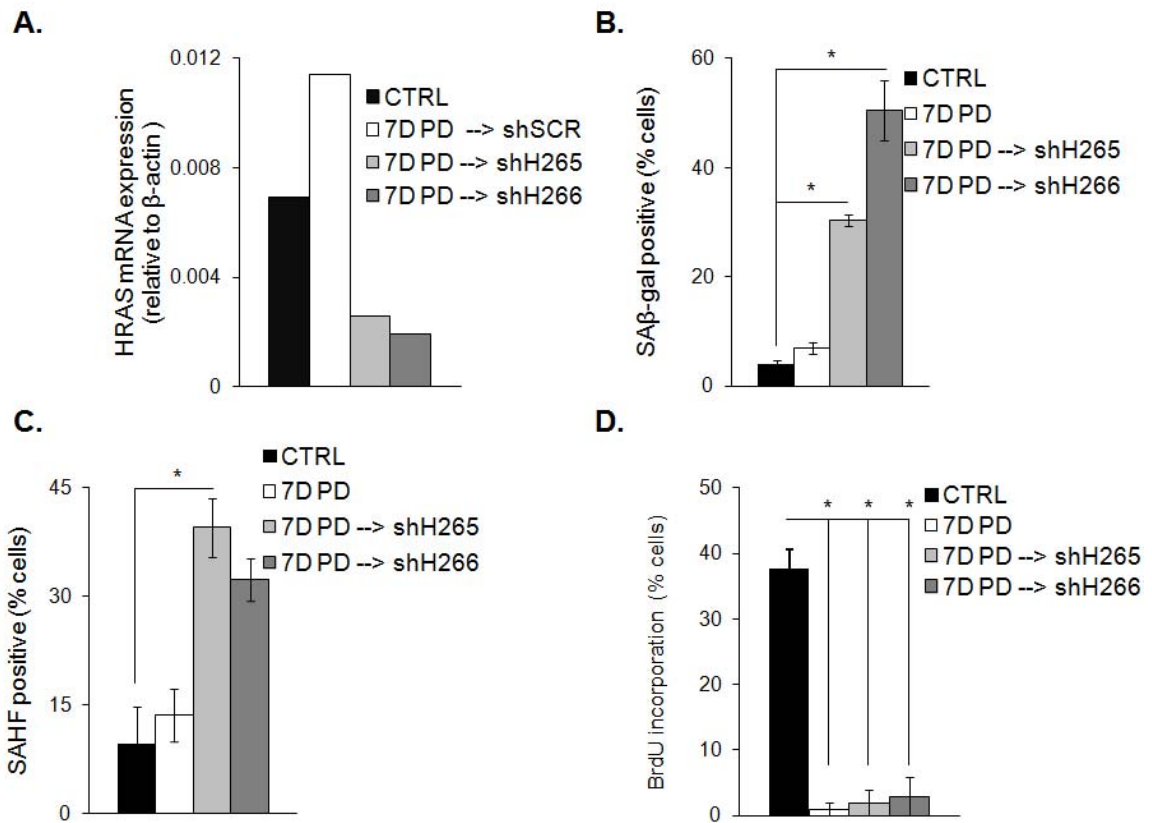


Figure 5.15 Reduction of HRAS is sufficient to drive geroconversion: the transition from quiescence into senescence.

LS8107 cells were treated with PD0332991 for seven days and then infected with lentiviral vectors targeting HRAS. PD treatment was maintained as viral selection occurred. The efficacy of knockdown was assessed by qPCR (A). The effect of reducing HRAS on the accumulation of SA- β -gal (B) or SAHF (C), or the ability of the cells to incorporate BrdU (D) was determined. * $p < 0.05$.

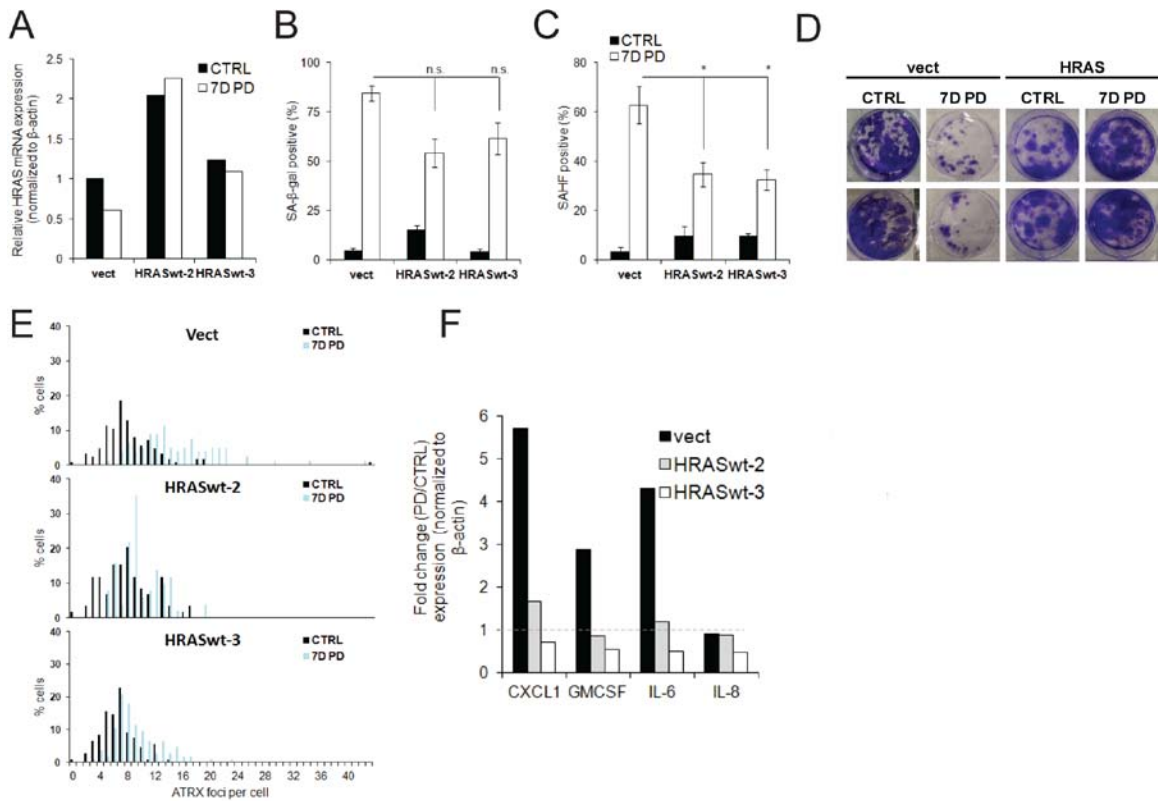


Figure 5.16 Enforcing expression of HRAS in LS8817 can prevent senescence induced by CDK4 inhibition.

Wild type *HRAS* (HRASwt) or a vector control (vect) was stably expressed in LS8817 cells two independent experiments. After four days of selection with puromycin, cells were treated with 1 μ M of PD0332991 for seven additional days (7D PD) and compared to cycling (CTRL) cells and analyzed for the following: *HRAS* mRNA levels measured by qPCR (A), SA- β -gal (B), HP1 γ SAHF (C), ability to return to cell cycle once PD0332991 once the dug was removed (D), number of ATRX foci on an individual basis (E) and induction of four SASP factors as measured by qPCR (F). * $p < 0.05$.

number of SA- β -gal positive cells that accumulated following CDK4i treatment, there was a significant reduction in SAHF-positive cells. Moreover, ATRX foci failed to accumulate to maximum levels, SASP factors were not induced at the mRNA level and the cells were improved in their capacity to return to cell cycle once the drug was withdrawn (Figure 5.16 B-F). Thus, enforcing *HRAS* expression can prevent CDK4i-induced senescence in LS8817 cells.

5.3 Discussion

By asking whether ATRX had a function in senescence beyond the SAHF, I identified that ATRX has a role in regulating PRC2 targets, as well as a role in binding to and repressing *HRAS* in TIS. Ectopically reducing *HRAS* could convert quiescence into senescence, indicating that this contributes to senescence induced by CDK4i and enforced expression of *HRAS* could prevent PD0332991-induced senescence, albeit not all markers of senescence – namely SA- β -gal accumulation – were attenuated. This suggests there are other mechanisms related to CDK4 inhibition that may contribute to evolution of this senescence marker; whatever these mechanisms may be, they are likely related to ATRX, as CDK4i (and doxorubicin) are significantly compromised in inducing SA- β -gal accumulation in ATRX-deficient WD/DDLS cell.

A loss of ATRX also resulted in a disturbance of EZH2 and SUZ12 target gene regulation following CDK4i treatment. EZH2 is a histone methyltransferase and is the enzymatic component of the polycomb repressive complex 2 (PRC2), contributing to efficient H3K27 methylation. SUZ12 is a zinc finger gene also found within PRC2 (Mills 2010; Hock 2012). PRC2 acts to methylate and maintain target gene repression through cell division, particularly during development (Comet et al. 2016). Regulation of this complex is also important for normal tissue maintenance, as EZH2 is frequently dysregulated in a number of cancers including breast, prostate, melanoma and bladder

cancer as well as in myeloid diseases like acute myeloid leukemia (Ernst et al. 2010). EZH2 can have pleiotropic effects, generally acting as an oncogene in solid tumors and a tumor suppressor in myeloid diseases (Volkel et al. 2015; Hock 2012; Ernst et al. 2010).

Activation of EZH2 in some cancers is thought to promote malignancy by repressing tumor suppressor genes (Karanikolas et al. 2009; Kim and Roberts 2016; Souroullas et al. 2016). Consistent with this, loss of EZH2 has been shown to drive senescence via reactivation of both p21 and p16 (Fan et al. 2011; Bracken et al. 2007). ATRX has been reported to direct the recruitment of EZH2 during X-chromosome inactivation (XCI) (Sarma et al. 2014). XCI has similarities to the formation in SAHF in that it involves facultative heterochromatin establishment, entire chromosome condensation (Funayama et al. 2006), and escape from repression of subset(s) of genes despite the overall global chromosomal accumulation of repressive marks (Balaton and Brown 2016). My current work indicates that ATRX has a role in regulating PRC2 target gene expression in senescence. This may be directly via ATRX targeting EZH2 to the DNA, as was seen in XCI, or may reflect a common set of genes that are regulated by both PRC2 (in the databases reported in GSEA and Enrichr) and ATRX (as I have observed in TIS).

Additionally, I found that ATRX controls *HRAS* expression in senescent cells. ATRX binds to the *HRAS* locus and represses gene expression. The *HRAS* locus is rich in G-quadruplex prone sequences and ATRX has been reported to affect gene expression from such sequences (Law et al. 2010). Repression of *HRAS* is sufficient to drive quiescent cells into senescence, suggesting that repressing *HRAS* may play an important role during TIS.

Unlike the accumulation of ATRX foci, the repression of *HRAS* by ATRX is likely TIS-specific and perhaps unique to transformed cells. I did not observe a reduction in *HRAS* levels in normal HDFs when they underwent replicative senescence, or via DNA damage (induced via both irradiation and doxorubicin treatment) (MK, unpublished data) despite their accumulation of ATRX foci. However, in the context of TIS, repression of *HRAS* may represent an alternative and/or combinatorial therapeutic opportunity; for a further discussion on this, see **6.2.3 Combinatorial therapies**. Furthermore, since ATRX seems to be involved in many different types of senescence, studying whether and how ATRX contributes to gene regulation in a context-specific manner may give insight into the unique and common (shared) elements of a wide range of senescence programs.

6. DISCUSSION & FUTURE DIRECTIONS

6.1 Overview

In my thesis studies, I investigated the mechanism of CDK4 inhibitor (CDK4i) induced senescence in transformed cells. In a pilot study, I identified that geroconversion was a clinically important pathway relevant to patient outcome, and I identified MDM2, ATRX and HRAS as mediators that control senescence during CDK4i-induced geroconversion. I also showed that ATRX plays a broader role participating in other forms of therapy induced senescence (TIS) and perhaps even in replicative or oncogene induced senescence.

When I began my work it was known that CDK4i could induce either quiescence or senescence in well differentiated and dedifferentiated liposarcoma (WD/DDLS), but we were unaware of the relationship between these states, the mechanisms that underlie cell fate, and whether or not this was clinically relevant. We now understand that CDK4 inhibition causes quiescence and that a distinct pathway from quiescence to senescence (i.e. geroconversion) involving MDM2 turnover, as well as ATRX and its ability to regulate both the SAHF and *HRAS* are important. Only a subset of cell lines engage the appropriate machinery to convert their growth arrest into irreversible senescence and elaborate the hallmarks associated with the senescent state (namely irreversibility, SA- β -gal, SAHF and SASP) (**Figure 6.01**).

More generally, I have identified the chromatin remodeling protein ATRX as a novel regulator of senescence (**Figure 6.01**). ATRX regulates the SAHF and also plays a direct role repressing the expression of *HRAS* during TIS in both CDK4i- and doxorubicin-treated cells. ATRX accumulates at nuclear foci in senescent cells, quite

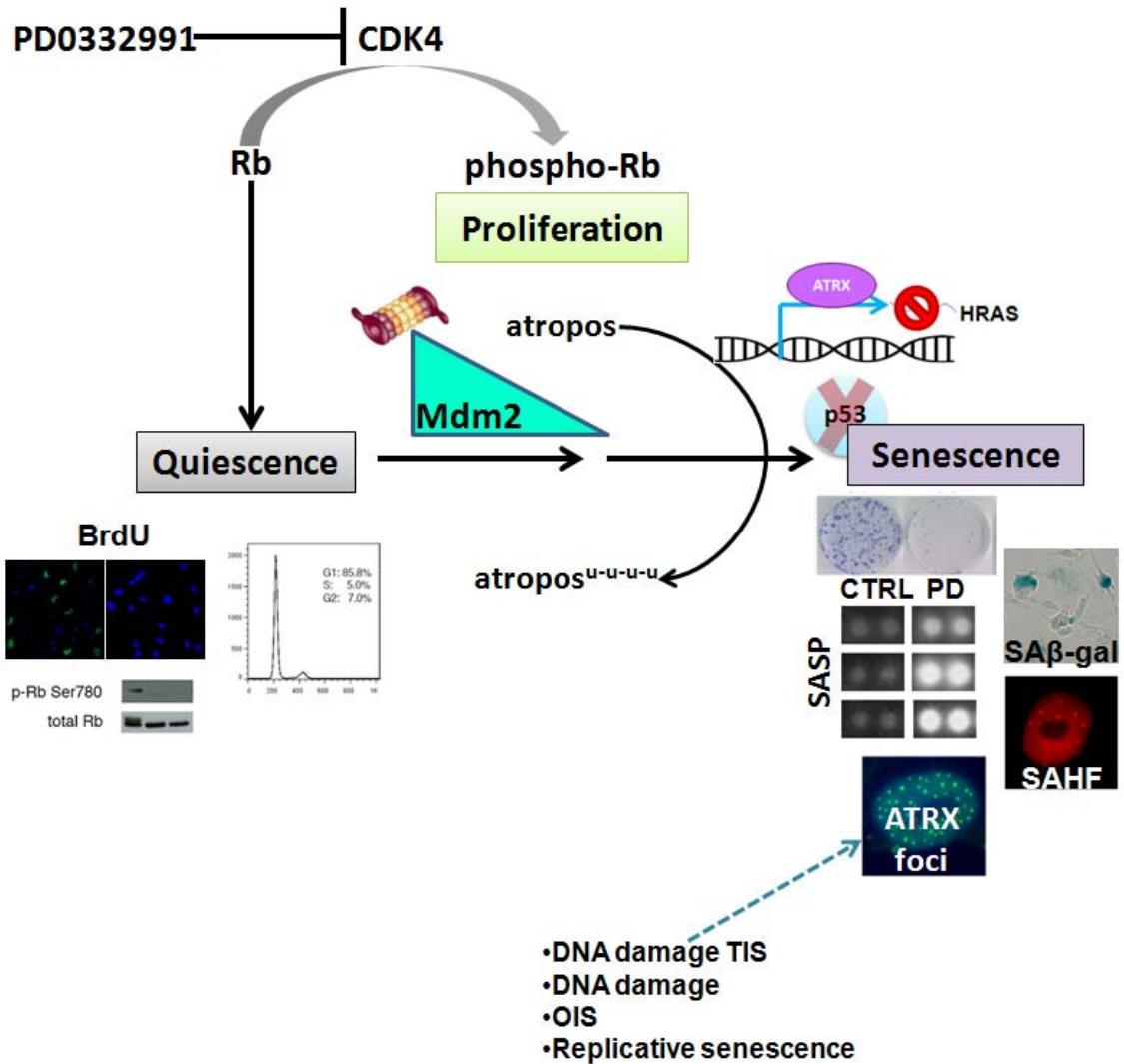


Figure 6.01 MDM2, ATRX and HRAS drive the transition from quiescence to senescence.

PD0332991 inhibits CDK4 activity; unphosphorylated Rb promotes quiescence, which is marked by a loss of BrdU incorporation, loss of phospho-Rb and 2c DNA content as assayed by flow cytometry. Entry into senescence depends on proteasomal turnover of MDM2 and is independent of p53. MDM2 ubiquitylates a currently unidentified substrate (termed atropos) in order to drive senescence. ATRX is also required to drive the transition from quiescence to senescence. ATRX binds to and represses *HRAS* to promote senescence. ATRX also has a role in regulating MDM2 turnover (not depicted). Senescence is characterized by accumulation of senescence associated β-galactosidase (SA-β-gal), senescence associated heterochromatic foci (SAHF), accumulation of ATRX foci, increased secretion of SASP factors and irreversibility of growth arrest (depicted by clonogenic outgrowth assays, blue). ATRX foci also form in senescent cells induced by a variety of different stimuli (lower; blue dashed arrow). ATRX is also required for the elaboration of senescence hallmarks induced by DNA damage therapy induced senescence (TIS).

early following growth arrest and specifically in cells that have embarked onto irreversibility, regardless of the cell type and the senescence stimulus. ATRX foci do not accumulate in any of the quiescent, autophagic or differentiated cells I have tested. Thus, ATRX may represent a novel, specific marker of cells that have embarked onto senescence.

By using an *in vitro* system of patient derived WD/DDLS cell lines to understand the clinical efficacy of CDK4i drugs we were able to uncover a unique situation where a single drug, in a single disease type, could lead to two different cellular fates: quiescence and senescence. This allowed me to dissect the molecular pathway governing these decisions, implicate new roles for MDM2, ATRX and HRAS in senescence and integrate this knowledge back into a clinically relevant application. Collectively, our work indicates that a “bedside to bench” paradigm – that is, taking a clinical observation back to the lab to be studied *in vitro* – can guide meaningful discovery at the molecular and cellular level, which not only gives us a clear understanding of fundamental biology, but ultimately can be applied clinically to maximize patient benefit.

6.2 Clinical Implications

Despite the heralded success of CDK4i drugs, their efficacy has been limited by a lack of a deep understanding of their molecular mechanisms of action and a lack of relevant clinical biomarkers. Our current work suggests that MDM2 is an indicator of the efficacy of CDK4i drugs in patients, with correlative data from a Phase II WD/DDLS trial supporting this. Certainly, this observation will have to be expanded in a larger patient cohort within WD/DDLS and to other diseases. The *in vitro* relationship between MDM2 down-regulation following CDK4i and senescence provides biologic rationale for why

reduced MDM2 levels would associate with a favorable clinical outcome, as senescence is generally deemed a favorable outcome of cytostatic drugs (Acosta and Gil 2012).

6.2.1 A prognostic biomarker for CDK4i

Although changes in MDM2 can be measured as soon as two weeks following the first treatment with a CDK4i, the holy grail of therapeutic biomarkers would be one that is prognostic and can dictate whether or not a patient should ever begin a particular treatment at all. My data indicate that the chromatin remodeling protein ATRX is involved in the post-translational regulation of MDM2 following CDK4 inhibition. We could not detect any mutations, deletions or amplifications of ATRX in our cell lines (AC, unpublished data); however, there was a striking association with response to CDK4i and our ability to detect ATRX using a Bethyl antibody that reacts to the C-terminal most region of the protein.

Using a combination of mass spectrometry and artificially modified peptides, I was able to map a phosphorylation event on S2487 of ATRX that likely accounts for this difference in reactivity ((Kovatcheva et al. 2015) and MK, unpublished data). We are currently working on developing monoclonal antibodies to specifically detect this modification. Such a reagent could hypothetically serve as a prognostic biomarker. Those patients whose tumor is positive for the S2487 phosphorylated moiety of ATRX would be predicted to exhibit a poor response to CDK4i, as we would expect MDM2 levels to not be reduced following treatment. While we do not yet know the kinase responsible for this modification, the serine residue maps to an SQ site. Such sites are preferentially phosphorylated by ATM/ATR-like kinases in response to DNA damage (Traven and Heierhorst 2005). We are further investigating the biology of this phosphorylation event and whether the kinase in question could be pharmacologically targeted to promote MDM2 turnover and ultimately ameliorate patient outcome.

How might ATRX regulate MDM2 levels? The regulation of MDM2 is extremely complex and is dictated by transcriptional and post-translational mechanisms that can be affected by post-translational modification (i.e. phosphorylation, ubiquitylation) and localization (e.g. Arf sequesters MDM2 in the nucleolus) (Riley and Lozano 2012; Weber et al. 1999; Manfredi 2010). We know that MDM2 levels decrease in response to CDK4i due to accelerated proteasomal turnover. While it is conceivable that ATRX is regulating the gene expression of a regulator of MDM2, this seems unlikely. ATRX foci do not begin to accumulate until 48 hours following CDK4i treatment, while a decrease in MDM2 levels is observed within the first 24 hours of treatment. Moreover, the correlation with the phospho-specific moiety is consistent with a model in which protein-protein interactions are variable (e.g. the release of E2F TFs is dictated by phosphorylation). Mary E. Klein, another graduate student in the lab, is investigating the mechanism of MDM2 regulation and it indeed seems to be related to sub-cellular localization and/or protein-protein interactions.

6.2.2 The MDM2 substrate

As discussed in **Chapter 3**, mullin compounds that target the MDM2-p53 interaction have been largely unsuccessful thus far, while compounds targeting the E3 ubiquitin ligase activity of MDM2 have yet to be tested in the clinic (Kitagaki et al. 2008; Selivanova 2014). However, my thesis work indicates that there is an alternative MDM2 substrate (i.e. not p53) that is important in regulating senescence. Given the ample evidence that senescence is a favorable clinical outcome in cancer therapy, there is untapped therapeutic potential in identifying what the non-p53 substrate(s) of MDM2 might be.

Traditionally, it is difficult to screen for ubiquitin ligase substrates on account of the overall abundance of the ubiquitin modification in the cell as well as the instability of many such substrates (due to the propensity for ubiquitination to promote proteasomal degradation) (Ciechanover 1994). However there are approaches to circumvent these problems, one of which has been termed the NEDDylator (Zhuang et al. 2013). This involves a catalytically inactive mutant of the E3 ligase of interest (i.e. MDM2) fused to Ubc12. Ubc12 is an E3 ligase for NEDD8; this ubiquitin-like protein modifier is much rarer than ubiquitin itself and is not associated with proteasomal targeting (Soucy et al. 2010). Currently, I am working on using an MDM2-NEDDylator system in combination with a 6x histidine- and biotin-tagged NEDD8 to purify putative substrates and identify them via mass spectrometry.

6.2.3 Combinatorial therapies

Also relevant to senescence and the clinical efficacy of CDK4i drugs is the observation that *HRAS* transcript levels are repressed in an ATRX-dependent fashion in senescent cells. Moreover, reducing *HRAS* via shRNA could convert quiescence to senescence, effectively altering the class of response to CDK4i drugs. It should be noted that *HRAS* is neither amplified nor activated in our cell lines (AC, unpublished data); thus, the effects of reducing *HRAS* do not seem to be due to dependence on elevated *HRAS*.

We were initially surprised to see *HRAS* repression as a driver of senescence, given the wealth of data in human diploid fibroblast studies that expression of activated *RAS*^{V12} mutants leads to oncogene induced senescence (OIS) (Dimauro and David 2010; Serrano et al. 1997). However, there is evidence that OIS induces an initial wave of

proliferation, followed by a potent and global suppression of Ras and its effector pathways (Courtois-Cox et al. 2006; Courtois-Cox et al. 2008). Without such suppression, cells did not undergo senescence. Moreover, reduced Ras-GTP loading (which effectively suppresses Ras signaling) led to induction of senescence in HDFs. Further evidence that Ras repression is relevant to senescence comes from studies in which inhibitors that affect Ras downstream signaling networks (e.g. Raf inhibitors, MEK inhibitors, PI3K inhibitors) can promote senescence in transformed cells (Axanova et al. 2010; Schick et al. 2015; Haferkamp et al. 2013; Ota et al. 2006).

There is evidence that combinations of CDK4i drugs with drugs that inhibit signaling through the RAS/MAPK pathway show broader benefit than CDK4 inhibition alone in a variety of circumstance (reviewed in (Hamilton and Infante 2016)). In breast cancer and breast cancer cell lines, CDK4i drugs have been used in combination with estrogen receptor antagonists and AKT inhibitors and reported to enhance their clinical or *in vitro* cytostatic activity (Vora et al. 2014). Other studies in pancreatic cancer also favor combinations of CDK4i with PI3-kinase (PI3K) and mTOR inhibitors (Franco et al. 2014). One model to explain this is that the hormone receptor antagonists/RTK/PI3K inhibitors are somehow dampening the mitogen-driven increase in cyclin D levels; cyclin D upregulation is perceived as a mechanism of acquired resistance to CDK4i therapy (reviewed in (Sherr et al. 2016)). Thus, combining CDK4 inhibitors with RTK inhibitors would more potently induce G1 growth arrest through the CDK4-Rb axis. An alternative model supported by the data presented here is that suppressing signaling through the RAS/MAPK pathway in a quiescent cell can induce its progression to senescence, improving the strength and consequences of the growth arrest.

How suppression of *HRAS* converts quiescence to senescence remains to be understood. We are currently exploring this through pharmacologic means using drugs such as tipifarnib, a farnesyl transferase inhibitor (FTI) that specifically inhibits HRAS but not KRAS or NRAS (which can be geranylgeranyl modified and maintain function in the presence of FTI) (End et al. 2001); Trametinib, which inhibits MEK1 and MEK2 thus silencing the Raf-mediated axis of Ras signaling (Zeiser 2014); and LY294002, which is a PI3K inhibitor (Gharbi et al. 2007). The results of these experiments will give insight into which signaling axes of Ras are relevant to senescence induction.

Of course, senescence induction is complex, multi-faceted and often times context-specific. Thus, mechanisms of CDK4i efficacy and/or resistance may be cell-type specific. Although we have evidence that MDM2 changes are relevant to CDK4i efficacy in cell types other than WD/DDLS and we did see decreases of *HRAS* in lung- and glioma-derived cell lines, such drug combinations will have to be tested in various different disease types. Ultimately, a more thorough understanding of these pathways can fuel rational combinatorial therapy design.

6.3 ATRX foci as a tool

Despite many decades of study, the senescence field still lacks reliable, unique markers that unequivocally define a senescent cell. It is possible that no such markers exist; in fact, what we refer to as senescence may simply be a collection of unique states that shares some common features and thus can never be defined by a single marker. On the other hand, it is possible that “core” senescence machinery does exist; that there are some elements common to all senescent cells, regardless of the cell or inducer type, against the backdrop of a constellation of context-specific hallmarks. My thesis work indicates that ATRX foci may in fact represent a commonly shared marker of senescence. Undoubtedly, the breadth of ATRX foci remains to be tested by other

groups, in a nearly limitless number of cell types and with inducers we have not yet employed. However, we are eager to see whether the senescence community (and beyond) will ascertain ATRX foci as a *bona fide* marker of senescence.

Thus, we see great utility in using ATRX foci as a screening tool and have ascertained with screening facilities that the foci are amenable to high content screens. This represents a significant conceptual advance in senescence screens which, up until now, have generally been carried out using growth arrest as the high throughput-compatible assay for senescence (Lahtela et al. 2013; Peeper et al. 2002; Shvarts et al. 2002; Wang et al. 2016). However, cell cycle arrest is not equivalent to senescence; ergo, such screens require manual validation with secondary senescence marker(s), thus limiting the scale and speed with which they can be conducted. ATRX foci, on the other hand, distinguish senescence from growth arrest, as ATRX is important in the transition from quiescence to senescence. Thus, these foci can be used to screen for compounds and/or genes that can both block and promote senescence without affecting proliferation.

Conceptually, senescence inducers can be identified by taking a cell line that is quiescent (for example, through serum starvation or in response to CDK4i) and treating it with a library of small molecules, FDA approved compounds, shRNAs sgRNAs, etc. Senescence inducers would promote ATRX foci formation within 4-7 days of treatment. Such screens could be used to identify combinatorial therapies to promote senescence in a setting where it is clinically favorable, like cancer therapy. ATRX screening could be used to rapidly and relatively inexpensively determine individual drug combinations, depending on the context. The same compounds and combinations could be concomitantly screened against untransformed cells. By identifying combinations that affect cancer cells but not normal cells, we could theoretically ameliorate some of the

detrimental side effects of TIS that are thought to be related to the senescence of normal tissue.

Senescence inhibitors, on the other hand – or senosuppressants as they may be termed – can be identified by screening libraries in cells that are known to undergo senescence with a particular treatment, like doxorubicin or CDK4i. Our data indicates that senescence induction and senescence maintenance are not equivalent; in fact, inactivating ATRX in a previously senescent cell did not drive it out of senescence. Thus, these screens must be conducted in either a parallel manner – where the senescence inducer and library are administered concomitantly – or in a sequential manner whereby the library precedes the senescence inducer. Once again we could simply screen for ATRX foci, this time looking for treatments and/or combinations that restrict the elaboration of ATRX foci. Understanding the relationships between quiescence, senescence and proliferation also have profound implications for stem cell biology. For example, we can understand molecularly how the maintenance and exhaustion of stem cell pools is regulated and how this affects physiology, or how to stimulate stem cell proliferation in a controlled fashion during a time of need, like following neuronal injury in order to facilitate tissue repair.

Senosuppressant screens would also be relevant in the context of age-related pathologies, which may be a direct consequence of senescence (and may be at least in part related to depletion of stem cell function). To date, the majority of age-related screens have been conducted by seeking lifespan extension in lower organisms such as nematodes or yeast (Ye et al. 2014; Collins et al. 2006; Yanos et al. 2012; Hamilton et al. 2005). Undoubtedly, such studies have taught us a great deal about genes related to aging, but there is some concern about their relevance in higher organisms (Mattison et al. 2012). ATRX foci provide an indirect way to gain genetic and/or pharmacologic

insight in the pathways that regulate senescence and – by association – perhaps aging in human tissue.

In the field of aging, there has been a great deal of interest in compounds that can kill senescent cells, termed senolytics. Indeed, such drugs have been identified and are currently being developed for clinical use. ABT-263 is a Bcl-2 family inhibitor that was developed to treat cancer, but has showed limited efficacy in clinical trials. ABT-263 has recently been shown to elicit apoptosis specifically in senescent cells and inhibit premature aging induced by total body irradiation in mice (Leverson et al. 2015) (Chang et al. 2016). Similarly, a combination of Dasatinib, another cancer drug traditionally used to treat leukemia, and quercetin, a naturally occurring flavonoid, has been shown to clear senescent cells and ameliorate pathologies in progeroid mice (Zhu et al. 2015).

However, even if such drugs do translate into human therapies, the approach to simply remove senescent cells from an organism is perhaps naïve, as senescence underlies both beneficial and detrimental biology. A clear understanding of what induces an aged or pathologic senescent cell, versus a therapy induced senescent cell, versus a developmental senescent cell (and so on) will be essential in order to precisely target pro- or anti-senescence therapies. The manipulation of senescent cells also quickly becomes precarious, as there is little understanding in what differentiates a senescent cell from a growth arrested cell. Thus, in restraining senescence we may inadvertently push cells into proliferation and promote malignancy. Furthermore, a lack of clear senescence markers limits our potential to accurately and specifically target senescent cells, potentially endangering normal cells. Employing ATRX as a marker that distinguishes between quiescence and senescence might ameliorate many of these concerns.

6.4 The molecular and temporal evolution of senescence

Currently, the molecular details between cell cycle exit and achieving the permanence of senescence are lacking (**Figure 1.1**). Through my thesis work, I have suggested ATRX as a common regulator of senescence. ATRX is a chromatin remodeling protein, and many groups agree on overall the importance of chromatin changes in senescence, albeit they may vary across different cell types and/or senescence inducers. It remains to be elucidated how senescence evolves over time. In the context of ATRX, this can perhaps be achieved by repeating ChIP-seq experiments at different temporal points to gain insight as to how these foci may be re-organizing. Indeed, there is evidence that the changes in chromatin modification in senescent cells are not novel histone marks, but rather a “rearrangement” of those existing modifications within the cell (Parry and Narita 2016; Chandra et al. 2012). Thus, ATRX may simply be re-distributed across the chromatin over time, regulating different genomic regions as necessary.

We also have yet to address how exactly ATRX remodels chromatin to influence senescence. The histone variant H3.3 is deposited in a replication-independent manner and is believed to contribute to chromatin maintenance in non-replicating senescent cells (Bush et al. 2013; Corpet et al. 2014; Duarte et al. 2014; Thakar et al. 2009; Yuen and Knoepfler 2013). ATRX has been shown to deposit H3.3 – in concert with an H3.3 histone chaperone, DAXX – at telomeric and pericentromeric regions of constitutive chromatin (Lewis et al. 2010). However, there is evidence from embryonic stem cells to suggest that DAXX and ATRX can have both shared and unique functions (He et al. 2015). It will be insightful to understand whether or not the senescence promoting activity of ATRX is related to DAXX and/or histone H3.3.

It will also be interesting to investigate whether these foci change once senescence has already been established; we know cytologically that the foci exist for weeks after a senescence inducer has been added, but we are unaware of whether their binding sites change and whether this impinges on gene expression. Furthermore, we know that ATRX (and SAHF) become dispensable for senescence maintenance once a true permanence of growth arrest has been induced. Is it possible to define the changes – whether they relate to chromatin changes, gene expression, SASP, etc. – that dictate the point of irreversibility of growth arrest? Is there a senescence restriction point, akin to the G1 phase restriction point?

To address questions like these, it is imperative to have a synchronous system that will allow us to study cells as they progress temporally and biologically into senescence. With our understanding of MDM2 regulation and how it mediates geroconversion in response to CDK4i, we have the capacity to generate such systems, hinging on a tightly controlled induction (and/or degradation) of MDM2. Using a combination of SA- β -gal, SAHF, ATRX foci and the irreversibility of growth arrest, we can begin to map the temporal regulation of senescence. Such systems can be coupled with ChIP-seq of ATRX and RNA-seq to investigate how gene expression changes over pseudotime (Trapnell et al. 2014), whether this is governed by ATRX (or perhaps other chromatin remodeling factors) and how it relates to irreversibility. Certainly, any pivotal genes and/or time frames identified from such a system will have to be validated in other models of senescence. It is highly unlikely that there will be a single unifying “senescence pathway”; just as there are many, many pathways towards differentiation, there may be an equal number of pathways to senescence. However, much like the Yamanaka factors described a core machinery required for pluripotency, we might hope

to describe a core machinery required for senescence. We believe such geroconversion systems are an appropriate place to begin to look for such machinery, should it exist.

Undoubtedly, a great deal of work remains to be done before we understand the transition from quiescence to senescence at the level required for a textbook; nevertheless, interactions between basic science, sequencing analyses and computational biology, drug development and screening, and clinical trials and materials argue that the questions will be solvable and their answers will have significant impact on the physiologic relevance of the transition from quiescence into senescence.

REFERENCES

- Acosta JC, Gil J (2012) Senescence: a new weapon for cancer therapy. *Trends in cell biology* 22 (4):211-219. doi:10.1016/j.tcb.2011.11.006
- Adams PD (2007) Remodeling chromatin for senescence. *Aging Cell* 6 (4):425-427. doi:10.1111/j.1474-9726.2007.00313.x
- Aguirre-Ghiso JA (2007) Models, mechanisms and clinical evidence for cancer dormancy. *Nature reviews Cancer* 7 (11):834-846. doi:10.1038/nrc2256
- Ahmad I, Patel R, Singh LB, Nixon C, Seywright M, Barnetson RJ, Brunton VG, Muller WJ, Edwards J, Sansom OJ, Leung HY (2011) HER2 overcomes PTEN (loss)-induced senescence to cause aggressive prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America* 108 (39):16392-16397. doi:10.1073/pnas.1101263108
- Aird KM, Zhang R (2013) Detection of senescence-associated heterochromatin foci (SAHF). *Methods in molecular biology* 965:185-196. doi:10.1007/978-1-62703-239-1_12
- Arango Duque G, Descoteaux A (2014) Macrophage cytokines: involvement in immunity and infectious diseases. *Frontiers in immunology* 5:491. doi:10.3389/fimmu.2014.00491
- Asghar U, Witkiewicz AK, Turner NC, Knudsen ES (2015) The history and future of targeting cyclin-dependent kinases in cancer therapy. *Nature reviews Drug discovery* 14 (2):130-146. doi:10.1038/nrd4504
- Axanova LS, Chen YQ, McCoy T, Sui G, Cramer SD (2010) 1,25-dihydroxyvitamin D(3) and PI3K/AKT inhibitors synergistically inhibit growth and induce senescence in prostate cancer cells. *The Prostate* 70 (15):1658-1671. doi:10.1002/pros.21201
- Bachmann MF, Kopf M, Marsland BJ (2006) Chemokines: more than just road signs. *Nature reviews Immunology* 6 (2):159-164. doi:10.1038/nri1776
- Baker DJ, Childs BG, Durik M, Wijers ME, Sieben CJ, Zhong J, Saltness RA, Jeganathan KB, Verzosa GC, Pezeshki A, Khazaie K, Miller JD, van Deursen JM (2016) Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature* 530 (7589):184-189. doi:10.1038/nature16932
- Baker DJ, Sedivy JM (2013) Probing the depths of cellular senescence. *The Journal of cell biology* 202 (1):11-13. doi:10.1083/jcb.201305155
- Baker DJ, Wijshake T, Tchkonja T, LeBrasseur NK, Childs BG, van de Sluis B, Kirkland JL, van Deursen JM (2011) Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 479 (7372):232-236. doi:10.1038/nature10600
- Baker SJ, Reddy EP (2012) CDK4: A Key Player in the Cell Cycle, Development, and Cancer. *Genes & cancer* 3 (11-12):658-669. doi:10.1177/1947601913478972
- Balaton BP, Brown CJ (2016) Escape Artists of the X Chromosome. *Trends in genetics : TIG* 32 (6):348-359. doi:10.1016/j.tig.2016.03.007
- Basehore MJ, Michaelson-Cohen R, Levy-Lahad E, Sismani C, Bird LM, Friez MJ, Walsh T, Abidi F, Holloway L, Skinner C, McGee S, Alexandrou A, Syrrou M, Patsalis PC, Raymond G, Wang T, Schwartz CE, King MC, Stevenson RE (2015) Alpha-thalassemia intellectual disability: variable phenotypic expression among males with a recurrent nonsense mutation - c.109C>T (p.R37X). *Clinical genetics* 87 (5):461-466. doi:10.1111/cge.12420
- Baughn LB, Di Liberto M, Wu K, Toogood PL, Louie T, Gottschalk R, Niesvizky R, Cho H, Ely S, Moore MA, Chen-Kiang S (2006) A novel orally active small molecule potently induces G1 arrest in primary myeloma cells and prevents tumor growth

- by specific inhibition of cyclin-dependent kinase 4/6. *Cancer research* 66 (15):7661-7667. doi:10.1158/0008-5472.CAN-06-1098
- Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P, Campisi J (2003) Reversal of human cellular senescence: roles of the p53 and p16 pathways. *The EMBO journal* 22 (16):4212-4222. doi:10.1093/emboj/cdg417
- Bellovin DI, Das B, Felsner DW (2013) Tumor dormancy, oncogene addiction, cellular senescence, and self-renewal programs. *Advances in experimental medicine and biology* 734:91-107. doi:10.1007/978-1-4614-1445-2_6
- Bent EH, Gilbert LA, Hemann MT (2016) A senescence secretory switch mediated by PI3K/AKT/mTOR activation controls chemoprotective endothelial secretory responses. *Genes & development* 30 (16):1811-1821. doi:10.1101/gad.284851.116
- Bentzinger CF, Wang YX, Rudnicki MA (2012) Building muscle: molecular regulation of myogenesis. *Cold Spring Harbor perspectives in biology* 4 (2). doi:10.1101/cshperspect.a008342
- Bernardes de Jesus B, Vera E, Schneeberger K, Tejera AM, Ayuso E, Bosch F, Blasco MA (2012) Telomerase gene therapy in adult and old mice delays aging and increases longevity without increasing cancer. *EMBO molecular medicine* 4 (8):691-704. doi:10.1002/emmm.201200245
- Bernardi R, Pandolfi PP (2007) Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. *Nature reviews Molecular cell biology* 8 (12):1006-1016. doi:10.1038/nrm2277
- Blagosklonny MV (2003) Apoptosis, proliferation, differentiation: in search of the order. *Seminars in cancer biology* 13 (2):97-105
- Blagosklonny MV (2011) Cell cycle arrest is not senescence. *Aging* 3 (2):94-101. doi:10.18632/aging.100281
- Blagosklonny MV (2014) Geroconversion: irreversible step to cellular senescence. *Cell Cycle* 13 (23):3628-3635. doi:10.4161/15384101.2014.985507
- Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, Greider CW (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* 91 (1):25-34
- Blomen VA, Boonstra J (2007) Cell fate determination during G1 phase progression. *Cellular and molecular life sciences : CMLS* 64 (23):3084-3104. doi:10.1007/s00018-007-7271-z
- Bosco EE, Wang Y, Xu H, Zilfou JT, Knudsen KE, Aronow BJ, Lowe SW, Knudsen ES (2007) The retinoblastoma tumor suppressor modifies the therapeutic response of breast cancer. *The Journal of clinical investigation* 117 (1):218-228. doi:10.1172/JCI28803
- Bracken AP, Kleine-Kohlbrecher D, Dietrich N, Pasini D, Gargiulo G, Beekman C, Theilgaard-Monch K, Minucci S, Porse BT, Marine JC, Hansen KH, Helin K (2007) The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes & development* 21 (5):525-530. doi:10.1101/gad.415507
- Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B, Stein H, Dorken B, Jenuwein T, Schmitt CA (2005) Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* 436 (7051):660-665. doi:10.1038/nature03841
- Bugs CA, Librelotto GR, Mombach JC (2011) A method to identify important dynamical states in Boolean models of regulatory networks: application to regulation of stomata closure by ABA in *A. thaliana*. *BMC genomics* 12 Suppl 4:S10. doi:10.1186/1471-2164-12-S4-S10

- Bursucker I, Rhodes JM, Goldman R (1982) Beta-galactosidase--an indicator of the maturational stage of mouse and human mononuclear phagocytes. *Journal of cellular physiology* 112 (3):385-390. doi:10.1002/jcp.1041120312
- Bush KM, Yuen BT, Barrilleaux BL, Riggs JW, O'Geen H, Cotterman RF, Knoepfler PS (2013) Endogenous mammalian histone H3.3 exhibits chromatin-related functions during development. *Epigenetics & chromatin* 6 (1):7. doi:10.1186/1756-8935-6-7
- Calabro V, Mansueto G, Parisi T, Vivo M, Calogero RA, La Mantia G (2002) The human MDM2 oncoprotein increases the transcriptional activity and the protein level of the p53 homolog p63. *The Journal of biological chemistry* 277 (4):2674-2681. doi:10.1074/jbc.M107173200
- Calado RT, Dumitriu B (2013) Telomere dynamics in mice and humans. *Seminars in hematology* 50 (2):165-174. doi:10.1053/j.seminhematol.2013.03.030
- Campisi J (1997a) Aging and cancer: the double-edged sword of replicative senescence. *Journal of the American Geriatrics Society* 45 (4):482-488
- Campisi J (1997b) The biology of replicative senescence. *European journal of cancer* 33 (5):703-709. doi:10.1016/S0959-8049(96)00058-5
- Campisi J, d'Adda di Fagagna F (2007) Cellular senescence: when bad things happen to good cells. *Nature reviews Molecular cell biology* 8 (9):729-740. doi:10.1038/nrm2233
- Cesare AJ, Reddel RR (2010) Alternative lengthening of telomeres: models, mechanisms and implications. *Nature reviews Genetics* 11 (5):319-330. doi:10.1038/nrg2763
- Chandra T, Kirschner K, Thuret JY, Pope BD, Ryba T, Newman S, Ahmed K, Samarajiwa SA, Salama R, Carroll T, Stark R, Janky R, Narita M, Xue L, Chicas A, Nunez S, Janknecht R, Hayashi-Takanaka Y, Wilson MD, Marshall A, Odom DT, Babu MM, Bazett-Jones DP, Tavaré S, Edwards PA, Lowe SW, Kimura H, Gilbert DM, Narita M (2012) Independence of repressive histone marks and chromatin compaction during senescent heterochromatic layer formation. *Molecular cell* 47 (2):203-214. doi:10.1016/j.molcel.2012.06.010
- Chandra T, Narita M (2013) High-order chromatin structure and the epigenome in SAHFs. *Nucleus* 4 (1):23-28. doi:10.4161/nucl.23189
- Chang J, Wang Y, Shao L, Laberge RM, Demaria M, Campisi J, Janakiraman K, Sharpless NE, Ding S, Feng W, Luo Y, Wang X, Aykin-Burns N, Krager K, Ponnappan U, Hauer-Jensen M, Meng A, Zhou D (2016) Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. *Nature medicine* 22 (1):78-83. doi:10.1038/nm.4010
- Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, Clark NR, Ma'ayan A (2013) Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC bioinformatics* 14:128. doi:10.1186/1471-2105-14-128
- Chen QM, Tu VC, Catania J, Burton M, Toussaint O, Dilley T (2000) Involvement of Rb family proteins, focal adhesion proteins and protein synthesis in senescent morphogenesis induced by hydrogen peroxide. *Journal of cell science* 113 (Pt 22):4087-4097
- Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP (2005) Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 436 (7051):725-730. doi:10.1038/nature03918
- Cheung TH, Rando TA (2013) Molecular regulation of stem cell quiescence. *Nature reviews Molecular cell biology* 14 (6):329-340. doi:10.1038/nrm3591

- Chicas A, Wang X, Zhang C, McCurrach M, Zhao Z, Mert O, Dickins RA, Narita M, Zhang M, Lowe SW (2010) Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. *Cancer cell* 17 (4):376-387. doi:10.1016/j.ccr.2010.01.023
- Chien Y, Scuoppo C, Wang X, Fang X, Balgley B, Bolden JE, Premssirup P, Luo W, Chicas A, Lee CS, Kogan SC, Lowe SW (2011) Control of the senescence-associated secretory phenotype by NF-kappaB promotes senescence and enhances chemosensitivity. *Genes & development* 25 (20):2125-2136. doi:10.1101/gad.17276711
- Childs BG, Baker DJ, Kirkland JL, Campisi J, van Deursen JM (2014) Senescence and apoptosis: dueling or complementary cell fates? *EMBO reports* 15 (11):1139-1153. doi:10.15252/embr.201439245
- Childs BG, Durik M, Baker DJ, van Deursen JM (2015) Cellular senescence in aging and age-related disease: from mechanisms to therapy. *Nature medicine* 21 (12):1424-1435. doi:10.1038/nm.4000
- Choi YJ, Li X, Hydbring P, Sanda T, Stefano J, Christie AL, Signoretti S, Look AT, Kung AL, von Boehmer H, Sicinski P (2012) The requirement for cyclin D function in tumor maintenance. *Cancer cell* 22 (4):438-451. doi:10.1016/j.ccr.2012.09.015
- Ciechanover A (1994) The ubiquitin-proteasome proteolytic pathway. *Cell* 79 (1):13-21
- Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, Benguria A, Zaballos A, Flores JM, Barbacid M, Beach D, Serrano M (2005) Tumour biology: senescence in premalignant tumours. *Nature* 436 (7051):642. doi:10.1038/436642a
- Collins JJ, Evason K, Kornfeld K (2006) Pharmacology of delayed aging and extended lifespan of *Caenorhabditis elegans*. *Experimental gerontology* 41 (10):1032-1039. doi:10.1016/j.exger.2006.06.038
- Comet I, Riising EM, Leblanc B, Helin K (2016) Maintaining cell identity: PRC2-mediated regulation of transcription and cancer. *Nature reviews Cancer*. doi:10.1038/nrc.2016.83
- Coppe JP, Desprez PY, Krtolica A, Campisi J (2010) The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annual review of pathology* 5:99-118. doi:10.1146/annurev-pathol-121808-102144
- Corpet A, Olbrich T, Gwerder M, Fink D, 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 (2):249-267. doi:10.4161/cc.26988
- Courtois-Cox S, Genter Williams SM, Reczek EE, Johnson BW, McGillicuddy LT, Johannessen CM, Hollstein PE, MacCollin M, Cichowski K (2006) A negative feedback signaling network underlies oncogene-induced senescence. *Cancer cell* 10 (6):459-472. doi:10.1016/j.ccr.2006.10.003
- Courtois-Cox S, Jones SL, Cichowski K (2008) Many roads lead to oncogene-induced senescence. *Oncogene* 27 (20):2801-2809. doi:10.1038/sj.onc.1210950
- Crago AM, Singer S (2011) Clinical and molecular approaches to well differentiated and dedifferentiated liposarcoma. *Current opinion in oncology* 23 (4):373-378. doi:10.1097/CCO.0b013e32834796e6
- d'Adda di Fagagna F (2008) Living on a break: cellular senescence as a DNA-damage response. *Nature reviews Cancer* 8 (7):512-522. doi:10.1038/nrc2440
- Dean JL, Thangavel C, McClendon AK, Reed CA, Knudsen ES (2010) Therapeutic CDK4/6 inhibition in breast cancer: key mechanisms of response and failure. *Oncogene* 29 (28):4018-4032. doi:10.1038/onc.2010.154

- Demaria M, Ohtani N, Youssef SA, Rodier F, Toussaint W, Mitchell JR, Laberge RM, Vijg J, Van Steeg H, Dolle ME, Hoeijmakers JH, de Bruin A, Hara E, Campisi J (2014) An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Developmental cell* 31 (6):722-733. doi:10.1016/j.devcel.2014.11.012
- Deshaies RJ, Joazeiro CA (2009) RING domain E3 ubiquitin ligases. *Annual review of biochemistry* 78:399-434. doi:10.1146/annurev.biochem.78.101807.093809
- Dhomen N, Reis-Filho JS, da Rocha Dias S, Hayward R, Savage K, Delmas V, Larue L, Pritchard C, Marais R (2009) Oncogenic Braf induces melanocyte senescence and melanoma in mice. *Cancer Cell* 15 (4):294-303. doi:10.1016/j.ccr.2009.02.022
- Di Micco R, Sulli G, Dobrev M, Lontos M, Botrugno OA, Gargiulo G, dal Zuffo R, Matti V, d'Ario G, Montani E, Mercurio C, Hahn WC, Gorgoulis V, Minucci S, d'Adda di Fagagna F (2011) Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. *Nature cell biology* 13 (3):292-302. doi:10.1038/ncb2170
- Dickson MA, Mahoney MR, Tap WD, D'Angelo SP, Keohan ML, Van Tine BA, Agulnik M, Horvath LE, Nair JS, Schwartz GK (2016) Phase II study of MLN8237 (Aisertib) in advanced/metastatic sarcoma. *Annals of oncology : official journal of the European Society for Medical Oncology* 27 (10):1855-1860. doi:10.1093/annonc/mdw281
- Dickson MA, Tap WD, Keohan ML, D'Angelo SP, Gounder MM, Antonescu CR, Landa J, Qin LX, Rathbone DD, Condy MM, Ustoyev Y, Crago AM, Singer S, Schwartz GK (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 (16):2024-2028. doi:10.1200/JCO.2012.46.5476
- Diez-Juan A, Andres V (2001) The growth suppressor p27(Kip1) protects against diet-induced atherosclerosis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 15 (11):1989-1995. doi:10.1096/fj.01-0130com
- Dilley RL, Verma P, Cho NW, Winters HD, Wondisford AR, Greenberg RA (2016) Break-induced telomere synthesis underlies alternative telomere maintenance. *Nature* 539 (7627):54-58. doi:10.1038/nature20099
- Dimauro T, David G (2009) Chromatin modifications: the driving force of senescence and aging? *Aging* 1 (2):182-190. doi:10.18632/aging.100023
- Dimauro T, David G (2010) Ras-induced senescence and its physiological relevance in cancer. *Current cancer drug targets* 10 (8):869-876
- Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 92 (20):9363-9367
- Duarte LF, Young AR, Wang Z, Wu HA, Panda T, Kou Y, Kapoor A, Hasson D, Mills NR, Ma'ayan A, Narita M, Bernstein E (2014) Histone H3.3 and its proteolytically processed form drive a cellular senescence programme. *Nature communications* 5:5210. doi:10.1038/ncomms6210
- End DW, Smets G, Todd AV, Applegate TL, Fuery CJ, Angibaud P, Venet M, Sanz G, Poignet H, Skrzat S, Devine A, Wouters W, Bowden C (2001) Characterization of the antitumor effects of the selective farnesyl protein transferase inhibitor R115777 in vivo and in vitro. *Cancer research* 61 (1):131-137

- Engelhardt M, Ozkaynak MF, Drullinsky P, Sandoval C, Tugal O, Jayabose S, Moore MA (1998) Telomerase activity and telomere length in pediatric patients with malignancies undergoing chemotherapy. *Leukemia* 12 (1):13-24
- Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, Waghorn K, Zoi K, Ross FM, Reiter A, Hochhaus A, Drexler HG, Duncombe A, Cervantes F, Oscier D, Boulwood J, Grand FH, Cross NC (2010) Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nature genetics* 42 (8):722-726. doi:10.1038/ng.621
- Ertel A, Dean JL, Rui H, Liu C, Witkiewicz AK, Knudsen KE, Knudsen ES (2010) RB-pathway disruption in breast cancer: differential association with disease subtypes, disease-specific prognosis and therapeutic response. *Cell cycle* 9 (20):4153-4163. doi:10.4161/cc.9.20.13454
- Eustermann S, Yang JC, Law MJ, Amos R, Chapman LM, Jelinska C, Garrick D, Clynes D, Gibbons RJ, Rhodes D, Higgs DR, Neuhaus D (2011) Combinatorial readout of histone H3 modifications specifies localization of ATRX to heterochromatin. *Nature structural & molecular biology* 18 (7):777-782. doi:10.1038/nsmb.2070
- Ewald JA, Desotelle JA, Wilding G, Jarrard DF (2010) Therapy-induced senescence in cancer. *Journal of the National Cancer Institute* 102 (20):1536-1546. doi:10.1093/jnci/djq364
- Fan T, Jiang S, Chung N, Alikhan A, Ni C, Lee CC, Hornyak TJ (2011) EZH2-dependent suppression of a cellular senescence phenotype in melanoma cells by inhibition of p21/CDKN1A expression. *Molecular cancer research : MCR* 9 (4):418-429. doi:10.1158/1541-7786.MCR-10-0511
- Finn RS, Aleshin A, Slamon DJ (2016a) Targeting the cyclin-dependent kinases (CDK) 4/6 in estrogen receptor-positive breast cancers. *Breast cancer research : BCR* 18 (1):17. doi:10.1186/s13058-015-0661-5
- Finn RS, Crown JP, Ettl J, Schmidt M, Bondarenko IM, Lang I, Pinter T, Boer K, Patel R, Randolph S, Kim ST, Huang X, Schnell P, Nadanaciva S, Bartlett CH, Slamon DJ (2016b) Efficacy and safety of palbociclib in combination with letrozole as first-line treatment of ER-positive, HER2-negative, advanced breast cancer: expanded analyses of subgroups from the randomized pivotal trial PALOMA-1/TRIO-18. *Breast cancer research : BCR* 18 (1):67. doi:10.1186/s13058-016-0721-5
- Finn RS, Crown JP, Lang I, Boer K, Bondarenko IM, Kulyk SO, Ettl J, Patel R, Pinter T, Schmidt M, Shparyk Y, Thummala AR, Voytko NL, Fowst C, Huang X, Kim ST, Randolph S, Slamon DJ (2015) The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. *The Lancet Oncology* 16 (1):25-35. doi:10.1016/S1470-2045(14)71159-3
- Finn RS, Dering J, Conklin D, Kalous O, Cohen DJ, Desai AJ, Ginther C, Atefi M, Chen I, Fowst C, Los G, Slamon DJ (2009) PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. *Breast cancer research : BCR* 11 (5):R77. doi:10.1186/bcr2419
- Flores JM, Martin-Caballero J, Garcia-Fernandez RA (2014) p21 and p27 a shared senescence history. *Cell cycle* 13 (11):1655-1656. doi:10.4161/cc.29147
- Foo RS, Chan LK, Kitsis RN, Bennett MR (2007) Ubiquitination and degradation of the anti-apoptotic protein ARC by MDM2. *The Journal of biological chemistry* 282 (8):5529-5535. doi:10.1074/jbc.M609046200

- Franceschi C, Capri M, Monti D, Giunta S, Olivieri F, Sevini F, Panourgia MP, Invidia L, Celani L, Scurti M, Cevenini E, Castellani GC, Salvioli S (2007) Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. *Mechanisms of ageing and development* 128 (1):92-105. doi:10.1016/j.mad.2006.11.016
- Franco J, Witkiewicz AK, Knudsen ES (2014) CDK4/6 inhibitors have potent activity in combination with pathway selective therapeutic agents in models of pancreatic cancer. *Oncotarget* 5 (15):6512-6525. doi:10.18632/oncotarget.2270
- Freund A, Orjalo AV, Desprez PY, Campisi J (2010) Inflammatory networks during cellular senescence: causes and consequences. *Trends in molecular medicine* 16 (5):238-246. doi:10.1016/j.molmed.2010.03.003
- Fridman AL, Tainsky MA (2008) Critical pathways in cellular senescence and immortalization revealed by gene expression profiling. *Oncogene* 27 (46):5975-5987. doi:10.1038/onc.2008.213
- Fry DW, Harvey PJ, Keller PR, Elliott WL, Meade M, Trachet E, Albassam M, Zheng X, Leopold WR, Pryer NK, Toogood PL (2004) Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. *Molecular cancer therapeutics* 3 (11):1427-1438
- Fumagalli M, Rossiello F, Clerici M, Barozzi S, Cittaro D, Kaplunov JM, Bucci G, Dobrova M, Matti V, Beausejour CM, Herbig U, Longhese MP, d'Adda di Fagagna F (2012) Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. *Nat Cell Biol* 14 (4):355-365. doi:10.1038/ncb2466
- Funayama R, Saito M, Tanobe H, Ishikawa F (2006) Loss of linker histone H1 in cellular senescence. *The Journal of cell biology* 175 (6):869-880. doi:10.1083/jcb.200604005
- Fuster JJ, Sanz-Gonzalez SM, Moll UM, Andres V (2007) Classic and novel roles of p53: prospects for anticancer therapy. *Trends in molecular medicine* 13 (5):192-199. doi:10.1016/j.molmed.2007.03.002
- Garcia-Prat L, Martinez-Vicente M, Perdiguero E, Ortet L, Rodriguez-Ubreva J, Rebollo E, Ruiz-Bonilla V, Gutarra S, Ballestar E, Serrano AL, Sandri M, Munoz-Canoves P (2016) Autophagy maintains stemness by preventing senescence. *Nature* 529 (7584):37-42. doi:10.1038/nature16187
- Gatti L, Supino R, Perego P, Pavesi R, Caserini C, Carenini N, Righetti SC, Zuco V, Zunino F (2002) Apoptosis and growth arrest induced by platinum compounds in U2-OS cells reflect a specific DNA damage recognition associated with a different p53-mediated response. *Cell death and differentiation* 9 (12):1352-1359. doi:10.1038/sj.cdd.4401109
- Geginat J, Sallusto F, Lanzavecchia A (2001) Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *The Journal of experimental medicine* 194 (12):1711-1719
- Geng YQ, Guan JT, Xu XH, Fu YC (2010) Senescence-associated beta-galactosidase activity expression in aging hippocampal neurons. *Biochemical and biophysical research communications* 396 (4):866-869. doi:10.1016/j.bbrc.2010.05.011
- Gey C, Seeger K (2013) Metabolic changes during cellular senescence investigated by proton NMR-spectroscopy. *Mechanisms of ageing and development* 134 (3-4):130-138. doi:10.1016/j.mad.2013.02.002
- Gharbi SI, Zvelebil MJ, Shuttleworth SJ, Hancox T, Saghir N, Timms JF, Waterfield MD (2007) Exploring the specificity of the PI3K family inhibitor LY294002. *The Biochemical journal* 404 (1):15-21. doi:10.1042/BJ20061489

- Giacinti C, Giordano A (2006) RB and cell cycle progression. *Oncogene* 25 (38):5220-5227. doi:10.1038/sj.onc.1209615
- Goldberg AD, Banaszynski LA, Noh KM, Lewis PW, Elsaesser SJ, Stadler S, Dewell S, Law M, Guo X, Li X, Wen D, Chapgier A, DeKolver RC, Miller JC, Lee YL, Boydston EA, Holmes MC, Gregory PD, Grealley JM, Rafii S, Yang C, Scambler PJ, Garrick D, Gibbons RJ, Higgs DR, Cristea IM, Urnov FD, Zheng D, Allis CD (2010) Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell* 140 (5):678-691. doi:10.1016/j.cell.2010.01.003
- Gonzalez-Navarro H, Abu Nabah YN, Vinue A, Andres-Manzano MJ, Collado M, Serrano M, Andres V (2010) p19(ARF) deficiency reduces macrophage and vascular smooth muscle cell apoptosis and aggravates atherosclerosis. *Journal of the American College of Cardiology* 55 (20):2258-2268. doi:10.1016/j.jacc.2010.01.026
- Haferkamp S, Borst A, Adam C, Becker TM, Motschenbacher S, Windhovel S, Hufnagel AL, Houben R, Meierjohann S (2013) Vemurafenib induces senescence features in melanoma cells. *The Journal of investigative dermatology* 133 (6):1601-1609. doi:10.1038/jid.2013.6
- Hamilton B, Dong Y, Shindo M, Liu W, Odell I, Ruvkun G, Lee SS (2005) A systematic RNAi screen for longevity genes in *C. elegans*. *Genes & development* 19 (13):1544-1555. doi:10.1101/gad.1308205
- Hamilton E, Infante JR (2016) Targeting CDK4/6 in patients with cancer. *Cancer treatment reviews* 45:129-138. doi:10.1016/j.ctrv.2016.03.002
- Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100 (1):57-70
- Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144 (5):646-674. doi:10.1016/j.cell.2011.02.013
- Hashizume R, Zhang A, Mueller S, Prados MD, Lulla RR, Goldman S, Saratsis AM, Mazar AP, Stegh AH, Cheng SY, Horbinski C, Haas-Kogan DA, Sarkaria JN, Waldman T, James CD (2016) Inhibition of DNA damage repair by the CDK4/6 inhibitor palbociclib delays irradiated intracranial atypical teratoid rhabdoid tumor and glioblastoma xenograft regrowth. *Neuro-oncology* 18 (11):1519-1528. doi:10.1093/neuonc/nov106
- Hayflick L (1965) The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res* 37:614-636
- He Q, Kim H, Huang R, Lu W, Tang M, Shi F, Yang D, Zhang X, Huang J, Liu D, Songyang Z (2015) The Daxx/Atrx Complex Protects Tandem Repetitive Elements during DNA Hypomethylation by Promoting H3K9 Trimethylation. *Cell stem cell* 17 (3):273-286. doi:10.1016/j.stem.2015.07.022
- Heaphy CM, de Wilde RF, Jiao Y, Klein AP, Edil BH, Shi C, Bettegowda C, Rodriguez FJ, Eberhart CG, Hebbar S, Offerhaus GJ, McLendon R, Rasheed BA, He Y, Yan H, Bigner DD, Oba-Shinjo SM, Marie SK, Riggins GJ, Kinzler KW, Vogelstein B, Hruban RH, Maitra A, Papadopoulos N, Meeker AK (2011) Altered telomeres in tumors with ATRX and DAXX mutations. *Science* 333 (6041):425. doi:10.1126/science.1207313
- Hennekam RC (2006) Hutchinson-Gilford progeria syndrome: review of the phenotype. *Am J Med Genet A* 140 (23):2603-2624. doi:10.1002/ajmg.a.31346
- Herbig U, Jobling WA, Chen BP, Chen DJ, Sedivy JM (2004) Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Molecular cell* 14 (4):501-513
- Hernandez-Vargas H, Palacios J, Moreno-Bueno G (2007a) Molecular profiling of docetaxel cytotoxicity in breast cancer cells: uncoupling of aberrant mitosis and apoptosis. *Oncogene* 26 (20):2902-2913. doi:10.1038/sj.onc.1210102

- Hernandez-Vargas H, Palacios J, Moreno-Bueno G (2007b) Telling cells how to die: docetaxel therapy in cancer cell lines. *Cell cycle* 6 (7):780-783. doi:10.4161/cc.6.7.4050
- Hewitt G, Jurk D, Marques FD, Correia-Melo C, Hardy T, Gackowska A, Anderson R, Taschuk M, Mann J, Passos JF (2012) Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. *Nat Commun* 3:708. doi:10.1038/ncomms1708
- Hobert O (2011) Regulation of terminal differentiation programs in the nervous system. *Annual review of cell and developmental biology* 27:681-696. doi:10.1146/annurev-cellbio-092910-154226
- Hochegger H, Takeda S, Hunt T (2008) Cyclin-dependent kinases and cell-cycle transitions: does one fit all? *Nature reviews Molecular cell biology* 9 (11):910-916. doi:10.1038/nrm2510
- Hock H (2012) A complex Polycomb issue: the two faces of EZH2 in cancer. *Genes & development* 26 (8):751-755. doi:10.1101/gad.191163.112
- Huang X, Di Liberto M, Jayabalan D, Liang J, Ely S, Bretz J, Shaffer AL, 3rd, Louie T, Chen I, Randolph S, Hahn WC, Staudt LM, Niesvizky R, Moore MA, Chen-Kiang S (2012) Prolonged early G(1) arrest by selective CDK4/CDK6 inhibition sensitizes myeloma cells to cytotoxic killing through cell cycle-coupled loss of IRF4. *Blood* 120 (5):1095-1106. doi:10.1182/blood-2012-03-415984
- Inuzuka H, Tseng A, Gao D, Zhai B, Zhang Q, Shaik S, Wan L, Ang XL, Mock C, Yin H, Stommel JM, Gygi S, Lahav G, Asara J, Xiao ZX, Kaelin WG, Jr., Harper JW, Wei W (2010) Phosphorylation by casein kinase I promotes the turnover of the Mdm2 oncoprotein via the SCF(beta-TRCP) ubiquitin ligase. *Cancer cell* 18 (2):147-159. doi:10.1016/j.ccr.2010.06.015
- Itahana K, Campisi J, Dimri GP (2007) Methods to detect biomarkers of cellular senescence: the senescence-associated beta-galactosidase assay. *Methods in molecular biology* 371:21-31
- Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JC, Trent JM, Staudt LM, Hudson J, Jr., Boguski MS, Lashkari D, Shalon D, Botstein D, Brown PO (1999) The transcriptional program in the response of human fibroblasts to serum. *Science* 283 (5398):83-87
- Jaskelioff M, Muller FL, Paik JH, Thomas E, Jiang S, Adams AC, Sahin E, Kost-Alimova M, Protopopov A, Cadinanos J, Horner JW, Maratos-Flier E, Depinho RA (2011) Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. *Nature* 469 (7328):102-106. doi:10.1038/nature09603
- Johnson SM, Torrice CD, Bell JF, Monahan KB, Jiang Q, Wang Y, Ramsey MR, Jin J, Wong KK, Su L, Zhou D, Sharpless NE (2010) Mitigation of hematologic radiation toxicity in mice through pharmacological quiescence induced by CDK4/6 inhibition. *The Journal of clinical investigation* 120 (7):2528-2536. doi:10.1172/JCI41402
- Joyner DE, Bastar JD, Randall RL (2006) Doxorubicin induces cell senescence preferentially over apoptosis in the FU-SY-1 synovial sarcoma cell line. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 24 (6):1163-1169. doi:10.1002/jor.20169
- Jun JI, Lau LF (2010) Cellular senescence controls fibrosis in wound healing. *Aging* 2 (9):627-631. doi:10.18632/aging.100201
- Karanikolas BD, Figueiredo ML, Wu L (2009) Polycomb group protein enhancer of zeste 2 is an oncogene that promotes the neoplastic transformation of a benign prostatic epithelial cell line. *Molecular cancer research : MCR* 7 (9):1456-1465. doi:10.1158/1541-7786.MCR-09-0121

- Kaur J, Debnath J (2015) Autophagy at the crossroads of catabolism and anabolism. *Nature reviews Molecular cell biology* 16 (8):461-472. doi:10.1038/nrm4024
- Kennedy AL, McBryan T, Enders GH, Johnson FB, Zhang R, Adams PD (2010) Senescent mouse cells fail to overtly regulate the HIRA histone chaperone and do not form robust Senescence Associated Heterochromatin Foci. *Cell division* 5:16. doi:10.1186/1747-1028-5-16
- Khanna AK (2009) Enhanced susceptibility of cyclin kinase inhibitor p21 knockout mice to high fat diet induced atherosclerosis. *Journal of biomedical science* 16:66. doi:10.1186/1423-0127-16-66
- Kim KH, Chen CC, Monzon RI, Lau LF (2013) Matricellular protein CCN1 promotes regression of liver fibrosis through induction of cellular senescence in hepatic myofibroblasts. *Molecular and cellular biology* 33 (10):2078-2090. doi:10.1128/MCB.00049-13
- Kim KH, Roberts CW (2016) Targeting EZH2 in cancer. *Nature medicine* 22 (2):128-134. doi:10.1038/nm.4036
- Kitagaki J, Agama KK, Pommier Y, Yang Y, Weissman AM (2008) Targeting tumor cells expressing p53 with a water-soluble inhibitor of Hdm2. *Molecular cancer therapeutics* 7 (8):2445-2454. doi:10.1158/1535-7163.MCT-08-0063
- Konecny GE, Winterhoff B, Kolarova T, Qi J, Manivong K, Dering J, Yang G, Chalukya M, Wang HJ, Anderson L, Kalli KR, Finn RS, Ginther C, Jones S, Velculescu VE, Riehle D, Cliby WA, Randolph S, Koehler M, Hartmann LC, Slamon DJ (2011) Expression of p16 and retinoblastoma determines response to CDK4/6 inhibition in ovarian cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 17 (6):1591-1602. doi:10.1158/1078-0432.CCR-10-2307
- Kong X, Feng D, Wang H, Hong F, Bertola A, Wang FS, Gao B (2012) Interleukin-22 induces hepatic stellate cell senescence and restricts liver fibrosis in mice. *Hepatology* 56 (3):1150-1159. doi:10.1002/hep.25744
- Kosar M, Bartkova J, Hubackova S, Hodny Z, Lukas J, Bartek J (2011) Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16(ink4a). *Cell cycle* 10 (3):457-468. doi:10.4161/cc.10.3.14707
- Kovatcheva M, Liu DD, Dickson MA, Klein ME, O'Connor R, Wilder FO, Socci ND, Tap WD, Schwartz GK, Singer S, Crago AM, Koff A (2015) MDM2 turnover and expression of ATRX determine the choice between quiescence and senescence in response to CDK4 inhibition. *Oncotarget* 6 (10):8226-8243. doi:10.18632/oncotarget.3364
- Kowalik TF, DeGregori J, Schwarz JK, Nevins JR (1995) E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *Journal of virology* 69 (4):2491-2500
- Krizhanovsky V, Xue W, Zender L, Yon M, Hernando E, Lowe SW (2008a) Implications of cellular senescence in tissue damage response, tumor suppression, and stem cell biology. *Cold Spring Harbor symposia on quantitative biology* 73:513-522. doi:10.1101/sqb.2008.73.048
- Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, Yee H, Zender L, Lowe SW (2008b) Senescence of activated stellate cells limits liver fibrosis. *Cell* 134 (4):657-667. doi:10.1016/j.cell.2008.06.049
- Kuilman T, Michaloglou C, Mooi WJ, Peeper DS (2010) The essence of senescence. *Genes & development* 24 (22):2463-2479. doi:10.1101/gad.1971610

- Kummar S, Gutierrez M, Doroshow JH, Murgo AJ (2006) Drug development in oncology: classical cytotoxics and molecularly targeted agents. *British journal of clinical pharmacology* 62 (1):15-26. doi:10.1111/j.1365-2125.2006.02713.x
- Kurz DJ, Decary S, Hong Y, Erusalimsky JD (2000) Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *Journal of cell science* 113 (Pt 20):3613-3622
- Laberge RM, Sun Y, Orjalo AV, Patil CK, Freund A, Zhou L, Curran SC, Davalos AR, Wilson-Edell KA, Liu S, Limbad C, Demaria M, Li P, Hubbard GB, Ikeno Y, Javors M, Desprez PY, Benz CC, Kapahi P, Nelson PS, Campisi J (2015) MTOR regulates the pro-tumorigenic senescence-associated secretory phenotype by promoting IL1A translation. *Nature cell biology* 17 (8):1049-1061. doi:10.1038/ncb3195
- Lackner DH, Hayashi MT, Cesare AJ, Karlseder J (2014) A genomics approach identifies senescence-specific gene expression regulation. *Aging cell* 13 (5):946-950. doi:10.1111/ace1.12234
- Lahtela J, Corson LB, Hemmes A, Brauer MJ, Koopal S, Lee J, Hunsaker TL, Jackson PK, Verschuren EW (2013) A high-content cellular senescence screen identifies candidate tumor suppressors, including EPHA3. *Cell cycle* 12 (4):625-634. doi:10.4161/cc.23515
- Lane DP, Brown CJ, Verma C, Cheek CF (2011) New insights into p53 based therapy. *Discovery medicine* 12 (63):107-117
- Law MJ, Lower KM, Voon HP, Hughes JR, Garrick D, Viprakasit V, Mitson M, De Gobbi M, Marra M, Morris A, Abbott A, Wilder SP, Taylor S, Santos GM, Cross J, Ayyub H, Jones S, Ragoussis J, Rhodes D, Dunham I, Higgs DR, Gibbons RJ (2010) ATR-X syndrome protein targets tandem repeats and influences allele-specific expression in a size-dependent manner. *Cell* 143 (3):367-378. doi:10.1016/j.cell.2010.09.023
- Lee BK, Bhinge AA, Iyer VR (2011) Wide-ranging functions of E2F4 in transcriptional activation and repression revealed by genome-wide analysis. *Nucleic acids research* 39 (9):3558-3573. doi:10.1093/nar/gkq1313
- Lee JC, Jeng YM, Liao JY, Tsai JH, Hsu HH, Yang CY (2015) Alternative lengthening of telomeres and loss of ATRX are frequent events in pleomorphic and dedifferentiated liposarcomas. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 28 (8):1064-1073. doi:10.1038/modpathol.2015.67
- Lee YK, Park NH, Lee H (2012) Prognostic value of alternative lengthening of telomeres-associated biomarkers in uterine sarcoma and uterine carcinosarcoma. *International journal of gynecological cancer : official journal of the International Gynecological Cancer Society* 22 (3):434-441. doi:10.1097/IGC.0b013e31823ca017
- Lehn S, Ferno M, Jirstrom K, Ryden L, Landberg G (2011) A non-functional retinoblastoma tumor suppressor (RB) pathway in premenopausal breast cancer is associated with resistance to tamoxifen. *Cell cycle* 10 (6):956-962. doi:10.4161/cc.10.6.15074
- Leng X, Connell-Crowley L, Goodrich D, Harper JW (1997) S-Phase entry upon ectopic expression of G1 cyclin-dependent kinases in the absence of retinoblastoma protein phosphorylation. *Current biology : CB* 7 (9):709-712
- Leontieva OV, Demidenko ZN, Gudkov AV, Blagosklonny MV (2011) Elimination of proliferating cells unmasks the shift from senescence to quiescence caused by rapamycin. *PloS one* 6 (10):e26126. doi:10.1371/journal.pone.0026126

- Leverson JD, Phillips DC, Mitten MJ, Boghaert ER, Diaz D, Tahir SK, Belmont LD, Nimmer P, Xiao Y, Ma XM, Lowes KN, Kovar P, Chen J, Jin S, Smith M, Xue J, Zhang H, Oleksijew A, Magoc TJ, Vaidya KS, Albert DH, Tarrant JM, La N, Wang L, Tao ZF, Wendt MD, Sampath D, Rosenberg SH, Tse C, Huang DC, Fairbrother WJ, Elmore SW, Souers AJ (2015) Exploiting selective BCL-2 family inhibitors to dissect cell survival dependencies and define improved strategies for cancer therapy. *Science translational medicine* 7 (279):279ra240. doi:10.1126/scitranslmed.aaa4642
- Lewis PW, Elsaesser SJ, Noh KM, Stadler SC, Allis CD (2010) Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. *Proceedings of the National Academy of Sciences of the United States of America* 107 (32):14075-14080. doi:10.1073/pnas.1008850107
- Li D, Marchenko ND, Moll UM (2011) SAHA shows preferential cytotoxicity in mutant p53 cancer cells by destabilizing mutant p53 through inhibition of the HDAC6-Hsp90 chaperone axis. *Cell death and differentiation* 18 (12):1904-1913. doi:10.1038/cdd.2011.71
- Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25 (14):1754-1760. doi:10.1093/bioinformatics/btp324
- Lin AW, Barradas M, Stone JC, van Aelst L, Serrano M, Lowe SW (1998) Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes & development* 12 (19):3008-3019
- Lin HK, Chen Z, Wang G, Nardella C, Lee SW, Chan CH, Yang WL, Wang J, Egia A, Nakayama KI, Cordon-Cardo C, Teruya-Feldstein J, Pandolfi PP (2010) Skp2 targeting suppresses tumorigenesis by Arf-p53-independent cellular senescence. *Nature* 464 (7287):374-379. doi:10.1038/nature08815
- Linares LK, Kiernan R, Triboulet R, Chable-Bessia C, Latreille D, Cuvier O, Lacroix M, Le Cam L, Coux O, Benkirane M (2007) Intrinsic ubiquitination activity of PCAF controls the stability of the oncoprotein Hdm2. *Nature cell biology* 9 (3):331-338. doi:10.1038/ncb1545
- Linke K, Mace PD, Smith CA, Vaux DL, Silke J, Day CL (2008) Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. *Cell death and differentiation* 15 (5):841-848. doi:10.1038/sj.cdd.4402309
- Liu Y, Sanoff HK, Cho H, Burd CE, Torrice C, Ibrahim JG, Thomas NE, Sharpless NE (2009) Expression of p16(INK4a) in peripheral blood T-cells is a biomarker of human aging. *Aging cell* 8 (4):439-448. doi:10.1111/j.1474-9726.2009.00489.x
- Longley DB, Harkin DP, Johnston PG (2003) 5-fluorouracil: mechanisms of action and clinical strategies. *Nature reviews Cancer* 3 (5):330-338. doi:10.1038/nrc1074
- Lord JM, Butcher S, Killampali V, Lascelles D, Salmon M (2001) Neutrophil ageing and immunesenescence. *Mechanisms of ageing and development* 122 (14):1521-1535
- Lovejoy CA, Li W, Reisenweber S, Thongthip S, Bruno J, de Lange T, De S, Petrini JH, Sung PA, Jasin M, Rosenbluh J, Zwang Y, Weir BA, Hatton C, Ivanova E, Macconail L, Hanna M, Hahn WC, Lue NF, Reddel RR, Jiao Y, Kinzler K, Vogelstein B, Papadopoulos N, Meeker AK, Consortium ALTSC (2012) Loss of ATRX, genome instability, and an altered DNA damage response are hallmarks of the alternative lengthening of telomeres pathway. *PLoS genetics* 8 (7):e1002772. doi:10.1371/journal.pgen.1002772

- Lujambio A (2016) To clear, or not to clear (senescent cells)? That is the question. *BioEssays : news and reviews in molecular, cellular and developmental biology* 38 Suppl 1:S56-64. doi:10.1002/bies.201670910
- Lujambio A, Akkari L, Simon J, Grace D, Tschaharganeh DF, Bolden JE, Zhao Z, Thapar V, Joyce JA, Krizhanovsky V, Lowe SW (2013) Non-cell-autonomous tumor suppression by p53. *Cell* 153 (2):449-460. doi:10.1016/j.cell.2013.03.020
- Lukas J, Herzinger T, Hansen K, Moroni MC, Resnitzky D, Helin K, Reed SI, Bartek J (1997) Cyclin E-induced S phase without activation of the pRb/E2F pathway. *Genes & development* 11 (11):1479-1492
- Lundberg AS, Hahn WC, Gupta P, Weinberg RA (2000) Genes involved in senescence and immortalization. *Current opinion in cell biology* 12 (6):705-709
- Malumbres M (2014) Cyclin-dependent kinases. *Genome biology* 15 (6):122
- Malumbres M, Barbacid M (2006) Is Cyclin D1-CDK4 kinase a bona fide cancer target? *Cancer cell* 9 (1):2-4. doi:10.1016/j.ccr.2005.12.026
- Malumbres M, Pevarello P, Barbacid M, Bischoff JR (2008) CDK inhibitors in cancer therapy: what is next? *Trends in pharmacological sciences* 29 (1):16-21. doi:10.1016/j.tips.2007.10.012
- Manfredi JJ (2010) The Mdm2-p53 relationship evolves: Mdm2 swings both ways as an oncogene and a tumor suppressor. *Genes & development* 24 (15):1580-1589. doi:10.1101/gad.1941710
- Massague J (2004) G1 cell-cycle control and cancer. *Nature* 432 (7015):298-306. doi:10.1038/nature03094
- Mattison JA, Roth GS, Beasley TM, Tilmont EM, Handy AM, Herbert RL, Longo DL, Allison DB, Young JE, Bryant M, Barnard D, Ward WF, Qi W, Ingram DK, de Cabo R (2012) Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. *Nature* 489 (7415):318-321. doi:10.1038/nature11432
- McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytzky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA (2010) The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome research* 20 (9):1297-1303. doi:10.1101/gr.107524.110
- Medema RH, Herrera RE, Lam F, Weinberg RA (1995) Growth suppression by p16ink4 requires functional retinoblastoma protein. *Proceedings of the National Academy of Sciences of the United States of America* 92 (14):6289-6293
- Meis-Kindblom JM, Sjogren H, Kindblom LG, Peydro-Mellquist A, Roijer E, Aman P, Stenman G (2001) Cytogenetic and molecular genetic analyses of liposarcoma and its soft tissue simulators: recognition of new variants and differential diagnosis. *Virchows Archiv : an international journal of pathology* 439 (2):141-151
- Menu E, Garcia J, Huang X, Di Liberto M, Toogood PL, Chen I, Vanderkerken K, Chen-Kiang S (2008) A novel therapeutic combination using PD 0332991 and bortezomib: study in the 5T33MM myeloma model. *Cancer research* 68 (14):5519-5523. doi:10.1158/0008-5472.CAN-07-6404
- Mercer J, Bennett M (2006) The role of p53 in atherosclerosis. *Cell cycle* 5 (17):1907-1909. doi:10.4161/cc.5.17.3166
- Mercer J, Figg N, Stoneman V, Braganza D, Bennett MR (2005) Endogenous p53 protects vascular smooth muscle cells from apoptosis and reduces atherosclerosis in ApoE knockout mice. *Circulation research* 96 (6):667-674. doi:10.1161/01.RES.0000161069.15577.ca
- Michaloglou C, Vredevelde LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS (2005) BRAFE600-associated

- senescence-like cell cycle arrest of human naevi. *Nature* 436 (7051):720-724. doi:10.1038/nature03890
- Michaud K, Solomon DA, Oermann E, Kim JS, Zhong WZ, Prados MD, Ozawa T, James CD, Waldman T (2010) Pharmacologic inhibition of cyclin-dependent kinases 4 and 6 arrests the growth of glioblastoma multiforme intracranial xenografts. *Cancer research* 70 (8):3228-3238. doi:10.1158/0008-5472.CAN-09-4559
- Millar AW, Lynch KP (2003) Rethinking clinical trials for cytostatic drugs. *Nature reviews Cancer* 3 (7):540-545. doi:10.1038/nrc1124
- Mills AA (2010) Throwing the cancer switch: reciprocal roles of polycomb and trithorax proteins. *Nature reviews Cancer* 10 (10):669-682. doi:10.1038/nrc2931
- Mitson M, Kelley LA, Sternberg MJ, Higgs DR, Gibbons RJ (2011) Functional significance of mutations in the Snf2 domain of ATRX. *Human molecular genetics* 20 (13):2603-2610. doi:10.1093/hmg/ddr163
- Mocchegiani E, Malavolta M (2004) NK and NKT cell functions in immunosenescence. *Aging cell* 3 (4):177-184. doi:10.1111/j.1474-9728.2004.00107.x
- Moll UM, Petrenko O (2003) The MDM2-p53 interaction. *Molecular cancer research* : MCR 1 (14):1001-1008
- 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, Serrano M (2013) Programmed cell senescence during mammalian embryonic development. *Cell* 155 (5):1104-1118. doi:10.1016/j.cell.2013.10.019
- Munoz-Espin D, Serrano M (2014) Cellular senescence: from physiology to pathology. *Nature reviews Molecular cell biology* 15 (7):482-496. doi:10.1038/nrm3823
- Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL (2011) Cyclin D as a therapeutic target in cancer. *Nature reviews Cancer* 11 (8):558-572. doi:10.1038/nrc3090
- Napier CE, Huschtscha LI, Harvey A, Bower K, Noble JR, Hendrickson EA, Reddel RR (2015) ATRX represses alternative lengthening of telomeres. *Oncotarget* 6 (18):16543-16558. doi:10.18632/oncotarget.3846
- Nardella C, Clohessy JG, Alimonti A, Pandolfi PP (2011) Pro-senescence therapy for cancer treatment. *Nature reviews Cancer* 11 (7):503-511. doi:10.1038/nrc3057
- Narita M, Narita M, Krizhanovskiy V, Nunez S, Chicas A, Hearn SA, Myers MP, Lowe SW (2006) A novel role for high-mobility group A proteins in cellular senescence and heterochromatin formation. *Cell* 126 (3):503-514. doi:10.1016/j.cell.2006.05.052
- Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 113 (6):703-716
- Naylor RM, Baker DJ, van Deursen JM (2013) Senescent cells: a novel therapeutic target for aging and age-related diseases. *Clinical pharmacology and therapeutics* 93 (1):105-116. doi:10.1038/clpt.2012.193
- Niesvizky R, Badros AZ, Costa LJ, Ely SA, Singhal SB, Stadtmauer EA, Haideri NA, Yacoub A, Hess G, Lentzsch S, Spicka I, Chanan-Khan AA, Raab MS, Tarantolo S, Vij R, Zonder JA, Huang X, Jayabalan D, Di Liberto M, Huang X, Jiang Y, Kim ST, Randolph S, Chen-Kiang S (2015) Phase 1/2 study of cyclin-dependent kinase (CDK)4/6 inhibitor palbociclib (PD-0332991) with bortezomib and dexamethasone in relapsed/refractory multiple myeloma. *Leukemia & lymphoma* 56 (12):3320-3328. doi:10.3109/10428194.2015.1030641
- O'Leary B, Finn RS, Turner NC (2016) Treating cancer with selective CDK4/6 inhibitors. *Nature reviews Clinical oncology* 13 (7):417-430. doi:10.1038/nrclinonc.2016.26

- Ohtani N, Takahashi A, Mann DJ, Hara E (2012) Cellular senescence: a double-edged sword in the fight against cancer. *Experimental dermatology* 21 Suppl 1:1-4. doi:10.1111/j.1600-0625.2012.01493.x
- Oki T, Nishimura K, Kitaura J, Togami K, Maehara A, Izawa K, Sakaue-Sawano A, Niida A, Miyano S, Aburatani H, Kiyonari H, Miyawaki A, Kitamura T (2014) A novel cell-cycle-indicator, mVenus-p27K-, identifies quiescent cells and visualizes G0-G1 transition. *Scientific reports* 4:4012. doi:10.1038/srep04012
- Ota H, Tokunaga E, Chang K, Hikasa M, Iijima K, Eto M, Kozaki K, Akishita M, Ouchi Y, Kaneki M (2006) Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras-MAPK signaling in human cancer cells. *Oncogene* 25 (2):176-185. doi:10.1038/sj.onc.1209049
- Overhoff MG, Garbe JC, Koh J, Stampfer MR, Beach DH, Bishop CL (2014) Cellular senescence mediated by p16INK4A-coupled miRNA pathways. *Nucleic acids research* 42 (3):1606-1618. doi:10.1093/nar/gkt1096
- Palm W, de Lange T (2008) How shelterin protects mammalian telomeres. *Annu Rev Genet* 42:301-334. doi:10.1146/annurev.genet.41.110306.130350
- Parry AJ, Narita M (2016) Old cells, new tricks: chromatin structure in senescence. *Mammalian genome : official journal of the International Mammalian Genome Society* 27 (7-8):320-331. doi:10.1007/s00335-016-9628-9
- Peeper DS, Shvarts A, Brummelkamp T, Douma S, Koh EY, Daley GQ, Bernards R (2002) A functional screen identifies hDRIL1 as an oncogene that rescues RAS-induced senescence. *Nature cell biology* 4 (2):148-153. doi:10.1038/ncb742
- Perez-Mancera PA, Young AR, Narita M (2014) Inside and out: the activities of senescence in cancer. *Nat Rev Cancer* 14 (8):547-558. doi:10.1038/nrc3773
- Pickart CM (2001) Mechanisms underlying ubiquitination. *Annual review of biochemistry* 70:503-533. doi:10.1146/annurev.biochem.70.1.503
- Prieur A, Besnard E, Babled A, Lemaître JM (2011) p53 and p16(INK4A) independent induction of senescence by chromatin-dependent alteration of S-phase progression. *Nat Commun* 2:473. doi:10.1038/ncomms1473
- Purvis JE, Karhohs KW, Mock C, Batchelor E, Loewer A, Lahav G (2012) p53 dynamics control cell fate. *Science* 336 (6087):1440-1444. doi:10.1126/science.1218351
- Puyol M, Martin A, Dubus P, Mulero F, Pizcueta P, Khan G, Guerra C, Santamaria D, Barbacid M (2010) A synthetic lethal interaction between K-Ras oncogenes and Cdk4 unveils a therapeutic strategy for non-small cell lung carcinoma. *Cancer cell* 18 (1):63-73. doi:10.1016/j.ccr.2010.05.025
- Qadeer ZA, Harcharik S, Valle-Garcia D, Chen C, Birge MB, Vardabasso C, Duarte LF, Bernstein E (2014) Decreased expression of the chromatin remodeler ATRX associates with melanoma progression. *The Journal of investigative dermatology* 134 (6):1768-1772. doi:10.1038/jid.2014.45
- Rai TS, Cole JJ, Nelson DM, Dikovskaya D, Faller WJ, Vizioli MG, Hewitt RN, Anannya O, McBryan T, Manoharan I, van Tuyn J, Morrice N, Pchelintsev NA, Ivanov A, Brock C, Drotar ME, Nixon C, Clark W, Sansom OJ, Anderson KI, King A, Blyth K, Adams PD (2014) HIRA orchestrates a dynamic chromatin landscape in senescence and is required for suppression of neoplasia. *Genes & development* 28 (24):2712-2725. doi:10.1101/gad.247528.114
- Rai TS, Puri A, McBryan T, Hoffman J, Tang Y, Pchelintsev NA, van Tuyn J, Marmorstein R, Schultz DC, Adams PD (2011) Human CABIN1 is a functional member of the human HIRA/UBN1/ASF1a histone H3.3 chaperone complex. *Molecular and cellular biology* 31 (19):4107-4118. doi:10.1128/MCB.05546-11
- Ratnakumar K, Duarte LF, LeRoy G, Hasson D, Smeets D, Vardabasso C, Bonisch C, Zeng T, Xiang B, Zhang DY, Li H, Wang X, Hake SB, Schermelleh L, Garcia BA,

- Bernstein E (2012) ATRX-mediated chromatin association of histone variant macroH2A1 regulates alpha-globin expression. *Genes & development* 26 (5):433-438. doi:10.1101/gad.179416.111
- Rayess H, Wang MB, Srivatsan ES (2012) Cellular senescence and tumor suppressor gene p16. *International journal of cancer* 130 (8):1715-1725. doi:10.1002/ijc.27316
- Ressler S, Bartkova J, Niederegger H, Bartek J, Scharffetter-Kochanek K, Jansen-Durr P, Wlaschek M (2006) p16INK4A is a robust in vivo biomarker of cellular aging in human skin. *Aging cell* 5 (5):379-389. doi:10.1111/j.1474-9726.2006.00231.x
- Rheinwald JG, Hahn WC, Ramsey MR, Wu JY, Guo Z, Tsao H, De Luca M, Catricala C, O'Toole KM (2002) A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. *Molecular and cellular biology* 22 (14):5157-5172
- Rhind N, Russell P (2012) Signaling pathways that regulate cell division. *Cold Spring Harbor perspectives in biology* 4 (10). doi:10.1101/cshperspect.a005942
- Riley MF, Lozano G (2012) The Many Faces of MDM2 Binding Partners. *Genes & cancer* 3 (3-4):226-239. doi:10.1177/1947601912455322
- Rodier F (2013) Detection of the senescence-associated secretory phenotype (SASP). *Methods in molecular biology* 965:165-173. doi:10.1007/978-1-62703-239-1_10
- Rodier F, Campisi J (2011) Four faces of cellular senescence. *The Journal of cell biology* 192 (4):547-556. doi:10.1083/jcb.201009094
- Rodier F, Campisi J, Bhaumik D (2007) Two faces of p53: aging and tumor suppression. *Nucleic acids research* 35 (22):7475-7484. doi:10.1093/nar/gkm744
- Romagosa C, Simonetti S, Lopez-Vicente L, Mazo A, Leonart ME, Castellvi J, Ramon y Cajal S (2011) p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. *Oncogene* 30 (18):2087-2097. doi:10.1038/onc.2010.614
- Roussel MF (1999) The INK4 family of cell cycle inhibitors in cancer. *Oncogene* 18 (38):5311-5317. doi:10.1038/sj.onc.1202998
- Rufini A, Tucci P, Celardo I, Melino G (2013) Senescence and aging: the critical roles of p53. *Oncogene* 32 (43):5129-5143. doi:10.1038/onc.2012.640
- Sansom OJ, Meniel V, Wilkins JA, Cole AM, Oien KA, Marsh V, Jamieson TJ, Guerra C, Ashton GH, Barbacid M, Clarke AR (2006) Loss of Apc allows phenotypic manifestation of the transforming properties of an endogenous K-ras oncogene in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 103 (38):14122-14127. doi:10.1073/pnas.0604130103
- Sanz-Gonzalez SM, Barquin L, Garcia-Cao I, Roque M, Gonzalez JM, Fuster JJ, Castells MT, Flores JM, Serrano M, Andres V (2007) Increased p53 gene dosage reduces neointimal thickening induced by mechanical injury but has no effect on native atherosclerosis. *Cardiovascular research* 75 (4):803-812. doi:10.1016/j.cardiores.2007.05.002
- Sarma K, Cifuentes-Rojas C, Ergun A, Del Rosario A, Jeon Y, White F, Sadreyev R, Lee JT (2014) ATRX directs binding of PRC2 to Xist RNA and Polycomb targets. *Cell* 159 (4):869-883. doi:10.1016/j.cell.2014.10.019
- Satyanarayana A, Kaldis P (2009) Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. *Oncogene* 28 (33):2925-2939. doi:10.1038/onc.2009.170
- Sawai CM, Freund J, Oh P, Ndiaye-Lobry D, Bretz JC, Strikoudis A, Genesca L, Trimarchi T, Kelliher MA, Clark M, Soulier J, Chen-Kiang S, Aifantis I (2012) Therapeutic targeting of the cyclin D3:CDK4/6 complex in T cell leukemia. *Cancer cell* 22 (4):452-465. doi:10.1016/j.ccr.2012.09.016

- Schick U, Kyula J, Barker H, Patel R, Zaidi S, Gregory C, Hafsi H, Roulstone V, Deutsch E, McLaughlin M, Harrington K (2015) Trametinib radiosensitises RAS- and BRAF-mutated melanoma by perturbing cell cycle and inducing senescence. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 117 (2):364-375. doi:10.1016/j.radonc.2015.06.026
- Schmitt CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM, Lowe SW (2002) A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell* 109 (3):335-346
- Schmolka N, Serre K, Grosso AR, Rei M, Pennington DJ, Gomes AQ, Silva-Santos B (2013) Epigenetic and transcriptional signatures of stable versus plastic differentiation of proinflammatory gammadelta T cell subsets. *Nature immunology* 14 (10):1093-1100. doi:10.1038/ni.2702
- Schwarze SR, Fu VX, Desotelle JA, Kenowski ML, Jarrard DF (2005) The identification of senescence-specific genes during the induction of senescence in prostate cancer cells. *Neoplasia* 7 (9):816-823
- Sdek P, Ying H, Zheng H, Margulis A, Tang X, Tian K, Xiao ZX (2004) The central acidic domain of MDM2 is critical in inhibition of retinoblastoma-mediated suppression of E2F and cell growth. *The Journal of biological chemistry* 279 (51):53317-53322. doi:10.1074/jbc.M406062200
- Selivanova G (2014) Wild type p53 reactivation: from lab bench to clinic. *FEBS letters* 588 (16):2628-2638. doi:10.1016/j.febslet.2014.03.049
- Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88 (5):593-602
- Sharpless NE (2004) Ink4a/Arf links senescence and aging. *Experimental gerontology* 39 (11-12):1751-1759. doi:10.1016/j.exger.2004.06.025
- Sharpless NE, DePinho RA (2004) Telomeres, stem cells, senescence, and cancer. *The Journal of clinical investigation* 113 (2):160-168. doi:10.1172/JCI20761
- Sharpless NE, Sherr CJ (2015) Forging a signature of in vivo senescence. *Nature reviews Cancer* 15 (7):397-408. doi:10.1038/nrc3960
- Sherr CJ, Beach D, Shapiro GI (2016) Targeting CDK4 and CDK6: From Discovery to Therapy. *Cancer discovery* 6 (4):353-367. doi:10.1158/2159-8290.CD-15-0894
- Shi D, Gu W (2012) Dual Roles of MDM2 in the Regulation of p53: Ubiquitination Dependent and Ubiquitination Independent Mechanisms of MDM2 Repression of p53 Activity. *Genes & cancer* 3 (3-4):240-248. doi:10.1177/1947601912455199
- Shvarts A, Brummelkamp TR, Scheeren F, Koh E, Daley GQ, Spits H, Bernards R (2002) A senescence rescue screen identifies BCL6 as an inhibitor of anti-proliferative p19(ARF)-p53 signaling. *Genes & development* 16 (6):681-686. doi:10.1101/gad.929302
- Singer S, Socci ND, Ambrosini G, Sambol E, Decarolis P, Wu Y, O'Connor R, Maki R, Viale A, Sander C, Schwartz GK, Antonescu CR (2007) Gene expression profiling of liposarcoma identifies distinct biological types/subtypes and potential therapeutic targets in well-differentiated and dedifferentiated liposarcoma. *Cancer research* 67 (14):6626-6636. doi:10.1158/0008-5472.CAN-07-0584
- Soucy TA, Dick LR, Smith PG, Milhollen MA, Brownell JE (2010) The NEDD8 Conjugation Pathway and Its Relevance in Cancer Biology and Therapy. *Genes & cancer* 1 (7):708-716. doi:10.1177/1947601910382898
- Souroullas GP, Jeck WR, Parker JS, Simon JM, Liu JY, Paulk J, Xiong J, Clark KS, Fedorow Y, Qi J, Burd CE, Bradner JE, Sharpless NE (2016) An oncogenic Ezh2 mutation induces tumors through global redistribution of histone 3 lysine 27 trimethylation. *Nature medicine* 22 (6):632-640. doi:10.1038/nm.4092

- Sousa-Victor P, Gutarra S, Garcia-Prat L, Rodriguez-Ubreva J, Ortet L, Ruiz-Bonilla V, Jardi M, Ballestar E, Gonzalez S, Serrano AL, Perdiguero E, Munoz-Canoves P (2014) Geriatric muscle stem cells switch reversible quiescence into senescence. *Nature* 506 (7488):316-321. doi:10.1038/nature13013
- Stavridis MP, Collins BJ, Storey KG (2010) Retinoic acid orchestrates fibroblast growth factor signalling to drive embryonic stem cell differentiation. *Development* 137 (6):881-890. doi:10.1242/dev.043117
- Stein GH, Drullinger LF, Soulard A, Dulic V (1999) Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Molecular and cellular biology* 19 (3):2109-2117
- Stevenson RE (1993) Alpha-Thalassemia X-Linked Intellectual Disability Syndrome. In: Pagon RA, Adam MP, Ardinger HH et al. (eds) *GeneReviews*(R). Seattle (WA),
- Stone S, Dayananth P, Kamb A (1996) Reversible, p16-mediated cell cycle arrest as protection from chemotherapy. *Cancer research* 56 (14):3199-3202
- Storer M, Mas A, Robert-Moreno A, Pecoraro M, Ortells MC, Di Giacomo V, Yosef R, Pilpel N, Krizhanovsky V, Sharpe J, Keyes WM (2013) Senescence is a developmental mechanism that contributes to embryonic growth and patterning. *Cell* 155 (5):1119-1130. doi:10.1016/j.cell.2013.10.041
- Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* 102 (43):15545-15550. doi:10.1073/pnas.0506580102
- Tchkonia T, Zhu Y, van Deursen J, Campisi J, Kirkland JL (2013) Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. *The Journal of clinical investigation* 123 (3):966-972. doi:10.1172/JCI64098
- Thakar A, Gupta P, Ishibashi T, Finn R, Silva-Moreno B, Uchiyama S, Fukui K, Tomschik M, Ausio J, Zlatanova J (2009) H2A.Z and H3.3 histone variants affect nucleosome structure: biochemical and biophysical studies. *Biochemistry* 48 (46):10852-10857. doi:10.1021/bi901129e
- Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, Lennon NJ, Livak KJ, Mikkelsen TS, Rinn JL (2014) The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nature biotechnology* 32 (4):381-386. doi:10.1038/nbt.2859
- Traven A, Heierhorst J (2005) SQ/TQ cluster domains: concentrated ATM/ATR kinase phosphorylation site regions in DNA-damage-response proteins. *BioEssays : news and reviews in molecular, cellular and developmental biology* 27 (4):397-407. doi:10.1002/bies.20204
- Tu Z, Aird KM, Zhang R (2013) Chromatin remodeling, BRCA1, SAHF and cellular senescence. *Cell cycle* 12 (11):1653-1654. doi:10.4161/cc.24986
- Turner NC, Ro J, Andre F, Loi S, Verma S, Iwata H, Harbeck N, Loibl S, Huang Bartlett C, Zhang K, Giorgetti C, Randolph S, Koehler M, Cristofanilli M, Group PS (2015) Palbociclib in Hormone-Receptor-Positive Advanced Breast Cancer. *The New England journal of medicine* 373 (3):209-219. doi:10.1056/NEJMoa1505270
- van Deursen JM (2014) The role of senescent cells in ageing. *Nature* 509 (7501):439-446. doi:10.1038/nature13193
- Vernier M, Bourdeau V, Gaumont-Leclerc MF, Moiseeva O, Begin V, Saad F, Mes-Masson AM, Ferbeyre G (2011) Regulation of E2Fs and senescence by PML nuclear bodies. *Genes & development* 25 (1):41-50. doi:10.1101/gad.1975111

- Vernier M, Ferbeyre G (2014) Complete senescence: RB and PML share the task. *Cell cycle* 13 (5):696. doi:10.4161/cc.28090
- Vigneron A, Vousden KH (2010) p53, ROS and senescence in the control of aging. *Aging* 2 (8):471-474. doi:10.18632/aging.100189
- Volkel P, Dupret B, Le Bourhis X, Angrand PO (2015) Diverse involvement of EZH2 in cancer epigenetics. *American journal of translational research* 7 (2):175-193
- Voon HP, Hughes JR, Rode C, De La Rosa-Velazquez IA, Jenuwein T, Feil R, Higgs DR, Gibbons RJ (2015) ATRX Plays a Key Role in Maintaining Silencing at Interstitial Heterochromatic Loci and Imprinted Genes. *Cell reports* 11 (3):405-418. doi:10.1016/j.celrep.2015.03.036
- Vora SR, Juric D, Kim N, Mino-Kenudson M, Huynh T, Costa C, Lockerman EL, Pollack SF, Liu M, Li X, Lehar J, Wiesmann M, Wartmann M, Chen Y, Cao ZA, Pinzon-Ortiz M, Kim S, Schlegel R, Huang A, Engelman JA (2014) CDK 4/6 inhibitors sensitize PIK3CA mutant breast cancer to PI3K inhibitors. *Cancer cell* 26 (1):136-149. doi:10.1016/j.ccr.2014.05.020
- Wang E (1995) Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. *Cancer research* 55 (11):2284-2292
- Wang Y, Xu Q, Sack L, Kang C, Elledge SJ (2016) A gain-of-function senescence bypass screen identifies the homeobox transcription factor DLX2 as a regulator of ATM-p53 signaling. *Genes & development* 30 (3):293-306. doi:10.1101/gad.271445.115
- Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, Chao P, Franz M, Grouios C, Kazi F, Lopes CT, Maitland A, Mostafavi S, Montojo J, Shao Q, Wright G, Bader GD, Morris Q (2010) The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic acids research* 38 (Web Server issue):W214-220. doi:10.1093/nar/gkq537
- Weber JD, Taylor LJ, Roussel MF, Sherr CJ, Bar-Sagi D (1999) Nucleolar Arf sequesters Mdm2 and activates p53. *Nature cell biology* 1 (1):20-26. doi:10.1038/8991
- Wong LH, McGhie JD, Sim M, Anderson MA, Ahn S, Hannan RD, George AJ, Morgan KA, Mann JR, Choo KH (2010) ATRX interacts with H3.3 in maintaining telomere structural integrity in pluripotent embryonic stem cells. *Genome research* 20 (3):351-360. doi:10.1101/gr.101477.109
- Wu YV, Okada T, DeCarolis P, Socci N, O'Connor R, Geha RC, Joy Somberg C, Antonescu C, Singer S (2012) Restoration of C/EBPalpha in dedifferentiated liposarcoma induces G2/M cell cycle arrest and apoptosis. *Genes, chromosomes & cancer* 51 (4):313-327. doi:10.1002/gcc.21917
- Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovskiy V, Cordon-Cardo C, Lowe SW (2007) Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 445 (7128):656-660. doi:10.1038/nature05529
- Yamaguchi H, Hung MC (2014) Regulation and Role of EZH2 in Cancer. *Cancer research and treatment : official journal of Korean Cancer Association* 46 (3):209-222. doi:10.4143/crt.2014.46.3.209
- Yang C, Boyson CA, Di Liberto M, Huang X, Hannah J, Dorn DC, Moore MA, Chen-Kiang S, Zhou P (2015) CDK4/6 Inhibitor PD 0332991 Sensitizes Acute Myeloid Leukemia to Cytarabine-Mediated Cytotoxicity. *Cancer research* 75 (9):1838-1845. doi:10.1158/0008-5472.CAN-14-2486

- Yanos ME, Bennett CF, Kaeberlein M (2012) Genome-Wide RNAi Longevity Screens in *Caenorhabditis elegans*. *Current genomics* 13 (7):508-518. doi:10.2174/138920212803251391
- Yao G, Lee TJ, Mori S, Nevins JR, You L (2008) A bistable Rb-E2F switch underlies the restriction point. *Nature cell biology* 10 (4):476-482. doi:10.1038/ncb1711
- Ye X, Linton JM, Schork NJ, Buck LB, Petrascheck M (2014) A pharmacological network for lifespan extension in *Caenorhabditis elegans*. *Aging cell* 13 (2):206-215. doi:10.1111/accel.12163
- Yim EK, Sheetz MP (2012) Force-dependent cell signaling in stem cell differentiation. *Stem cell research & therapy* 3 (5):41. doi:10.1186/scrt132
- Yoshida A, Lee EK, Diehl JA (2016) Induction of Therapeutic Senescence in Vemurafenib-Resistant Melanoma by Extended Inhibition of CDK4/6. *Cancer research* 76 (10):2990-3002. doi:10.1158/0008-5472.CAN-15-2931
- Young AR, Narita M (2010) Connecting autophagy to senescence in pathophysiology. *Current opinion in cell biology* 22 (2):234-240. doi:10.1016/j.ceb.2009.12.005
- Yuen BT, Knoepfler PS (2013) Histone H3.3 mutations: a variant path to cancer. *Cancer cell* 24 (5):567-574. doi:10.1016/j.ccr.2013.09.015
- Yun K, Wold B (1996) Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Current opinion in cell biology* 8 (6):877-889
- Zeiser R (2014) Trametinib. Recent results in cancer research *Fortschritte der Krebsforschung Progres dans les recherches sur le cancer* 201:241-248. doi:10.1007/978-3-642-54490-3_15
- Zeuner A, Francescangeli F, Contavalli P, Zapparelli G, Apuzzo T, Eramo A, Baiocchi M, De Angelis ML, Biffoni M, Sette G, Todaro M, Stassi G, De Maria R (2014) Elimination of quiescent/slow-proliferating cancer stem cells by Bcl-XL inhibition in non-small cell lung cancer. *Cell death and differentiation* 21 (12):1877-1888. doi:10.1038/cdd.2014.105
- Zhang R, Chen W, Adams PD (2007) Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol Cell Biol* 27 (6):2343-2358. doi:10.1128/MCB.02019-06
- Zhang R, Poustovoitov MV, Ye X, Santos HA, Chen W, Daganzo SM, Erzberger JP, Serebriiskii IG, Canutescu AA, Dunbrack RL, Pehrson JR, Berger JM, Kaufman PD, Adams PD (2005) Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. *Developmental cell* 8 (1):19-30. doi:10.1016/j.devcel.2004.10.019
- Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS (2008) Model-based analysis of ChIP-Seq (MACS). *Genome biology* 9 (9):R137. doi:10.1186/gb-2008-9-9-r137
- Zhu Y, Tchkonja T, Pirtskhalava T, Gower AC, Ding H, Giorgadze N, Palmer AK, Ikeno Y, Hubbard GB, Lenburg M, O'Hara SP, LaRusso NF, Miller JD, Roos CM, Verzosa GC, LeBrasseur NK, Wren JD, Farr JN, Khosla S, Stout MB, McGowan SJ, Fuhrmann-Stroissnigg H, Gurkar AU, Zhao J, Colangelo D, Dorransoro A, Ling YY, Barghouthy AS, Navarro DC, Sano T, Robbins PD, Niedernhofer LJ, Kirkland JL (2015) The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. *Aging cell* 14 (4):644-658. doi:10.1111/accel.12344
- Zhuang M, Guan S, Wang H, Burlingame AL, Wells JA (2013) Substrates of IAP ubiquitin ligases identified with a designed orthogonal E3 ligase, the NEDDylator. *Molecular cell* 49 (2):273-282. doi:10.1016/j.molcel.2012.10.022
- Ziv O, Glaser B, Dor Y (2013) The plastic pancreas. *Developmental cell* 26 (1):3-7. doi:10.1016/j.devcel.2013.06.013