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This thesis is dedicated to any patients who may get cured with treatments manifesting from this work.

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Abbreviations

4-HT: 4-hydroxy Tamoxifen
ANRIL: Antisense ncRNA in the *INK4* locus
ARHAGP18: Rho GTPase Activating Protein 18
ATM: Ataxia Telangiectasia Mutated
ATR: Ataxia Telangiectasia and Rad3-related protein
BDNF-AS: Brain-Derived Neurotrophic Factor-Antisense Transcript
Cas9: CRISPR Associated Protein
CCF: Cytoplasmic Chromatin Fractions
CDKN2B-AS1: Cyclin Dependent Kinase inhibitor 2B-Antisense Transcript 1
cDNA: Complementary DNA
ChIP: Chromatin Immunoprecipitation
COLDAIR: Cold-Induced Long Antisense Intergenic RNA
COOLAIR: Cold Assisted Intronic Non-coding RNA
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
DAPI: 4,6-diamidino-2-phenylindole
DDR: DNA Damage Response
DNA: Deoxyribonucleic Acid
dsRNA: Double Stranded RNA
FAIRE: Formaldehyde-Assisted Isolation of Regulatory Elements
FLC: Flowering Locus C
GRO-seq: Global Run-on sequencing
GWAS: Genome Wide Association Studies
H3K27me3: Trimethylation on 27^{th} lysine residue of Histone 3
H3K9me2: Dimethylation on 9^{th} lysine residue of Histone 3
HDF: Human Diploid Fibroblast
HOTAIR: HOX transcript antisense RNA
HOX: Homeobox
HOXC: Antisense to Homeobox C
HP1: Heterochromatin Protein 1
hTERT: human Telomerase Reverse Transcriptase
iPSCs: Induced Pluripotent Stem Cells
LAMA2: Laminin, Alpha 2
lncRNA: Long Non-coding RNA
miR: MicroRNAs
mRNA: messenger RNA
mTOR: mammalian Target Of Rafamycin
MTREC: Mtl1-Red1 core
MYC: Myelocytomatosis

ncRNA: Non-Coding RNA
NET-seq: Native Elongating Transcript sequencing
NK Cells: Natural Killer Cells
PANDA: Promoter of CDKN1A antisense DNA damage activated (RNA)
PI3K: Phosphatidylinositol 3-kinases
PML NB: Premyelocytic Leukemia Nuclear Bodies
POD: Premyelocytic Oncogenic Domain
PRC2: Polycomb Response Complex 2
PRO-seq: Precision nuclear Run-On and Sequencing assay
PTEN: Phosphatase and tensin homolog
REV3L: Protein reversionless 3-like also known as DNA polymerase zeta catalytic subunit (POLZ).
RNA-seq: RNA sequencing
RNA: Ribonucleic Acid
RNAi; RNA interference
ROS: Reactive Oxygen Species
SAFA: Scaffold-Attachment-Factor A
SAHF: Senescence Associated Heterochromatic Foci
SAL: Senescence Associated Long noncoding RNA
SAPD: Senescence Associated Protein Degradation
SASP: Senescence Associated Secretory Phenotype
siRNA: Small Interfering RNA
SMS: Senescence Messaging Secretome
SRCAP: Snf2 Related CBP Activator Protein
START: Senescence Triggered Antisense Read-through Transcripts
TAM: Tumour Associated Macrophages
TERC: Telomeric RNA Component
TERRA: Telomeric repeat containing RNA
TLR: Toll Like Receptor
TRAMP: Trf4-Air2-Mtr4 polyadenylation
TRF2: Telomeric repeat-binding factor 2
TSG: Tumour Suppressor Gene
Tsix: X (inactive)-specific transcript, opposite strand
TSS: Tumour Suppressor Gene
UPP: Ubiquitin Proteasome Pathway
VAD: Vlinc Antisense to DDAH1
Vlinc: Very large Intergenic non-coding RNA
XIC: X-chromosome Inactivation Centre
XIST: X-inactive specific transcript

Résumé

La sénescence, qui est un mécanisme antitumoral majeur, est définie comme un état d'arrêt irréversible de la prolifération cellulaire en réponse à un stress comme l'activation illégitime d'oncogènes. Les cellules qui entrent en sénescence subissent de profonds changements de leur épigénome.
Les ARNs antisens sont suspectés de joue des rôles importants dans le contrôle du destin cellulaire et dans des processus cellulaires variés. Dans la levure, le variant d'histone H2A.Z co-opère avec les machineries du RNAi et de l'hétérochromatine pour réprimer sur les loci de gènes convergents l'apparition d'antisens dus à des défauts de terminaison de transcription de un des deux gènes.
Chez les mammifères, l'existence et la régulation de tels transcrits antisens restent inconnues. De façon intéressante, la déplétion du variant d'histones H2A.Z est connue pour induire la sénescence. Nous nous sommes donc demandés si la sénescence est accompagnée de la régulation de tels transcrits antisens sur les gènes convergents, si la régulation par H2A.Z est conservée et si ces transcrits pouvaient avoir un rôle fonctionnel.
Dans un modèle de sénescence induite par les oncogènes in vitro, nous avons identifiés par RNA-Seq brin spécifiques plusieurs loci de gènes convergents où des ARN antisens pourraient être générés par des défauts de terminaison de transcription sur le gène convergent. Des analyses en profondeurs sur deux loci ont confirmé que les transcrits antisens sont effectivement générés par un tel mécanisme (appelé "read-through transcriptionnel"). Nous avons appelé ces antisens START RNAs (pour " *S*enescence *T*riggered *A*ntisense *R*ead-through *T*ranscripts). Nous avons par la suite montré que ces STARTs répriment l'expression du gène pour lequel ils sont antisens. Finalement, nous avons montré qu'ils sont réprimés par H2A.Z dans les cellules en prolifération. Nous proposons donc un modèle où la progression en sénescence s'accompagne d'une diminution de H2A.Z, ce qui se traduit par l'induction de transcrits antisens régulateurs sur une famille de loci de gènes convergents dus à des défauts de contrôle de la terminaison de la transcription.

“In science, we have to be very brave. Actually we should not believe in text books, or we should not believe in what elderly people, your teachers says to you, because that’s the main job of a scientist- we want to re-write text books. Sometimes stupid idea may cause a breakthrough. So I think it’s important not to stop young minds.”

- Shinya Yamanaka

Table of Contents

PART I: INTRODUCTION

A. Cellular Senescence

1. ***Introduction on Cellular Senescence*** ***8***
2. ***Causes and effectors of Cellular Senescence*** ***10***
3. ***Markers of Senescence*** ***15***
   a. ***Acidic β-galactosidase*** ***16***
   b. ***p16INK4A*** ***16***
   c. ***DNA damage foci and typical chromatin alterations*** ***21***
   d. ***The SASP*** ***25***
   e. ***Telomere shortening and dysfunction*** ***26***
   f. ***The combination of Senescence markers*** ***28***
4. ***Various conditions for Senescence Induction*** ***30***
   a. ***Replicative Senescence in vitro*** ***30***
   b. ***Oncogene Induced Senescence (OIS) in vitro*** ***33***
   c. ***Tumor suppressor loss mediated Senescence in vitro*** ***35***
   d. ***Other inducers of Senescence in-vitro*** ***37***
   e. ***Replicative Senescence in vivo*** ***38***
   f. ***OIS in vivo*** ***41***
   g. ***OIS in the context of nevus and melanoma formation*** ***42***
   h. ***Tumor suppressor loss mediated Senescence in vivo*** ***44***
5. ***Oncogene-induced reversible growth arrest*** ***46***
6. ***Immuno-surveillance of senescent cells*** ***49***

B. Non-coding Antisense RNAs

1. ***Introduction on Antisense Transcripts*** ***53***
2. ***Detection of antisense transcripts*** ***57***
3. ***Expression of antisense transcripts*** ***58***
4. ***An insight into antisense functionalities*** ***59***
5. ***RNA Polymerase II termination pathways*** ***68***
6. ***Generation of Pervasive Antisense RNA at convergent genes*** ***73***

C. Implications of Non-coding RNAs in Cellular Senescence

1. ***Noncoding RNAs and Cardinal Tumour Suppressors*** ***80***
    a. ***Senescence Associated(SA) microRNAs in p53/p21 pathway*** ***80***
    b. ***Senescence Associated long noncoding (SAL) RNA affecting the p53/p21 pathway*** ***82***
    c. ***SA-microRNAs in the pRB/p16 Pathway*** ***83***
    d. ***SAL-RNAs in the pRB/p16 Pathway*** ***84***
2. ***Role of ncRNAs in various traits of senescence*** ***86***
    a. ***SASP ncRNAs*** ***86***
    b. ***ncRNAs Involved in Telomere Metabolism*** ***87***
    c. ***Other ncRNAs associated to Senescence Traits*** ***88***

PART II: RESULTS

1. ***Background, rationale and objectives of my PhD project In a nutshell*** ***92***
2. ***The Manuscript***
3. ***Additional Data*** ***95***

PART III: DISCUSSION

1. ***Discussion on various facets of my PhD project***
    a. ***A putative senescence landscape*** ***99***
    b. ***The surveillance machineries at work*** ***102***
    c. ***Cause and Effect*** ***105***
    d. ***The players at the cross-roads of the 2 phenotypes*** ***106***
    e. ***Mechanistic insight of the associated growth arrest*** ***108***
    f. ***The proof of read-through harboured startling revelations*** ***109***
    g. ***Therapeutic angle*** ***110***
    h. ***The Holy Grail in the mystery of aging?*** ***112***
    i. ***Model*** ***114***
    j. ***Conjecture*** ***117***

Reference ***118***

Supplementary Table ***145***

Part 1: Introduction

A. Cellular Senescence

Societies have traditionally taken three approaches in handling recidivist criminals: exile, execution and lifetime imprisonment. It seems that human cells use similar strategies to prevent rogue cells harbouring dangerous mutations from turning into fully-fledged cancers. Epithelial tissue, such as that lining the airways and intestines, continuously renews and sloughs off, thereby sentencing some precancerous cells to extra-corporeal exile. There is also a cellular version of the death penalty — apoptosis, a well-established anticancer mechanism. In 2005, a series of very elegant studies (Michaloglou et al., 2005; Chen et al., 2005; Collado et al., 2005; Braig et al., 2005) provided the first in vivo evidence that the body can subject potential cancer cells to the equivalent of a life-sentence: cellular senescence, thereby repudiating senescence from being denounced as a mere cell culture artefact (Sharpless and Depinho 2005).

1. Introduction on Cellular Senescence:

Cellular senescence refers to the specific phenomenon wherein a proliferation-competent cell undergoes permanent growth arrest in response to various cellular stresses. The senescent state is accompanied by a failure to re-enter the cell division cycle in response to mitogenic stimulation and by an acquired resistance to oncogenic challenge. These properties of stress-induced irreversible proliferative arrest and resistance to both mitogenic and oncogenic stimuli, provide the best formal definition of the senescent state (Sharpless and Sherr 2015).

The concept of cellular senescence was kindled in the limelight with path-breaking observations that human diploid cell types have a specific replicative limit in culture, although they can remain viable and metabolically active after assuming a stable, non-dividing state, a phenomenon commonly termed as “Hayflick Limit” (Hayflick and Moorhead 1961). This is because continued cell propagation calls for telomeric attrition, which eventually leads to stable proliferative

arrest (Harley et al., 1990; Bodnar et al., 1998). Cultured primary mouse embryonic fibroblasts also exhibits such defined replicative capacity (Todaro et al., 1963) and undergo senescence, although telomere attrition wasn't found to responsible to this end (Blasco et al., 1997). Instead chronic activation of cardinal tumour suppressors such as the retinoblastoma protein (RB) and the transcription factor p53 were found to be the culprit, in a way that mimics proliferative arrest in response to non-physiological conditions of tissue culture or oncogene challenge (herein referred to as oncogene-induced senescence (OIS) (Harvey et al., 1991; Serrano et al., 1997; Kamijo et al., 1997; Wright and Shay 2000; Sherr and DePinho 2000). Emerging data are leading to acknowledge that additional diverse senescence stimuli include reactive oxygen species (ROS), other DNA-damaging and the unfolded protein response (Kuilman et al., 2010; Rodier and Campisi 2011; Campisi 2013).

Various stimuli that are known to activate senescence are cancer-associated stresses, and the acquired resistance of senescent cells from the onslaught of oncogenic transformation supports a role for senescence in preventing cancer progression and this barrier has been found to be indefinite or permanent, lasting for a lifetime. (Kuilman et al., 2010; Rodier and Campisi 2011; Campisi 2013; Collado, Blasco and Serrano 2007; Kim and Sharpless 2006) To this lines, repression of RB–p53 signaling in proliferating cells can bypass the onset of cellular senescence, whereas established senescent human cells in culture resist oncogenic insults that attempt to force cell cycle re-entry, such as the introduction of telomerase or the simian vacuolating virus 40 (SV40) large T-antigen (Shay et al., 1991), which inactivates both the RB and the p53 pathways. This observation is consistent with an earlier finding where Lena Gurdon and colleague for the first time demonstrated the inability of senescent cells to phosphorylate RB when challenged with large T-antigen (Stein et al., 1990). By contrast, experimental co-inactivation of the G1 cyclin-dependent kinase (CDK) inhibitor p16INK4A and p53 in some senescent human cells (Beauséjour et al., 2003) or conditional deletion of *Rb1* in mouse embryonic fibroblasts (Sage et al., 2003) have been reported to reverse senescence, permitting

re-entry into the cell cycle. Nonetheless, senescence is irreversible in the sense that known physiological stimuli cannot force senescent cells to re-enter the cell cycle (Campisi 2013, Sherr and Sharpless 2015).

With senescence at the nexus of so many diverse research areas, and with a surfeit of high-profile papers focused on the subject, a troubling issue for the field has been obscured: namely, that markers to identify, quantify and characterize senescent cells in an intact organism are nonspecific and unreliable. This lack of a uniform definition of what constitutes senescence promotes confusion and controversy, and continues to raise numerous conceptual problems (Sharpless and Sherr 2015).

2. Causes and effectors of Cellular Senescence

Present conception of senescence and its effector programs like diverse stress erupts largely from the in-vitro work i.e. from cell culture based studies. In addition to telomere erosion, several other tumour-associated stresses have been shown to induce a senescent growth arrest *in-vitro*, including certain DNA lesions and reactive oxygen species (ROS) (Nardella et al., 2011; Sedelnikov et al., 2004; von Zglinicki 2002). The cross-roads of the two types of stress have certain common telomere damage trigger that activate the DNA damage response (DDR), a signalling pathway in which ATM or ATR kinases block cell-cycle progression through stabilization of p53 and transcriptional activation of the cyclin-dependent kinase (Cdk) inhibitor p21. Besides these, as introduced before, oncogenic challenges in elegant *in-vivo* studies also elicit such permanent growth arrest. Oncogenic Ras acts through overexpression of Cdc6 and suppression of nucleotide metabolism, causing aberrant DNA replication, formation of double stranded DNA breaks (DSBs) and activation of the DDR pathway (Aird et al., 2013; Di Micoo et al., 2006)

However, senescence mediated via E2F3 activation or c-Myc inhibition is DDR-independent and involves p19Arf and p16Ink4a while inhibition of PTEN have been shown to drive senescence on p53, mTOR dependent and DDR independent pathway (Sedelnikov et al., 2004, Lazzirini et al., 2005; Chen et al., 2005). BRAF (V600E) is also DDR-independent and induces senescence exploiting a metabolic pathway with prominent machinery like mitochondrial pyruvate dehydrogenase (PDH) at work (Kaplon et al., 2013). Several other studies underscored that senescence is closely linked to profound metabolic changes (Kondoh et al., 2005; Dorr et al., 2013). On the contrary a repertoire of studies identified that repression of tumour suppressors can also lead to induction of senescence, which includes RB, PTEN, NF1 and VHL (Kim and Sharpless 2006; Lazzirini et al., 2005). Of these, RB inactivation engages the DDR, whereas the others are DDR-independent and act through p19Arf and p16Ink4a. Moreover there is a sort of hierarchy in the antitumorigenic role displayed by various components of the INK4 locus. To this end it has been shown that loss of p16 alone can drive tumorigenesis even in the backdrop of unalterted p19 expression, suggesting that p19 is a recessive Tumour Suppressor Gene (TSG) (Sharpless et al., 2001). More over these two genes displays antigonastc role in certain backgrounds. To this end a study dealing with aging mouse model where in the mouse strain used was deficient of BubR1 (a gene that codes for mitotic checkpoint protein and hence undergoes premature separation of sister chromosomes and develop progressive aneuploidy along with various progeroid phenotypes) showed that loss of p16 ameliorated aging phenotypoe however loss of p19 exacerbated senescence and consequenty augmented aging and aging related phenotype (Baker et al., 2008). The complexity of the subject is further elevated by the fact of differential propensity in the usage of tumour suppressor machinery as murine cells are found to be prone in the usage of p19 than towards induction of senescence than p14 by human cells (Ben-Porath et al., 2005) which highlights the species-specific differences in the mode of senescence induction. Prolonged exposure to interferon-b also induces senescence, demonstrating that chronic viral-sensing like sensor is also crucial in mitigating

mitogenic signaling outside the context of neoplastic transformation (Moiseeva et al., 2006). Other, less broadly studied inducers of senescence include epigenetic, nucleolar and mitotic spindle stresses. For example, genome-wide chromatin decompression by exposure to histone deacetylase inhibitors triggers senescence via a p21- dependent mechanism (Romanov et al., 2010). Epigenetic stress can also have specific effectors that promote senescence may be the INK4a/ARF locus, which in proliferating cells is repressed by polycomb group-mediated H3K27 methylation and H2AK119 ubiquitination (Lapak and Burd 2014). Nucleolar stress caused by RNA polymerase I inhibitors triggers a robust p53-mediated senescence response (Hein et al., 2012). Senescence can also be elicited by suboptimal expression of proteins implicated in spindle formation or mitotic checkpoint control, including human TACC3 and murine BubR1, Bub3 and Rae1, all of which engage p53 and p21 independently of the DDR, often in combination with p16Ink4a (Baker et al., 2004; Schmidt et al., 2010). It is highly likely that additional stressors and mechanisms that drive cells into senescence will be uncovered given the rapidly evolving nature of the field. Chronic influx of proinflammatory cytokines and chemokines is an emerging feature of senescent cells irrespective of senescence mediators or effector pathways (Fig. 1). Although p21-p53 and p16 nexus both qualified to be two major archetypal inducers of senescence and but these two pathways can be differentially exploited, however may ultimately become engaged upon sustained senescence. For example, DNA damage initially halts cell-cycle progression through p53-mediated induction of p21, but persisted lesion calls for urgency by activating p16Ink4a through p38-MAPK-mediated mitochondrial dysfunction (Freund et al., 2011; Passos et al., 2010). The extent to which effector mechanisms of *in-vitro* senescence apply to in vivo senescence has not been tested extensively. Genetic experiments using knockout strains for each of these tumour suppressors that dissected how senescent cells accumulate in these tissues and contribute to their deterioration, established that p16Ink4a is an effector of senescence and ageing. On the other hand inactivation of p21 has been shown to improve stem cell function in intestinal crypts and bone marrow in mutant mice with short telo-

meres (Choudhury et al., 2007). This indicated that in situations where irreparable damage produces a sustained and robust p53 response, p21 acts to promote tissue deterioration by executing senescence. However, in the context of organismal ageing, individual cells experience multiple cellular pressures, including various kinds of genotoxic, proteotoxic and mitotic stresses (Seigel and Amon 2012). Thus, to advance our understanding of these processes, it will be imperative to have an uncoupled and unbiased approach to senescence and aging and to examine how combinations of diverse senescence-promoting stressors impact the actions of the various downstream effector pathways and the characteristics of the resulting senescent phenotypes. Furthermore, while cellular senescence is well recognized as an in vivo tumour suppressive mechanism, its irreversibility remains a topic of debate. Certain evidence indicates that BRAF (V600E) oncogene-induced senescence (OIS) can be reversed by activation of phosphatidylinositol 3-kinase (PI3K) or inhibition of PDH (Fig. 1) (Kaplon et al., 2013; Vredeveld et al., 2012). Although there is a claim of having senescent cells successfully dedifferentiated into pluripotent stem cells (Lapasset et al., 2011), overwhelming studies from very prominent laboratories have shown senescence as potential impediment to cellular reprogramming as discussed later (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009; Zhao et al., 2013).

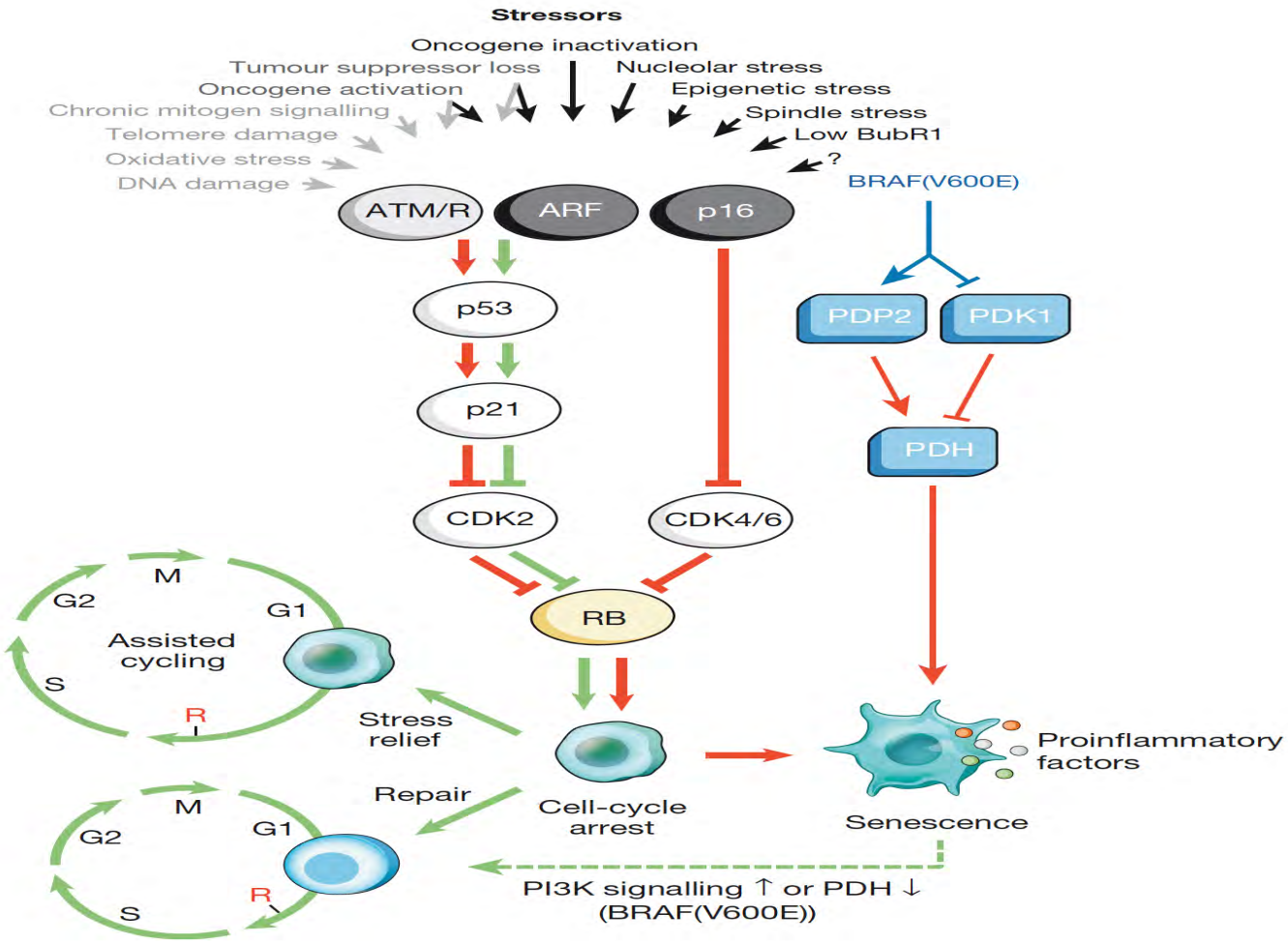

Fig.1 Senescence-inducing stimuli and main effector pathways.
A variety of cell-intrinsic and -extrinsic stresses can activate the cellular senescence program. These stressors engage various cellular signalling cascades but ultimately activate p53, p16Ink4a, or both. Stress types that activate p53 through DDR signalling are indicated with grey text and arrows (ROS elicit the DDR by perturbing gene transcription and DNA replication, as well as by shortening telomeres). Activated p53 induces p21, which induces a temporal cell-cycle arrest by inhibiting cyclin E–Cdk2. p16Ink4a also inhibits cell-cycle progression but does so by targeting cyclin D–Cdk4 and cyclin D–Cdk6 complexes. Both p21 and p16Ink4a act by preventing the inactivation of Rb, thus resulting in continued repression of E2F target genes required for S-phase onset. Upon severe stress (red arrows), temporally arrested cells transition into a senescent growth arrest through a mechanism that is currently incompletely understood. Cells exposed to mild damage that can be successfully repaired may resume normal cell-cycle progression. On the other hand, cells exposed to moderate stress that is chronic in nature or that leaves permanent damage may resume proliferation through reliance on stress support pathways (green arrows). This phenomenon (termed assisted cycling) is enabled by p53-mediated activation of p21. Thus, the p53–p21 pathway can either antagonize or synergize with p16Ink4a in senescence depending on the type and level of stress. BRAF(V600E) is unusual in that it establishes senescence through a metabolic effector pathway. BRAF(V600E) activates PDH by inducing PDP2 and inhibiting PDK1 expression, promoting a shift from glycolysis to oxidative phosphorylation that creates senescence-inducing redox stress. Cells undergoing senescence induce an inflammatory transcriptome regardless of the senescence inducing stress (coloured dots represent various SASP factors). Red and green connectors indicate ‘senescence-promoting’ and ‘senescence-preventing’ activities, respectively, and their thickness represents their relative importance. The dashed green connector denotes a ‘senescence-reversing’ mechanism. (Deursen 2014)

3. Markers of Senescence

Lessons acquired from cell culture experiments have identified senescent cells to labeled under the category of adherent class of cells and exhibits distinct morphology pattern- flattening, vacuolization and accumulation of stress granules (Kuilman et al., 2010; Rodier and Campisi 2011; Campisi 2013). Increases in cell size relative respective to proliferating cells in culture may reflect a continuation of anabolic processes that have refuted to be ceased, such as protein and membrane synthesis, in these cells that have decided to undergo permanent exit from the cell cycle. Senescent cells routinely express senescence-associated β-galactosidase (SAβ-gal) and p16INK4A, and with exception of p53 expressing cells, secrete proinflammatory cytokines including interleukin-1 (IL-1), IL-6, IL-8, vascular endothelial growth factor A (VEGFA) and certain invasive EMT mediators like matrix metalloproteinases (MMPs) and these components constitutes the core of Senescence Messaging Secretome (SMS) and phenotype of cells exhibiting such property and commonly referred to as Senecence Associated Secretory Phenotype (SASP) (Fig.3). (Kuilman et al., 2010; Rodier and Campisi 2011; Campisi 2013; Coppe et al., 2008; Rodier et al., 2009). Certain human OIS cells exhibit an atypical feature of heterochromatin patterning that is present in discrete nuclear subdomains, commonly referred to as senescence-associated heterochromatic foci (SAHFs) and are generally found to be associated with S-phase-promoting gene loci, such as E2F target genes (Narita et al., 2003). Additionally, a prominent feature of many senescent cells is an chronic and persistent DNA-damage response (Bartkova et al., 2006; Di Micco et al., 2006; Rodier et

al., 2011; Fumagalli et al., 2014). Although many of the features described above are widely but not uniformly observed in cultured senescent cells, numerous problems arise when trying to use them to describe senescence in living animals. (Sherr and Sharpless 2015).

a. Acidic β-galactosidase. Owing to the simplicity of the assay and detection of it in tissues, SAβ-gal activity has been one of the most familiarly used senescence signature. It is measured at pH 6.0 with the artificial substrate X-gal (Dimri et al., 1995; Itahana, Campisi and Dimri 2007). Endogenous β-galactosidase (encoded by the *GLB1* gene) in humans is a lysosomal enzyme with optimal activity displayed at pH 4.0–4.5, so its detection at suboptimal pH 6.0 connotes its very high level of expression in senescent cells (Kurz et al., 2000; Lee et al., 2006). Gangliosides, keratin sulfate and various glycoproteins have been the canonical β-galactosidase substrates.

b. p16INK4A. From the *in-vivo* data one can opine that p16 has been one of the cliché biomarker for senescence. P16 inhibits cyclin D-dependent CDK4 and CDK6 (Serrano, Hannon and Beach 1993). *CDKN2A* that codes for p16 is located at a close proximity with *CDKN2B*, which encodes a second INK4 family member, p15INK4B (Serrano and Beach 1994). Interestingly although the transcriptional machinery efficiently integrates exons 2 and 3 of the *CDKN2A,* thereby generating distinct transcripts originating from another upstream promoter and exon, however their coding sequences gets translated in an alternative reading frame (ARF) (Quelle et al., 1995) resulting in the generation of p14ARF in and p19ARF protein in human and mouse respectively

(Fig.2), a potent tumour suppressor that acts upstream of p53 (Serrano et al., 1997; Pomerantz et al., 1998; Zhang et al., 1998; Kamijo et al., 1998). This information underlines the fact that both RB and p53 are regulated by products of the *CDKN2A* locus: p16INK4A and ARF, respectively. However there seems to be a division of labour between these two tumour suppressors in the context of cellular senescence. For example, p19ARF expression serves as a critical hallmark of mouse embryonic fibroblast exhibiting replicative senescence, whereas p16INK4A is a key regulation of in-vitro senescence and especially Oncogene Induced Senescence in human cells (Chandler and Peter 2013). It is important to point out that in malignant human tumours, deletion and silencing of the entire *CDKN2A–CDKN2B* locus and mutations inactivating p16INK4A are among the most frequent genetic events (Bignell et al 2010; Beroukhim et al., 2010), implying that, inactivation of *RB1* and *TP53* mediated via concurrent loss of *CDKN2A–CDKN2B* arms the cells to bypass tumour-suppressive restraints that are imposed by senescence. Moreover elevated p16 levels have often been found to be associated to wound healing responses or clastogen exposure that involves imminent inflammatory response (Burd et al., 2013; Waaijer et al., 2012; Sorrentino et al., 2014; Natarajan et al., 2003; Jun and Lao 2010). The transient p16INK4A expression followed by the disappearance of p16INK4A-positive cells during healing and tissue repair raises the question of whether these cells were senescent and eliminated (for example, through clearance by the immune system) or whether non-senescent immune cells entering a wound are a source of p16INK4A expression, or both (Sherr and Sharpless 2015). In regard to the study conducted by Sharpless cnd Colleagues that documents elevated p16 expression in the

Tumour Associated macrophages (TAMs) that accumulates in the implanted tumours fails to didn't resolve a similar interim question whether these are genuine senescent tumour cells or merely expressing p16INK4A as consequence of acute stress response that generally are characterized by inflammation and macrophage differentiation alike to above mentioned example of p16INK4A activation with wounding (Demaria et al., 2014; Burd et al., 2013; Natarajan et al., 2003; Jun and Lau 2010; Jun and Lau 2010).

Concomitant elevation in p16INK4A expression levels has been detected in lymphocytes upon aging. Abundant p16INK4A expression in peripheral blood T cells from aged humans and mice (Lemster et al 2008; Liu et al., 2009; Liu et al., 2011) is associated with a marked decline in their proliferative capacity, a defect that can, in part, be rescued through T cell-specific *p16* inactivation (Liu et al., 2011; Migliaccio et al., 2005) which sheds considerable light on exacerbating effect of growth arrested T-cells that plausibly cripples its from clearing senescent thereby encouraging the prolong secretion of proinflammatory cytokines that fits with emerging model of "inflamaging" besides the fact that these hyporeplicative T-cell neither displays SAHF nor SASP, two of the commonly used hallmarks of senescence, especially in the in-vitro context. In the context of p16INK4A-expressing lymphocytes there appears to be lot contradicting opinion regarding whether these hyporeplicative lymphocytes are indeed senescent, or simply depicts a functionally perturbed cell state (Lemster et al., 2008; Migliaccio et al., 2005; Jaruga et al., 2000; Shi et al., 2014; Di Mitri et al., 2011; Liu and Sharpless 2009; Akbar and Henson 2011) while some argued that they these cells enters a sort of hibernation and feigns the state durable growth arrest as they still harness the capability of entering the

cell cycle (Jaruga et al., 2000; Shi et al 2014).

However certain *in-vivo* situations has evoked greater concerns with p16INK4A being readily detected in non-senescent cells: for example, it is expressed at particularly high levels in cells with inactivated RB (Aird et al., 2013; Serrano, Hannon and Beach 1993; Shapiro et al., 1995) including many cancer cells (Shapiro et al., 1995; Khleif et al., 1996; Nakao et al., 1997; Witkiewicz et al., 2011). Hence, even with clinching evidence of p16INK4A expression, the growth arrest can still be blunted by mutations that inactivate downstream effectors of the senescence programme such as RB. This is consistent with the above cited work from Lena Gurdon's laboratory that demonstrated using large T-antigen that senescent cells fails to make phosphorylate the Rb. Prima facie of such situations the matter has been further complicated by the unavailability of good p16 antibody for immunohistochemistry to facilitate its detection and distribution in mice on varied settings of senescence.

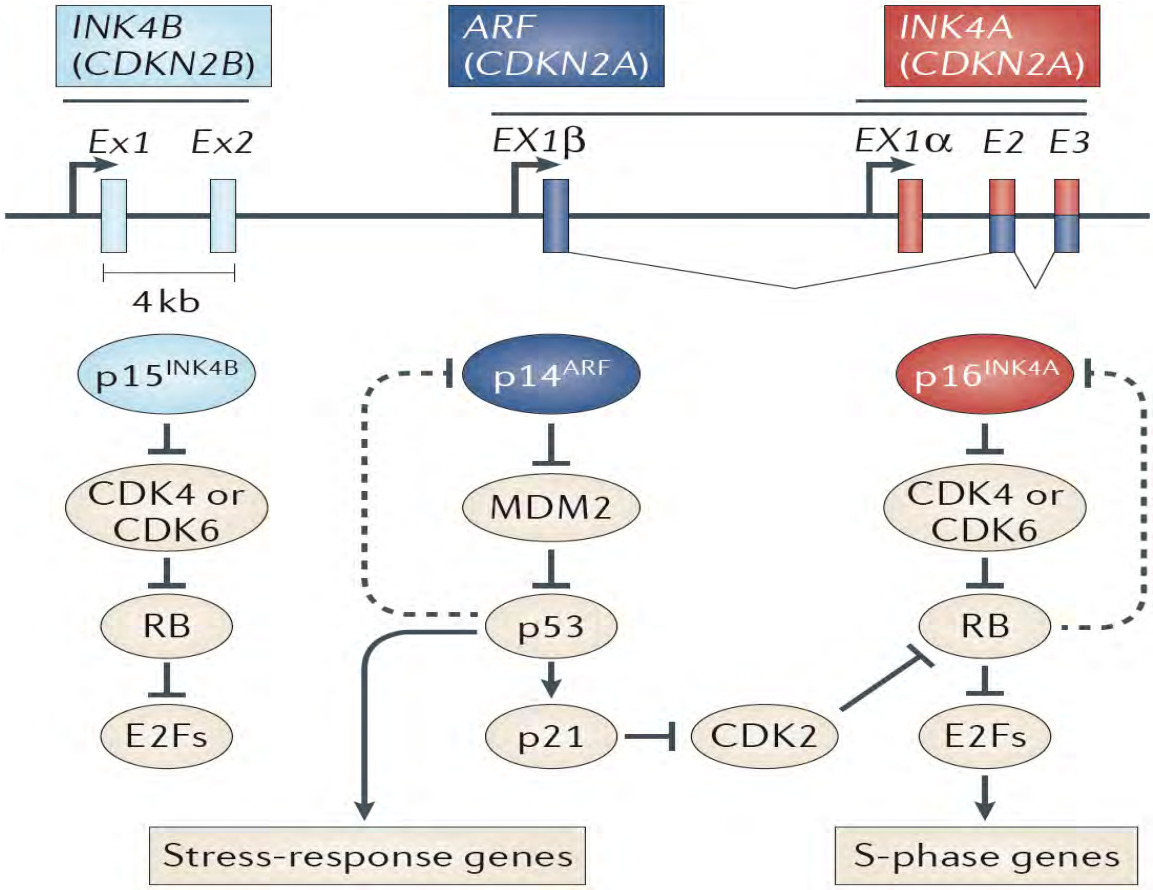

Fig.2 The *CDKN2A–CDKN2B* locus. The cyclin-dependent kinase inhibitor 2A (*CDKN2A*)–*CDKN2B* locus, which is less than 50 kb in overall length, encodes three tumour-suppressor proteins. Exons within the locus are indicated by coloured vertical bars, and the three promoters are indicated by arrows. The *CDKN2B* gene (which encodes p15INK4B) is specified by two exons (light blue). The *CDKN2A* gene encodes both p14ARF (p19ARF in mice; three dark blue exons) and p16INK4A (three red exons). RNAs transcribed from alternative first exons (designated Ex1β for *ARF* and Ex1α for *INK4A*) are spliced to mRNA sequences encoded by exons 2 and 3 of the *INK4A* gene, thereby generating two transcripts that are translated in alternative reading frames. The p16INK4A and p15INK4B proteins inhibit cyclin D-dependent CDK4 and CDK6 to prevent phosphorylation of the retinoblastoma protein (RB). The hypophosphorylated form of RB sequesters E2F transcription factors, preventing them from coordinately activating a suite of genes that are required for DNA replication (as shown for *INK4A* only). The ARF protein binds to the MDM2 E3 ubiquitin ligase to prevent p53 polyubiquitylation and to facilitate p53 activation. In turn, the p53 transcription factor regulates an extensive group of genes that are commonly induced by cellular stress. These include the CDK2 inhibitor p21CIP1, which inhibits CDK2-mediated RB phosphorylation during progression through the G1 phase of the cell division cycle. Inactivation of p53 leads to *ARF* induction, whereas inactivation of RB induces *INK4A* expression. These negative feedback loops are depicted by dashed lines. Silencing of the *CDKN2A– CDKN2B* locus in stem cells, or its frequent deletion in cancer cells, abrogates the tumour-suppressive functions of RB and p53 to facilitate cellular self-renewal. (Sharpless and Sherr 2015)

c. DNA damage foci and typical chromatin alterations: One of the markers that almost invariable correlate with senescence induction is DNA damage. DNA damage and these type of senescent are generally classified as DNA damage dependent senescence that can be driven by errors erupting from DNA replication during S phase, cell intrinsic factors like ROS (Reactive Oxygen Species) and natural extrinsic genotoxic insults such as UV or ionizing radiations, and therapeutically upon exposure to chemotherapeutic drugs. DNA-damage foci that are characterized by phosphorylated γH2AX appearance or activation of 53BP1, are generally detected at dysfunctional telomere of replicating senescent cells (d'Adda di Fagagna et al., 2003; Takai et al., 2003) indicating that replicative senescence to a large extent are driven by a coordinated role of telomeric attrition and its damage. In precancerous lesions, replicative errors or high ROS levels can drive DNA damage and in turn elicit cellular response by activating p53 and p21CIP1 that can either precipitate as apoptosis or senescence (Halazonetis, Gorgoulis and Bartek 2008) depending on the cell type and veracity of the lesion. However it should be noted that such foci have been detected at other sites than that of telomere that also culminates to activation of ataxia telangiectasia mutated (ATM)–p53–p21CIP1 signalling cascade of inducing effective growth arrest (Bartkova et al., 2006; Di Micco et al., 2006; Rodier et al., 2011; Fumagalli et al., 2014; Nakamura et al., 2008). However it has been shown that components that can have serious consequence on the integrity of chromatin structure like chromatin remodelers or histone modifiers like histone deacetylase inhibitors can also can also activate DNA damage signaling cascade besides p16INK4A and SAβ-gal without any detectable telomere dysfunction or overt DNA damage

(Bakkenist and Kastan 2003; Munro et al., 2004).

Altered nuclear phenotype and consequently variability in the gene expression pattern patterns are often observed in cells undergoing senescence besides down-regulation of cell cycle genes, up-regulation of senescence marker genes, and senescence-associated alteration of the secretome. Thus, it is possible that senescence is under the constraints of some unique chromatin modification that beholds the gene regulatory mechanisms. For example, p16, a functional biomarker of senescence is negatively regulated by histone H3 Lys 27 trimethylation (H3K27me3) and its docking proteins of the polycomb group (Jacobs et al., 1999), which can be countered by JMJD3, the H3K27 demethylase (Agger et al., 2009).

The tumor suppressor promyelocytic leukemia (PML), a component of PML nuclear bodies (PML NBs, also known as promyelocytic oncogenic domains [PODs]), is a marker of senescence: During senescence, PML is up-regulated, the size and number of PML NBs are increased and PML contributes to senescence via upregulation of p53 and Rb (Ferbeyre et al., 2000; Pearson et al., 2000). It has also been shown that Rb is involved in silencing of E2F target genes during senescence through its physical association with AGO2 (a key component of RNA induced silencing complex [RISC]) and microRNA (e.g., let-7) (Benhamed et al., 2012).

To a staunch exception with mouse, human (Kosar et al., 2011; Di Micco et al 2011; Kennedy et al., 2010) senescent cells in in-vitro displays vivid 4,6-diamidino-2-phenylindole (DAPI)-stained foci that are detected to bear various hallmarks of constitutive heterochromatin such as H3K9me3 and binding of Heterochromatin Protein 1 and most critically HMGA1 to chromatin

(Narita et al., 2003) besides other factors that has less relevance in the light of heterochromatin constituency like E2F. Such structure has been termed as Senescence Associated Heterochromatic Foci (SAHF). Furthermore SAHF harbouring these repressive chromatin marks and are presumed to have formed during spatial repositioning of pre-existing heterochromatin (Chandra et al., 2012; Chandra and Narita 2013). However SAHFs per se may not serve as a senescence marker as certain cell types (like BJ) does not display SAHF owing to low p16 expression. Strikingly enrichment of H3K9me3 and HP1 at E2F have been documented in certain SAHF-independent settings of senescence. Translocation of HIRA (a histone chaperone) to PML (promyelocytic leukemia nuclear) bodies lays the foundation stone for the primary step of chromatin condensation (Zhang et al., 2005). These subnuclear organelles are thought to serve as a staging ground for the formation of HIRA/ASF1A-containing complexes, which are subsequently incorporated into chromatin and play an essential role in instigating SAHF formation. RNAi depletion of either ASF1A or HMGA1 (a SAHF component) leads to a partial bypass of senescence (Zhang et al., 2005; Narita et al., 2006).

Paradoxically depending on the experimental backdrop, interference in the expression of genes like p53, C/EBPb, or interleukin-6 (IL-6)—also reduces SAHF-positivity, however that consequently abrogates senescence (Kuilman et al 2008; Chan et al; 2005; Ye et al., 2007; Zhang et al., 2005). Moreover, these foci as discussed above serves to depict certain categories of senescence (like OIS), and they are limited to cell types expressing p16 and often can be dispensable for cellular senescence (Agger et al., 2009; Koser et al., 2011).

While SAHFs are characterized microscopically (Cecco et al., 2013), a study recently identified another type of senescence associated chromatin alteration using a biochemical approach: formaldehyde-assisted isolation of regulatory elements (FAIRE), a method for genome-wide mapping of open chromatin regions. They showed, in replicative senescent HDFs, an overall condensation of chromatin in euchromatic regions, with the exception of some specific genes. On the other hand, chromatin of repetitive sequences, including major classes of retrotransposon as well as pericentromeric regions, which are highly condensed in normal cells, tend to become open in senescent cells. The decondensation of these heterochromatic regions is associated with expression of transposable elements (such as LINE1 elements) and their transposition, particularly at later time points after the induction of senescence. They also showed the decondensation of pericentromeric heterochromatin in late senescent cells. It has been recently shown the loss of chromatin during senescence (Ivanov et al., 2013). They found that reduction of LMNB1 and loss of nuclear envelope integrity in senescent cells are associated with the appearance of cytoplasmic chromatin fragments (CCFs). These are targeted to autophagy for degradation, resulting in low histone content. A reduction of total histone content was seen within the deeper (or ‘‘mature’’) portions of nevi, a model for in vivo OIS (Ivanov et al., 2013). Thus, similar to the redistribution of the relative condensation of chromatin mentioned above, this reduction in histone content appears to be a late event during senescence. The physiological significance of these chromatin alterations that are associated with late or deep senescence remains to be elucidated, or they might be considered ‘‘degenerative’’ alterations as the investigators suggest (Cecco et al., 2013;

Ivanov et al., 2013). On the background of this context it is noteworthy to point out that In addition to autophagy, the second major protein degradative pathway—the proteasome—also has functional relevance to the phenotype. It was recently shown that aberrant Ras/ERK signaling leads to senescence-associated protein degradation (SAPD), whose targets include proteins required for cell cycle progression, cell migration, mitochondrial functions, RNA metabolism, and cell signaling (Deschеˆnes-Simard et al 2013). Inactivation of individual SAPD targets is sufficient for senescence induction. Thus, in addition to autophagic ‘‘bulk’’ degradation, ‘‘selective’’ proteasomal protein degradation also plays important roles in senescence.

d. The SASP Although incompetent with respect to cell division, senescent cells still secretes a plethora of factors that plays different role in cellular microenvironment that includes metalloproteinase that degrade the extracellular matrix (MMPs), immune modulators and proinflammatory cytokines where in nuclear factor-κB (NF-κB) has been shown to be a master regulators of these secretome (Chien et al. 2011). Collectively this phenotype was designated as the senescence-associated secretory phenotype (SASP) or these factors are collectively termed as senescence messaging secretome (SMS) (Campisi 2013; Collado, Blasco and Serrano 2007, Kim and Sharpless 2006). SASP have turned out to be a double-edged sword in respect to tumorigenesis as studies conducted in different laboratories showed that SASP can be both pro as well as anti-tumorigenic. Although counter-intuitive, initially around 10 years ago, a seminal work conducted in Campisi’s laboratory documented that these secreted factors from senescent cells can promote tumorigenesis,

where co-incubation of senescent fibroblast with breast cancers cells subcutaneously in nude mice, the breast cancer cells were found to grow more rapidly. The SASP factors have been shown to be the part of plethora of senescence associated scenarios including telomeric attrition mediated compromised replicative capabilities (Acosta et al., 2013), elicitation of immune system and its consequence on tissue morphology (Xue et al., 2007; Krizhanovsky et al., 2008; Lujambio et al., 2013) in wound healing responses (Xue et al., 2007; Krizhanovsky et al., 2008; Lujambio et al., 2013).

e. Telomere shortening and dysfunction. As discussed earlier every cells are evolutionary bestowed with a specified replicative signature true to the cell type in question and the inability to add telomeric repeats to chromosome ends beyond the contrained limit eventually leads to telomere deprotection and a DNA-damage response that limits cellular proliferative lifespan (Hayflick 1965; Todaro and Green 1963; Blackburn 1991; Lansdorp 2009; Artandi and DePinho 2010; Shay and Wright 2011). As a proof of concept, in turn, enforced expression of telomerase can bypass replicative senescence and maintain chromosomal integrity. Similarly, in mice that are engineered to undergo telomere attrition and exhibit premature ageing phenotypes (like G3 Terc-/- mice; Rudolph et al 1999), reactivation of telomerase can reverse degenerative traits (Jaskelioff et al., 2011). However at this junction it is important to point out that these do not readily exhibit signs of ageing until they are intercrossed for several generations to allow telomere attrition (Todaro and Green 1963; Rudolph et al., 1999) as multiple telomeric repeats acts as a savior by buffering the phenotype of reduced telomerase will only show com-

promised phenotype once the telomeric repeat reaches a critical threshold. Hence this observation makes it clear that telomeric attrition cannot be attributed to be the sole reason of replicative senescence atleast in mice, a phenotype principally found to be driven by p16 in mice and unlike as in human. Many pathophysiological features of genetic disorders that are commonly associated to dysfunctional telomere in human closely overlaps with certain aspects that are commonly encountered in early ageing, such as pulmonary fibrosis, bone marrow failure and cirrhosis (Lansdorp et al., 2009; Armanios et al., 2013), highlighting the relevance of studying telomeric structure and functionality to unravel to what extent cellular senescence phonotype overlaps to organismal ageing. However the most precise assays such as flow-cytometric fluorescence *in situ* hybridization, require assessment of telomere integrity of each individual chromosome, and therefore are technically cumbersome and require substantial numbers of viable cells can be a pitfall for experiments with limited cell number. Furthermore as with the expression of p16INK4A, telomere shortening and dysfunction can occur in non-senescent cells, and senescence can be triggered by many stresses that are independent of telomere shortening in human cells *in-vitro*.

f. The combination of Senescence markers

Given the shortcomings of isolated senescence biomarkers described above, as well as the fact that senescence can occur in response to diverse stimuli, act through different mechanisms and, seemingly, spread by intercellular signaling within stressed tissues, it has become increasingly clear that no single marker will faithfully represent senescence (Sherr and Sharpless 2015). Hence, most investigators agree that expression of a combination of generally used senescence markers should be used to define senescent cells *in vivo* (Sherr and Sharpless 2015). A well assumed suggestion has been to affirm cell cycle arrest and then superimpose “at least two additional senescence markers, the choice of which may vary for different settings” (Sherr and Depinho 2000). Going with the publication in the field of senescence the most overtly used combination relies on SAβ-gal and p16INK4A; however, other combinations of p16INK4A and SASP markers, have been used. However the filed have not reached to any consensus on the combination of markers that can qualify to ascertain senescent cells, however a recent review by Deursen underlined that test of irreversibility or the permanent exit to be the gold standard for identifying senescent cells (Deursen 2015).

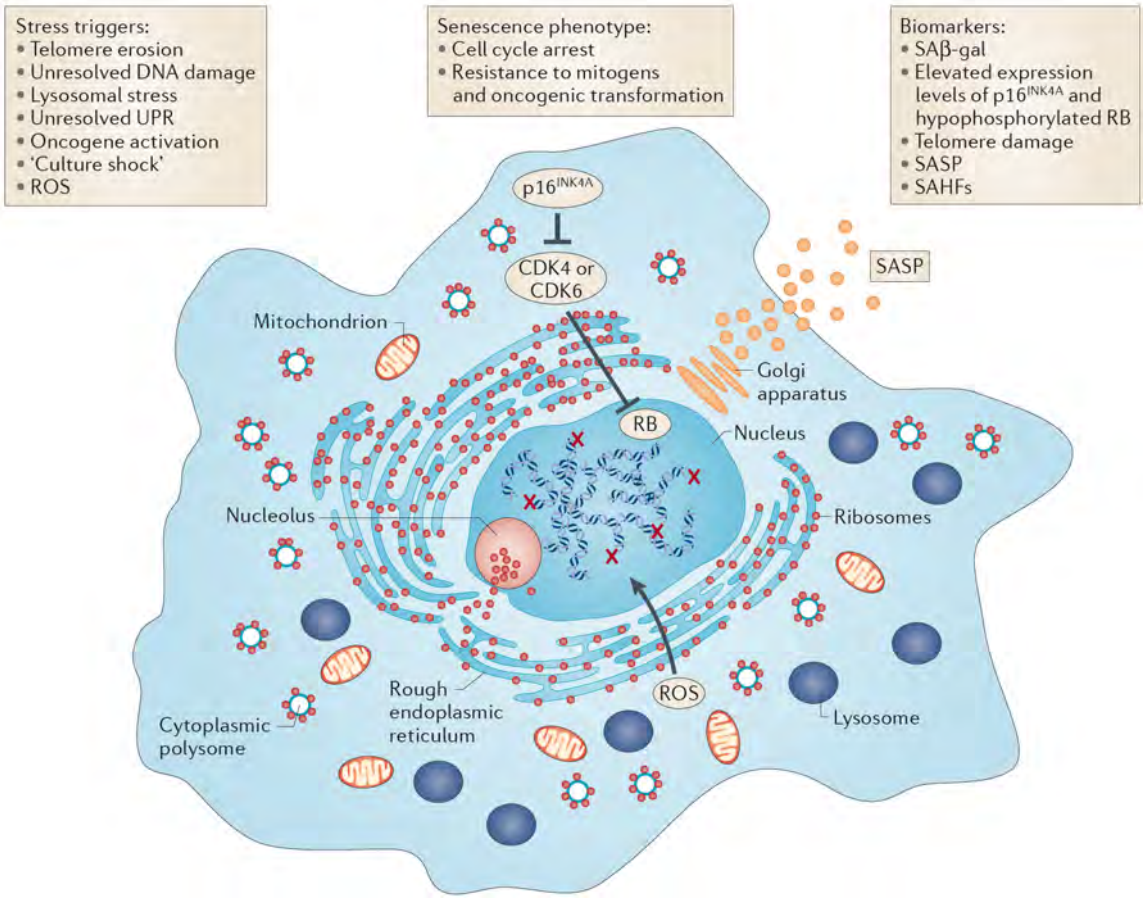

Fig.3 Stress triggers and biomarkers of senescence. We define permanent growth arrest in response to various cellular stresses as the *sine qua non* of cellular senescence. The senescent state is accompanied by a failure to re-enter the cell division cycle in response to mitogens and by an acquired resistance to oncogenic challenge. Many forms of cellular stress can trigger cellular senescence and lead to the expression of the most commonly used biomarkers. The schematic depicts different organelles, including: the nucleus, containing damaged chromatin (X); the nucleolus; rough endoplasmic reticulum and 'free' cytoplasmic polysomes, each with ribosomes; mitochondria with internal cristae producing reactive oxygen species (ROS); and abundant lysosomes expressing β-galactosidase. Vesicles emanating from the Golgi apparatus contain secreted cytokines and chemokines that can impinge on surrounding cells (known as the senescence-associated secretory phenotype (SASP)), leading to paracrine signalling. The cyclin-dependent kinase (CDK) inhibitor p16INK4A in the cytoplasm prevents CDK4 and CDK6 from assembling into functional holoenzymes with their allosteric regulators, the D-type cyclins (not shown); p16INK4A binding to CDKs prevents the import of active kinases into the nucleus and inhibits the phosphorylation of nuclear retinoblastoma protein (RB). SAβ-gal, senescence-associated β-galactosidase; SAHFs, senescence-associated heterochromatic foci; UPR, unfolded protein response. (Sherr and Sharpless 2015)

4. Various conditions for Senescence Induction

Work from various laboratories have lead us to realize that cellular senescence is essentially as stress response that can occur in various conditions like Replicative Senescence can be induced as a consequence of telomeric attrition whereas Oncogene Induced Senescence has evolved as an essential fail safe mechanism of cell that counteracts tumorigenesis at the onslaught of oncogenic challenges.

a. Replicative Senescence in-vitro

Preliminary observations of a study that engaged explanation of primary cells from human tissue was that such cells exhibit a predefined finite proliferative limit and hence are ‘‘mortal.’’ In fact, their proliferative capacity upon explanation consistently displays three phases: phase I, corresponding to a period of little proliferation before the first passage, during which the culture establishes; phase II, characterized by rapid cell proliferation; and phase III, during which proliferation gradually grinds to a complete halt (Hayflick and Moorhead 1961). Commenting on the possible causes of the transition to phase III, Hayflick hypothesized that ‘‘The finite lifetime of diploid cell strains *in-vitro* may be an expression of aging or senescence at the cellular level” (Hayflick 1965). The term cellular senescence therefore denotes a phase of exhaustive proliferative capacity, despite continued viability and metabolic activity. Consistent with Hayflick’s proposal, we now know that, with the propagation of human cells in culture, telomeres (the protective chromosomal termini) are progressively shortened, ultimately causing cells to reach their ‘‘Hayflick limit.’’ This

barrier has been termed replicative (cellular) senescence, since it is brought about by saturated replicative capacity. Telomeric attrition attributes to failure of DNA polymerase to completely replicate the lagging strands. Thus, telomeres act as a molecular clock, reflecting the replicative history of a primary cell (Harley et al., 1990). When telomeres reach a critical minimal length, their protective structure is disrupted. This triggers a DNA damage response (DDR), which is associated with the appearance of foci that stain positive for γH2AX (a phosphorylated form of the histone variant H2A.X) and the DDR proteins 53BP1, NBS1, and MDC1. Moreover, the DNA damage kinases ATM and ATR are activated in senescent cells (D'Adda di Fagagna 2008). Following persistent DDR, these kinases activate CHK1 and CHK2 kinases. Communication between DDR-associated factors and the cell cycle machinery is brought about by phosphorylation and activation of several cell cycle proteins, including CDC25 (a family of phosphatases) and p53. Furthermore, differential expression of p53 isoforms has been linked to replicative senescence (Fujita et al., 2009). Together, these changes can induce a transient proliferation arrest, allowing cells to repair their damage. However, if the DNA damage exceeds a certain threshold, cells are destined to undergo either apoptosis or senescence and this decision differs with cell types. The factors bringing about this differential outcome have remained largely elusive, but the cell type and the intensity and duration of the signal, as well as the nature of the damage, are likely to be important determinants (D'Adda di Fagagna 2008). In addition to p53, prolonged replicative arrest also known to activate RB tumor suppressor and its signaling partners, including p16INK4A (a cyclin-dependent kinase inhibitor acting upstream of RB). Indeed, activation of both

the p53 and p16INK4A–RB pathways is essential for induction of senescence in a variety of human cell strains (Ben-Porath and Weinberg 2005). The relative contribution of these cascades to senescence depends on the cell strain: While some are significantly delayed in their onset of senescence upon inactivation of p16INK4A alone, others require a deficiency in p53 or in p53 as well as p16INK4A for the abrogation of senescence. However, such escape offers futile advantage as it predisposes the cell to the onslaught of a tougher quality control machinery known as telomeric crisis, resulting in chromosomal instability and death (Shay and Wright 2005).

The dependence of replicative senescence on telomere shortening is evident from its bypass by the ectopic expression of the catalytic subunit of the telomerase holoenzyme (hTERT), which elongates telomeres, thereby abrogating the effect of the end replication problem (Bodnar et al., 1998). The limited life span of most primary human cells is explained by the fact that, in contrast to stem cells, telomerase is not expressed in human somatic cells, so they are unable to maintain telomeres at a sufficient length to circumvent a DDR (Harley et al 1990). Therefore, the ectopic expression of hTERT is a common practice *in-vitro*, allowing for the immortalization of primary human cells. Likewise, tumor cells often express telomerase (Shay and Bacchetti 1997), or elongate their telomeres through a mechanism termed alternative lengthening of telomeres (ALT) (Muntoni and Reddel 2005).

b. Oncogene Induced Senescence (OIS) in vitro

Early break-through studies on mutant HRAS (HRASV12) led the startling revelations that, although it can immortally transform most mammalian cell lines and collaborate with immortalizing genes in oncogenically transforming primary cells, it induces p53 as well as p16 mediated cell cycle arrest when it is introduced alone into primary cells (and at least in one immortal rat fibroblast cell line) (Serrano et al., 1997), thereby highlighting the in-built fail-safe mechanism of a cell at work to conceal tumorigenesis. This study noted the striking phenotypic resemblance of such non proliferating cells to those in replicative senescence, and this phenomenon has eventually come to be known as OIS. Unlike replicative senescence, OIS cannot be bypassed by expression of hTERT, confirming its independence from telomere attrition, however a recent study has indicated that oncogenes such as Ras can induce telomere dysfunction, including telomere attrition in primary fibroblasts, and that OIS is not stable in cells with high telomerase activity (Suram et al., 2012). One of the hallmark features shared by cells undergoing replicative senescence and OIS is the critical involvement of the p53 and p16INK4A–RB pathways, at least in certain settings. In murine cells, functional inactivation of p53 or its direct upstream regulator, p19ARF, is sufficient to bypass RASV12-induced senescence (Serrano et al., 1997; Kamijo et al., 1997). In human cells, p16INK4A seems to play a more prominent role than p53, as some cells depend solely on p16INK4A for OIS (Ben-Porath and Weinberg 2005). Owing to such specific tumour suppressor dependency, the ease of inducing OIS differs with cell types. It is important to point out that OIS induced through BRAF can

refute the necessity of such tumour suppressor activity and can mediate OIS through metabolic pathways, such as the activation of Pyruvate Dehydrogenase. Moreover OIS mechanisms do not seem to be universal across cell types and genetic contexts. The signaling routes relaying OIS by RASV12 versus BRAFE600 also exemplify this: Whereas RASV12-induced senescence can be bypassed by abrogation of the p16INK4A–RB pathway (Serrano et al., 1997), BRAFE600-triggered senescence cannot be bypassed by functional inactivation of p16INK4A (Michaloglou et al., 2005). In a support for existence senescence independent of p53 module and DNA damage signaling, a very elegant study has been shown that depletion of S phase kinase-associated protein 2 (Skp2) can suppress tumorigenesis through Arf-p53 independent induction of cellular senescence (Lin et al., 2010). In the light of Ras induced OIS, it is interesting to note that a study has lead us to reckon on how ROS mediates ERK induction of p38, a component generally found to associated with chronic inflammation through TLR signaling, in mediating OIS response (Dolado et al., 2007). In the similar lines another study have highlighted the critical role played by the p38, Tip60 and PRAK signaling cascade in mediating Ras induced OIS, wherein the activation of PRAK through acetylation of Tip60 depends on the p38 mediated phosphorylation of Tip60 (Zheng et al., 2013), although potential role of Tip60 as tumour suppressor by mediating the oncogene induced DNA damage response had been shown earlier (Gorrini et al., 2007). Recent evidence suggests the relevance of OIS also in the context of induced pluripotency *in-vitro*. As the INK4A/ARF proteins and p53 limit the efficiency of iPS cell formation, it has been suggested that cellular senescence counteracts the induced conversion of primary cells into pluripotent

stem cells (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009; Zhao et al., 2013). Alternatively, increased proliferation rates associated with p53 loss may result in accelerated kinetics of iPSC formation (Hanna et al., 2009). If extrapolated to in vivo setting, one could imagine that cancer stem cells arise from a similar reprogramming process (Krizhanovsky and Lowe 2009) preferably in aging tissue where the status of p53 are largely blunted with the passage of time. This observation indicates that cellular senescence might suppress tumor formation not only by inducing a persistent cell cycle arrest, but also plausibly by limiting the generation of cancer stem cells.

c. Tumor suppressor loss mediated Senescence in vitro

Similar to oncogene mutation or overexpression, loss of a tumor suppressor can also trigger senescence. This was demonstrated in-vitro using human primary fibroblast through loss of NF1, a protein that converts RAS to its inactive GDP-bound form thereby mediating activation of oncogenic RAS. A strength of this approach is that it does not rely on the ectopic expression of RAS. In the setting of reduced NF1, RAS activation produced a marked, but transient, activation of the downstream ERK and AKT pathways followed by a profound shutdown of this signaling temporally accompanied by the appearance of senescence. This global abrogation of ERK and PI3K signaling is associated with the activation of a broad transcriptional program of negative regulators of the RAS pathway, including Sproutys, RasGAPs, and

DUSP6/MKP3 (an ERK phosphatase). The authors observed a similar response following ectopic expression of mutant RAS or of mutant B-RAF, a downstream target of RAS that activates ERK MAPK signaling, showing that these effects were not specific to NF1 deletion (Fig.4). Additionally, the authors confirmed many of their findings using small-molecule inhibitors of ERK or PI3K signaling. Curiously, the authors also show that abnormal activation of PI3K signaling also leads to senescence, a result suggesting that different circuitries mediate OIS depending on the inciting oncogene (Courtois-Cox et al., 2006).

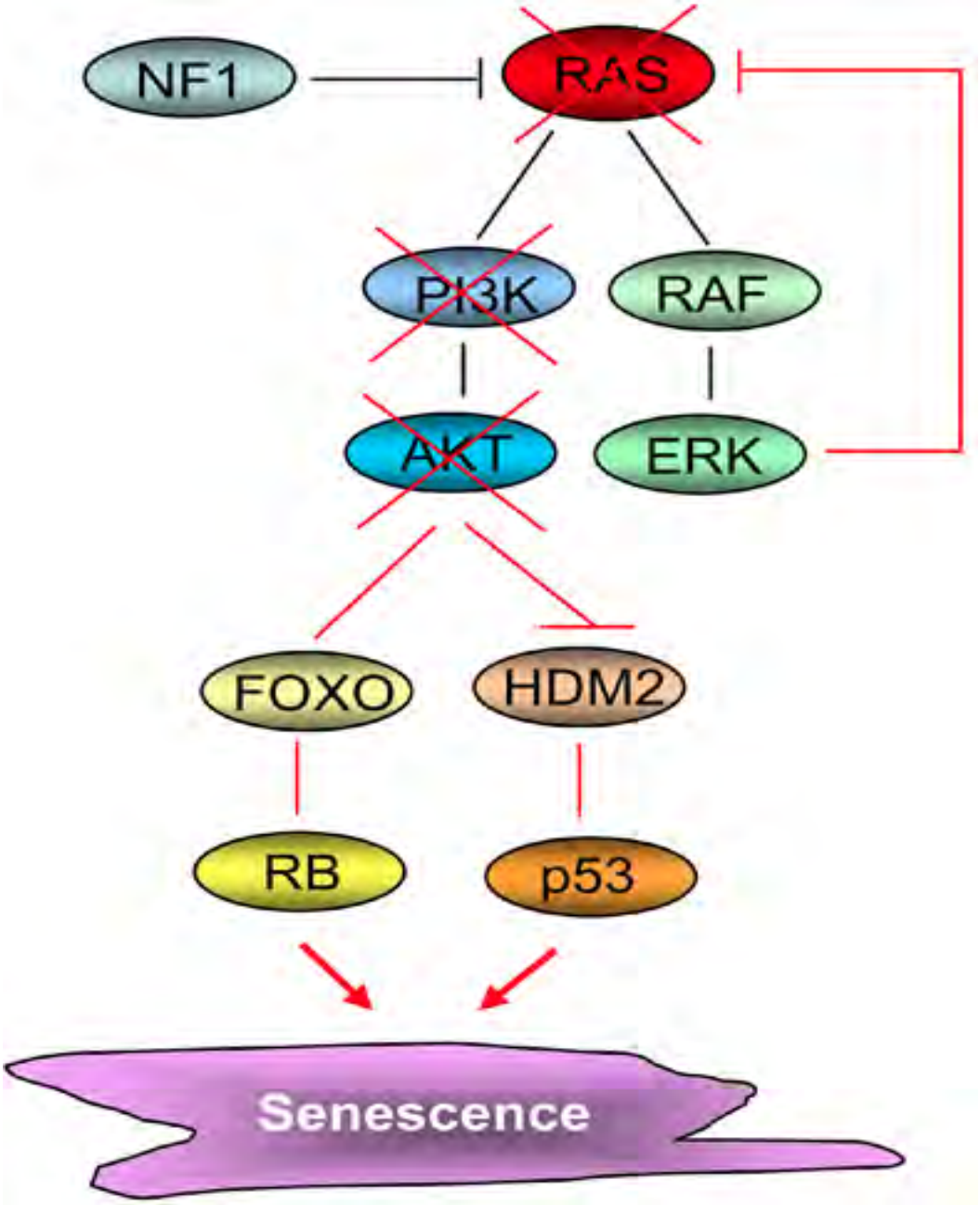

Fig.4 A model of demonstrating loss of tumour suppressor mediated OIS.
Activated RAS leads to growth arrest (mediated by loss of function mutation of a tumour suppressor NF1) as a result of potent negative feedback that abrogates ERK and PI3K signaling (Adopted from Bardeesy and Sharpless 2006).

d. Other Inducers of Senescence in-vitro

Besides these canonical mediators, tampering the epigenetic landscape can also deploy cellular senescence. To this end, work from Livingston's group has shown that shRNA mediated depletion of p400, chaperon for the histone variant H2A.Z, drives a p53-p21 dependent prematured senescence response, with loss of p53 or p21 was found to rescue the phenotype to normalcy (Chan et al., 2005). In the similar lines, it has also been shown that H2A.Z suppresses p21 dependent elicitation of senescence response as depletion of H2A.Z was found to enhance the p21 mediated senescence induction in manner similar to its chaperon p400 (Gevry et al., 2007), highlighting H2A.Z as a regulator of p21 expression. As p21 is known to play a capital role in mediating replicative senescence, it remain unknown whether the senescence mediated through depletion of p400 or H2A.Z relates to replicative senescence. Encouragingly, from the perspective of Therapy Induced Senescence (TIS), various epigenetic drugs were found to drive proficient senescence response in varied settings of solid tumours. For example DU145 and LNCaP cancer cells cultured continuously in 400 nM 5-azacytidine, a drug makes the cell inhibitory to DNA methylation become senescent within 7 days (Schwarze et al., 2005), whereas MCF7 breast and H1299 lung cancer cells triggers a senescent-like growth arrest owing to attenuated Ras signaling within 10 days after a 24-hour exposure to 100 nM Sirtinol, a class III HDAC inhibitor (Ota et al., 2006) by counteracting the effects of Sirtuin 1 (SIRT1). It needs to be emphasized that the epigenetic facilitators of senescence are not limited to the above-mentioned examples.

e. Replicative Senescence in vivo

Studies showing elevated SAb- GAL activity in the skin of elderly people (Herbig et al., 2006), have lead to a general opinion that senescent cells accumulates in aging tissues. In addition, a small but significant decrease in telomere length has been detected in aged tissue, although this has been observed in certain cell types. These studies, which relied on single senescence markers, were later backed by studies showing that several DNA damage and heterochromatinization markers—including 53BP1, γH2AX, phospho-Ser 1981-ATM, HP-1b, and HIRA—are increased in dermal fibroblasts from aging baboons when compared to the younger counterpart. DNA damage markers were shown to co-localize with telomeres, and this correlated with increased p16INK4A expression (Herbig et al., 2006), suggesting that dysfunctional telomeres can create DNA damage signals leading to the onset of senescence also in vivo. Besides, recent data indicates that end of chromosome are refractory to the DNA repair machinery leading to the elicitation of persistent DDR response and the eventual precipitation of senescence. Conversely, beneficial aspects of replicative senescence have also been unraveled, including some that are unrelated to cancer. In a mouse model of liver fibrosis, senescent cells were detected, as evidenced by positivity for SA-b-GAL, p16INK4A, p53, p21CIP1, and HMGA1 (Kuilman et al., 2013). These senescent cells are found to be derived activated hepatocellular stem cells and thus limit the progression of fibrosis (Krizhanovsky et al 2008). In addition, two reports (Cosme-Blanco et al., 2007; Felder and Greider 2007) have provided evidence backing the proposal by Hayflick (Hayflick 1965) that replicative se-

nescence limits tumorigenesis. Both studies report that mice lacking the RNA component of telomerase (mTR) display decreased tumor formation in the context of either one or two copies of mutant p53R172P replacing endogenous p53 (Cosme-Blanco et al., 2007) or Em-MYC/ BCL2-driven lymphomagenesis (Krizhanovsky et al 2008). The lack of telomerase activity had been shown previously to result in an induction of apoptosis, but, in these two models, this response was abrogated owing to expression of the p53 mutant or BCL2, respectively. These studies show that cells display extensive induction of senescence in intestine, kidney, and spleen (Cosme-Blanco et al., 2007), or in microlymphomas (Feldser and Greider 2007). This has provided the first direct evidence of replicative senescence preventing tumorigenesis, at least in the context of an apoptosis block. Telomere shortening or dysfunction initially triggers a robust stress response that, through the induction of cyclin dependent kinase inhibitors that impedes the replicative potential of cells. As such, this aspect of telomere biology is likely to act as a potent anti-cancer mechanism. This is consistent with a significant weakness in dealing with the burden of tumorigensis on the backdrop of deficient telomeric activity (mTR-/) when coupled with INK4a/Arf loss (Greenberg et al., 1999). In the absence of p53, however, dysfunctional telomere instead promotes tumorigenesis (Chin et al., 1999). This could occur through the inability of cells to activate a full-throttled DDR, and through the perturbation of the apoptosis and senescence arms (Kuilman et al., 2013).

f. OIS in vivo

The past decade have witnessed an overwhelming number of studies demonstrating senescence in both murine and human lesions *in-vivo*. Senescence markers have been demonstrated in several contexts in which oncogenes or tumor suppressor genes were perturbed (Fig. 1). Schmitt and colleagues (Braig et al., 2005) reported on the tumor-suppressive role of the chromatin-remodeling enzyme Suv39h1 in NRAS-driven lymphomagenesis, which correlates with bypass of OIS *in-vitro* in Suv39h1$^{-/-}$ splenocytes. Serrano and coworker (Collado, Blasco and Serrano 2007) showed that conditional expression of KRASV12 from its endogenous locus in mice results in the emergence of lung adenomas, as well as premalignant pancreatic intraductal neoplasia, most of which fail to progress to malignancy. The adenomatous stage is specifically found to be characterized by a low proliferative index and decorated by emergence of several senescence markers, including SA-b-GAL, p15INK4B, p16INK4A, and several new markers. Peeper and coworkers (Michaloglou et al., 2005) showed that congenital human melanocytic nevi, which frequently harbor activating BRAF or NRAS mutations, display several characteristics of senescence. In addition, murine papillomas driven by HRASV12 expression or induced upon DMBA/TPA treatment express several senescence markers (Collado, Blasco and Serrano 2007; Chen et al., 2005). Recent evidence indicates that Myc, known mainly for its proapoptotic function, drives a subset of murine lymphomas into senescence through stromal secretion of TGFb (Reimann et al 2010), providing support for the premise that senescence in the *in-vivo* settings is not solely implemented in a cell au-

tonomous manner. Moreover, expression of several senescence-associated genes is reduced when tumors progress beyond the senescent stage (Collado, Blasco and Serrano 2007; Chen et al., 2005; Lazzerini Denchi et al., 2005). However, particularly for cell cycle-regulating genes like p16INK4A, abundant expression may, in fact, be maintained during malignancy, which is explained either by the occurrence of other (epi)genetic aberrations including events occurring in parallel and downstream like blunted RB expression. In link with TIS, it is important to point out that application of DNA Methylation inhibitors (DNMTi) in the settings of Acute Leukemia or myelodysplastic syndrome have lead to encouraging outcomes (Griffiths et al., 2013).

g. OIS in the context of nevus and melanoma formation

Melanocytic nevi constitutes a category of benign tumors that depicts a continence against progression toward melanoma thereby offering a favorable *in-vivo* settings for investigating OIS in humans, mice, and fish. In 2002, $BRAF^{V599E}$ was identified as a frequent mutation in human cancer, predominantly in melanoma (Davies et al., 2002). However interestingly in the following year a study published that the same mutation is present in the large majority of nevi (Pollock et al 2003). Hence in spite of the presence of an oncogenic BRAF (or, in some cases, NRAS) allele, an important and common feature of nevi is their exceedingly low proliferative activity. This characteristic is typically maintained for decades until the lesion gradually disappears. Nevi express elevated levels of p16INK4A and display increased SA-b-GAL activity

(Michaloglou et al 2005). Arguing against a role for replicative senescence, it was found that telomere length in nevi is indistinguishable from that in normal skin. This strongly suggests that nevi undergo OIS in vivo. Secreted IGFBP7 has been reported to play a central role in the initiation and maintenance of the senescent state of nevus cells (Wajapeyee et al., 2008) however the veracity of this data was challenged in a successive study. Melanoma formation was strongly accelerated in Cdkn2a or p53- deficient backgrounds. Recently, two advanced mouse models have been created that more closely resemble spontaneous mutation of the oncogene by conditionally expressing $Braf^{V600E}$ from its endogenous promoter (Dankort et al., 2009; Dhomen et al 2009). In these models, specific expression of mutant BRAF in the melanocytic compartment results in the formation of nevus-like benign lesions, which, depending on the model used, remain stable for several months to more than a year. Importantly, these melanocytic cell groups express several senescence markers, corroborating earlier observations on RAS^{G12V} that physiological expression levels of an activated oncogene can also give rise to senescence in neoplasms. In line with the idea that p16INK4A is not always strictly required for OIS in vivo (Michaloglou et al 2005) nevus formation was unimpaired in a p16INK4A-deficient background, although increased melanoma penetrance and reduced latency were observed (Dhomen et al 2009). In contrast, in combination with a mutant Braf knock-in allele, loss of Pten produced aggressive tumors much resembling human metastatic melanoma (Dankort et al., 2009). These elegant models have convincingly shown that mutation of BRAF can drive nevus formation, and that a specific secondary lesion (e.g., Pten loss) collaborates to drive fulminant melanoma.

h. Tumor suppressor loss mediated senescence in vivo

The first demonstration of tumor suppressor (PTEN) loss-induced senescence in vivo was demonstrated from the work of Pandolfi's lab (Chen et al., 2005). Exploring the evolution of prostate cancer, they discovered senescence in early-stage prostate abnormalities in humans and in mice engineered to sustain prostate-specific deletion of the PTEN tumour-suppressor gene. However, in contrast to the situation in melanocytes, prostate OIS is dependent on p53, and co-deletion of PTEN and p53 cancelled senescence, promoting full blown prostate cancer. In the similar lines, work from the same lab showed that Pten-loss Induced Cellular senescence (PICS) could be exploited as a pro-senescence therapeutic intervention (Fig.5) (Alimonti et al., 2010).

As mentioned earlier, loss of NF1 has been demonstrated to be associated to OIS even in *in-vivo* settings in the study (Courtois-Cox et al., 2006). To this end, the authors took advantage of tumors from human patients with neurofibromatosis type I, an autosomal dominant syndrome that results from inheritance of a single mutant allele of nf1. In such patients, loss of NF1 potentiates RAS activation leading to neurofibromatosis (Fig.4). However, such tumors were detected to nurture low malignant potential. Correspondingly, the authors show that tumors from neurofibromatosis type I patients exhibit features of senescence (e.g., increased p16INK4A expression and senescence-associated β-galactosidase activity). Therefore, these data suggest that OIS in response to acute NF1 inactivation occurs in humans and is a barrier to malignancy in patients with neurofibromatosis type I.

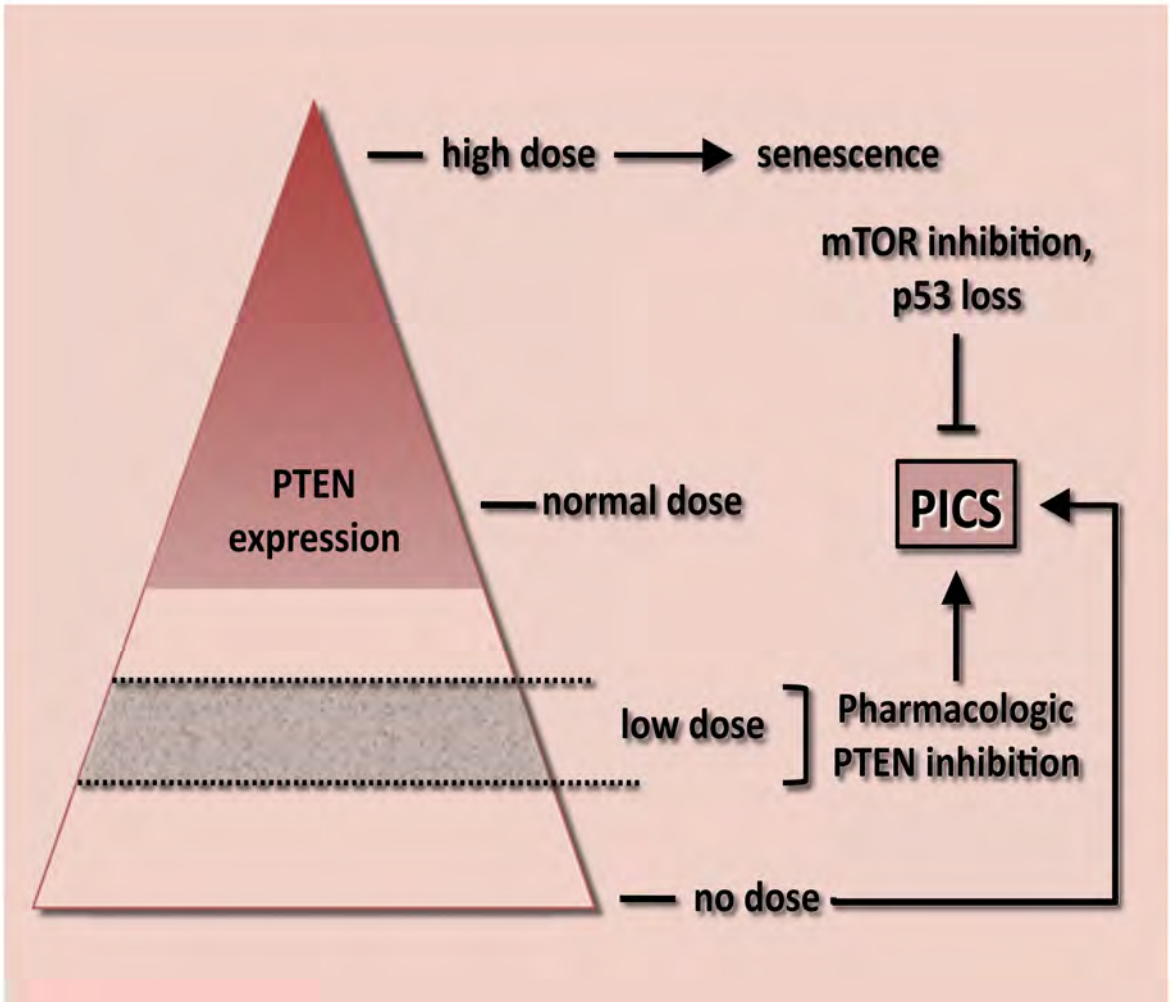

Fig.5 Pharmacologic Induction of PICS:
Loss of both gene copies (‘‘no dose’’) of PTEN sets in motion a senescence program, ‘‘PICS,’’ in a p53- and mTOR-dependent fashion. It occurs in the absence of a DNA damage response and can be established also in already arrested cells. Also over-expression of PTEN (‘‘high dose’’), or inactivation of PI3K, can cause senescence. Cells with a ‘‘low dose’’ (30%–50% of the normal dose in WT cells) can be forced to enter senescence upon pharmacologic inhibition of PTEN. (Peeper 2010)

5. Oncogene-induced reversible growth arrest

The fact that vast majority of nevi are restricted from their progression to melanoma speaks volumes in favour of robustness of OIS as a cancer counteractive strategy. However this observation also underlines the fact that cells in a very small fraction of nevi can probably evade the clutches of OIS and as such can drive fulminant melanoma. Work from Bosenberg laboratory demonstrated that disruption of *Cdkn2a* (*Ink4a/Arf)* locus alone is not sufficient to permit $Braf^{V600E}$-induced melanoma, suggesting that activated Raf signaling engages an anti-proliferative response independent of Cdkn2a signaling, however, the combination of Braf activation and Cdkn2a loss was sufficient for nevi to manifest as melanoma, presumably in the setting of additional stochastic events (Damsky et al., 2015). Unbiased RNA seq analysis revealed that mTOR signaling was strongly downregulated in growth arrested nevi but gets upregulated with progression to melanoma. In order to explore Braf-induced senescence, Bosenberg and colleagues inactivated the Lkb1 (Stk11) tumor suppressor in melanocytes with elevated levels of Braf (Damsky et al., 2015). Although depletion of Lkb1 promoted bypass of OIS, these cells didn't reveal a propensity to be transformed to melanoma. However parallel depletion of Lkb1 and Cdkn2a allowed melanoma formation indicating active Raf engages anti-proliferative effects via mTOR repression and Cdkn2a activation. The authors also noted a repressive effect of Lkb1 on melanoma metastasis. To corroborate the role of mTOR, the authors took advantage of well-characterized *Pten;Braf* melanoma model, wherein concomitant *Pten* inactivation and *Braf* activation potently cooperate to rapidly induce murine cancers.

By interfering with specific components of the mTORC1 or mTORC2 complex, this study demonstrated that coactivation of mTORC1 and mTORc2 stands imperative for melanoma formation (Damsky et al., 2015). The study also highlighted the potential role of miRNA99/100 mediation depletion of IGF2-mTOR cascade in impeding nevi from progressing to fulminant melanoma formation (Fig.6) (Damsky et al., 2015). Such examples would suggest that melanocyte senescence is the impermanent kind, leading to the question of whether such ephemeral growth arrest is “real senescence” or a less deep, “pseudo-senescence” (Souroullas and Sharpless 2015). However existence of rare clonal population of non-senescent Braf expressing cells in nevi that might go into a sort of hibernation and can re-surface on a backdrop of pref-erable settings (such as mTOR activation) cannot be ruled out. (Souroullas and Sharpless 2015).

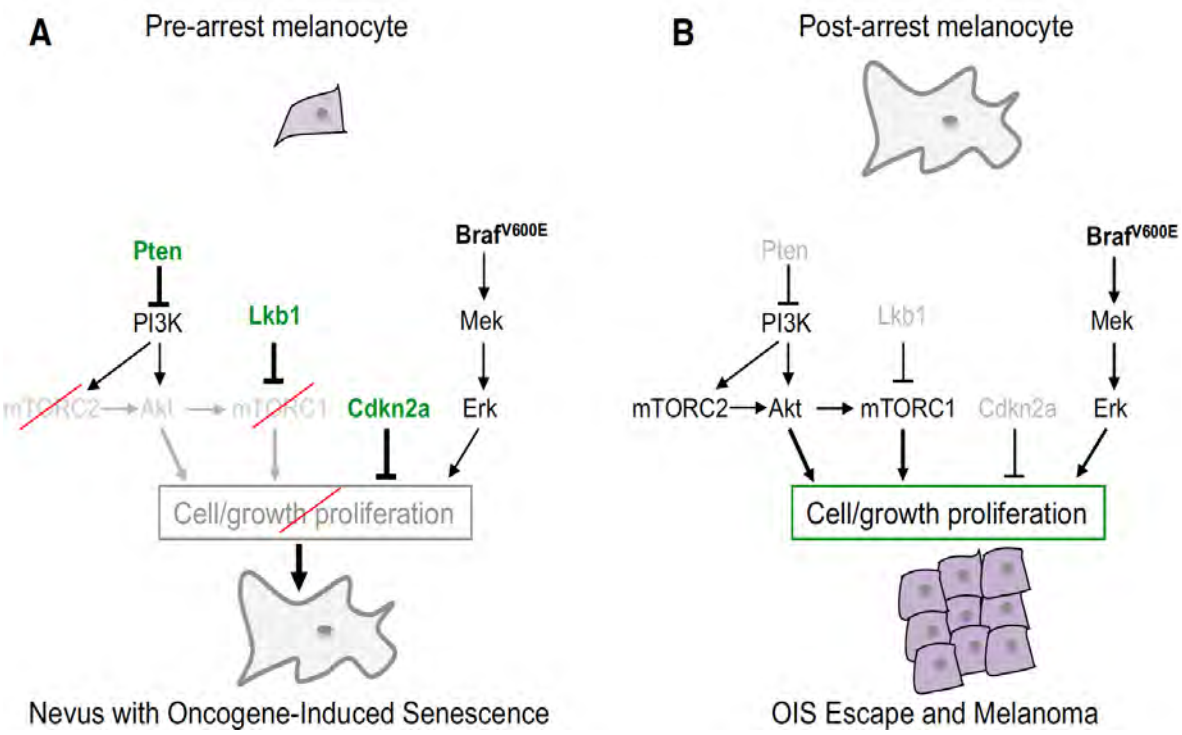

Fig.6 Bypassing of OIS in Melanocytes and Progression to Melanoma:
(A) In the presence of activated *Braf*^{V600E}, expression of Pten, Lkb1, and Ink4a/Arf (Cdkn2a locus) in melanocytes inhibits both mTORC1 and mTORC2 signaling, impeding cell growth and proliferation, resulting in oncogene-induced senescence.
(B) Inactivating mutations of Pten, Lkb1, and Cdkn2a in postarrested melanocytes result in elevated mTORC1/2 signaling and enhanced proliferation, bypassing senescence, and promoting transformation to melanoma. (Souroullas and Sharpless 2015)

6. Immuno-surveillance of senescent cells

Regarding tumor prevention, cellular senescence has long been regarded as a cell autonomous mechanism that exploit the various tumour suppressors for its induction and maintenance regime i.e. suppressing tumor development through the induction of a stable cell cycle arrest (Lowe at al 2004). In a very elegant study conducted Zender laboratory revealed expression of oncogenic NrasG12V alone is sufficient to trigger a robust oncogene induced senescence in otherwise normal mouse hepatocytes *in-vivo* (Kang et al., 2011), highlighting the relevance of senescence induction in pre-neoplastic lesions. A plethora of chemokines and cytokines secreted from premalignant senescent hepatocytes led to the attraction of innate as well as adaptive immune cells, which were found in the vicinity to senescent hepatocytes, suggesting that precancerous senescent hepatocytes could be subjected to immune clearance. Indeed, time course analyses revealed a rapid turnover of precancerous senescent hepatocytes, which was designated as immune-surveillance of precancerous senescent cells. The interesting proof of concept was gathered from the observation that severe combined immune-deficient (SCID) mice and CD4 knockout mice revealed compromised potential in the clearance of the NrasG12V expressing senescent hepatocytes indicating that functional senescence surveillance was dependent on an intact innate and adaptive immune response. Strikingly, the aborted axis of immune clearance of NrasG12V expressing pre-cancerous cells in these mice resulted in the development of hepatocellular carcinomas at later time points, further showing that immuno-surveillance is a critical pre-requisite that needs to be met in orto

limit the tumorigenesis of pre-neoplastic lesion. This data of senescence specific surveillance at work has been further supported from the fact that the mice mutant for NrasG12V hepatocytes harboured CD4 T-cells but not in NrasG12V expressing p19Arf knockout mice, in which senescence induction has been blunted. Furthermore, CD4 T-cells have been found to be dependent solely on monocytes and freshly replenished macrophages to efficiently kill senescent hepatocytes, nullifying any possible intervention from the kufer cells. Collectively, these results showed that oncogene induced senescence (OIS) plays an important role in the eliciting a sharp influx of specific immune responses against antigens expressed in precancerous cells and that a continuum of CD4 T cell mediated immune clearance of pre-malignant senescent cells is crucial to suppress the trajectory towards hepatocellular carcinoma (Fig.7) (Kang et al., 2011). These data established a novel cell non-autonomous role of the cellular senescence program (Kang et al., 2011). The discussed data are also particularly interesting against the background of an ongoing scientific debate regarding the stability of the senescence associated cell cycle arrest. While early *in-vitro* studies suggested the senescence associated proliferative arrest to be irreversible (Zhu et al 1998), subsequent studies provided evidence that under certain circumstances as discussed previously in the case of melanocytic nevi, a subfraction of cells may escape the senescence arrest and re-enter the cell cycle (Beauséjour et al., 2003; Coppe et al., 2008; Kuilman et al., 2008). Obviously, the escape of even a small subfraction of precancerous cells can have far-reaching consequences *in-vivo*, as there will be strong selection for such cells with subsequent tumor development potential. Melanocytic nevi contain large numbers of senescent melano-

cytes (Michaloglou et al 2005), however, in general these nevi are not subject to any kind of immune clearance (Hoenicke and Zender 2012). It remain an open question that whether any alternative surveillance machineries deployed in nevi to limit melanogenesis and if yes then the fact that certain nevi have been demonstrated to harbor the capacity to escape the from the senescence onslaught, opens a further question how the surveillance machinery are circumvented by this clonal escapist population.

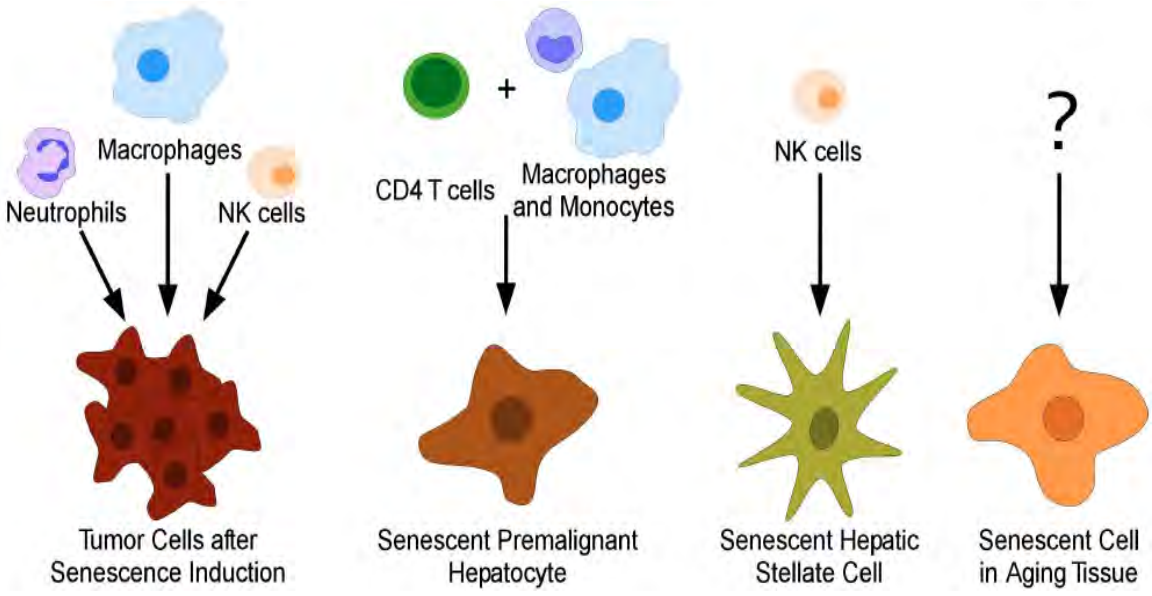

Fig.7 Schematic representation of immune responses against senescent cells in different disease settings. Upon senescence induction in established liver carcinomas, an innate immune response is triggered and senescent cells are cleared by macrophages, neutrophils and natural killer cells (NK cells) as demonstrated from the work of Xue at al., 2007. (first column). In contrast, premalignant senescent hepatocytes, induced by aberrant activation of oncogenic Nras, are subject to an antigen specific CD4 T cell mediated immune response, which also involves monocytes/macrophages (second column). NK cell mediated clearance of senescent hepatic stellate cells was shown to be crucial to restrict the progression of liver fibrosis in chronically damaged livers (third column). Future work is needed to address whether immune responses against senescent cells in aging tissues occur and which components of the immune system are involved. (Hoenicke and Zender 2012)

B. Non-coding Antisense RNAs

1. Antisense transcripts- Introduction

Over the last decade or so, emerging data from various laboratories have led us to appreciate unprecedented complexities in the regulation of gene expression. With the advent of non-coding RNAs especially with lessons learnt from wide spread occurrence of antisense transcripts we now realize that the system deviates much from the very simplified Central Dogma of genome regulation as it extrapolates beyond the protein coding potential. These noncoding or antisense transcripts that gets generated in course of transcription are deprived of protein coding abilities and yet play a major role in gene regulation (Eddy et al., 2001; Pontier and Gribnau 2011; Rinn et al., 2007; Camblong et al., 2007; Hingay et al., 2006; Kawano et al., 2007; Beltran et al., 2008). One of the most cliché examples of such non-coding antisense transcripts is the occurrence of a very early developmentally programmed (in the post-implantation stages of the embryonic development) XIST (X-inactive specific transcript) that originates from the XIC (X-chromosome Inactivation Centre) in females and plays an integral role in dosage compensation by inactivation one of two X chromosomes.

The non-coding RNAs erupt in various sizes and these regulatory RNA devoid of protein coding potential are dissected into two major classes, non-coding RNA less than 200 nucleotides as small RNAs, while the others are termed as long non-coding RNAs (Fig.8) (lncRNAs). Technological advances have led

us to identify large number of lncRNAs and antisense transcripts (Guttman and Rinn 2012; Marcer and Mattick 2013; Mattick 2009; Esteller 2011).

The class of RNAs that are transcribed in an opposite direction on the other strand to that of its sense transcripts are designated as Antisense Transcripts (Fig.8). Hence in principle antisense transcripts can erupt from genes either harbouring or with compromised protein-coding potential. The study of gene regulation by antisense transcription is particularly intriguing, as they implement their function by the virtue of the orientation of their genomic alignment. However it has also been observed that the strict complementarity of the antisense transcripts to its sense counterpart does not stand to be pre-requisite in order to execute their regulatory function as partial complementarity in certain settings have been detected to do the job convincingly (Su et al., 2007; Wilkening et al., 2013; Pelechano et al., 2013).

One of the key difference that bifurcates antisense RNA from non-coding RNAs stems from the fact that antisense RNAs do not inherently lack the protein coding potential, however their genomic orientation to these connoted sense transcripts obliterate the expression of its complementary sense transcripts (Shearwin, Callen and Egan 2005) whereas non-coding RNA irrespective of their structural orientation to the genes they affect are devoid of potential to get translated. However like lncRNAs, antisense transcripts also displays specific domains that enable them to interact with DNA, RNA and protein (Guttman and Rinn 2012; Mercer and Mattick 2013).

Over last decade overwhelming number of studies indicated wide-spread occurrence of such antisense transcripts in varied species Katayama et al., 2005; David et al., 2006; Kampa et al., 2004). However the much of these

transcripts are not evolutionarily conserved both in terms of structure and function (Yassour et al., 2010; Rhind et al., 2011; Goodman, Daugharthy and Kim 2012; Kutter et al., 2012; Raghavan et al., 2012; Nicolas et al., 2012). This species specific variation have large complicated the matters for structural and evolutionary biologist as that makes one wonder how divergent are the structures of these antisense transcripts as well as the mechanism of gene regulation untaken by these transcripts and how these non-coding RNAs have undergone differential selection to mediate key cellular processes from species to species. (Pontier and Gribnau 2011; Rinn et al., 2007; Camblong et al., 2007; Hingay et al., 2006; Kawano et al., 2007; Beltran et al., 2008; Carrieri et al., 2012). More over it has been shown that around 30% of the human genome displays antisense transcription potentials. However the ratio of sense to antisense transcripts have detected to be abruptly low as antisense transcripts are in general 10 times less abundant that its corresponding sense (Ozsolak et al., 2010). This observation raises an important question as to unravel the mechanistic underpinnings of how does this inadequate number of antisense transcripts mediate its function with high efficiency and precision in most of the settings. It is important to mention here that antisense transcripts not only exerts its impact on transcription, but certain studies have appreciated its role in post-transcriptional regulation as well.

In context of the cellular localization these antisense transcripts are quite obviously detected in nucleus (Derien et al 2012), with an appreciable number adhered to chromatin (Chu et al., 2011; Zhao et al., 2010) and to a certain extent in mitochondria where they play a crucial role in the regulation of extrachromosomal genes and maternal inheritance (Djabali et al., 2012).

In order to unleash the complete potential of these non-coding RNAs, studies are currently underway to throw light on their differential expression in varied settings of cell fate and more importantly if a signature or bar codes of non-coding antisense transcript is created to specify lineage identity.

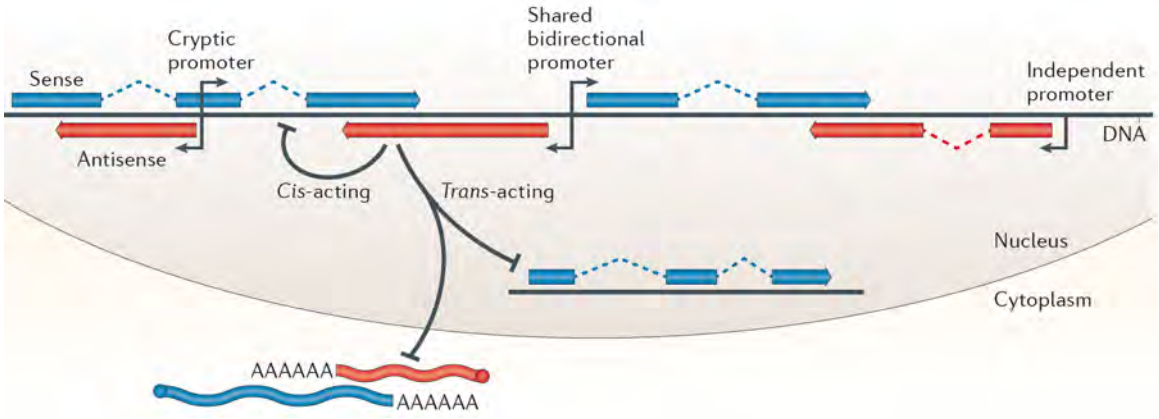

Fig.8 Classification of Antisense Transcripts: Antisense transcripts can be classified according to different criteria, such as their origin, genomic orientation, mode of action, length, stability and even the species in which they are expressed. These transcripts have been found to originate from independent promoters, shared bidirectional promoters or cryptic promoters that are situated within genes (see the figure). According to their orientation with respect to sense genes, they can be further classified as head-to-head, tail-to-tail or internal (that is, when they are fully covered by the sense transcripts). Antisense transcripts can exert their function locally, distally, in *cis* or in *trans,* and they can also function in multiple subcellular compartments. *Cis*-acting mechanisms of these transcripts can act either locally (for example, in promoter–gene interactions) or distally (for example, in enhancer–gene interactions). *Trans*-acting mechanisms can also act either locally (for example, antisense transcripts affecting the allele from which they originated and/or any additional allele) or distally (for example, antisense transcripts affecting other genes). Moreover, antisense transcripts can be classified into short (<200 nucleotides) and long (>200 nucleotides) non-coding RNAs (ncRNAs), and stable or unstable RNAs.
Short ncRNAs are accepted as fundamental players in gene regulation. Although they are widespread among eukaryotes, relevant differences exist among species; for example, PIWI-interacting RNAs (piRNAs) are found in animals but not in plants or fungi. Species-specific differences in mechanisms of action might be expected when these mechanisms depend on an accessory machinery, such as the RNA interference machinery, that is not present in all species. As an example, the pairing of sense–antisense transcripts and their consequent degradation by RNase III in Gram-positive bacteria are not seen in Gram-negative bacteria, which suggests a different processing pattern of double-stranded RNAs. Similarly, any effect of an antisense transcript that is mediated by DNA methylation is not expected to function in *Saccharomyces cerevisiae*, in which the appropriate DNA methylation machinery is lacking. However, mechanisms of action that are based on the general and highly conserved transcription machinery — for example, transcriptional interference by chromatin modifications — are more likely to be conserved across species (Pelechano and Steinmetz 2013).

2. Detection of antisense transcripts

Technological advances in genomics have largely driven identification of antisense transcripts to a large extent. One of the impediments that invariably comes to the forefront while study antisense transcripts is to make sure to take their orientation of transcription in consideration. Overtly used approaches like canonical RNA-sequencing, exome sequence or microarray do not imbibe the feature to distinguish strand specific transcripts. Such shortcomings have led to the discovery of various techniques dedicated to enable the detection validation and quantify strand specific transcripts (Xu et al., 2011; Xu et al., 2009; van Dijk et al., 2011; Wilkening et al., 2013; Pelechano et al., 2013; Perocchi, Xu, Clauder-Munster and Steinmetz 2007; Levin et al., 2010). These techniques mainly relies on capturing the process of active transcription using techniques such as global run-on sequencing (GRO-seq) (Core, Waterfall and Lis 2008) and by a relatively recent implemented techniques like native elongating transcript sequencing (NET-seq) (Churchman and Weissman 2011) and Precision nuclear Run-On and Sequencing assay (PRO-seq) (Kwak et al., 2013) that helps to identify strand specific antisense transcripts via the detection of RNA Polymerase II engagement upon transcription at the base pair resolution with precision.

3. Expression of antisense transcripts

The transcript itself or an aftermath of transcription as in the case of pervasive transcription can either mediate the function of an antisense transcript. Antisense RNA can get generated at varied genomic venues as diverse as possible. This can range anything from the divergent genes (Sigova et al., 2013; Neil et al., 2009; Xu et al., 2009; Seila et al., 2008; Core, Waterfall and Lis 2008) to genes sharing convergent orientations (discussed in details later). Moreover these antisense transcripts can get generated at cryptic promoters and additionally various epigenetic players like Sin3b, a histone deacetylase complex, as shown in yeast can regulate the accumulation of such antisense transcripts and in turn can mediate certain key cellular process like DNA damage response (Nicolas et al., 2007). Antisense transcripts associated to bidirectional promoters of divergent (head to head) genes are generally more readily detected in gene-dense topologies (Neil et al., 2009; Xu et al., 2009). However the frequency of such bidirectional promoter associated antisense transcripts varies from species to species with *Drosophila* having a distinctly low number of such antisense transcripts owing to low number of bidirectional promoters (Core et al., 2012). Interestingly it is of note that these antisense transcripts do not always arise from DNA template but can also arise from originate from RNA-dependent RNA polymerase (RdRP) activity (Lehmann, Brueckner and Cramer 2007; Wagner et al., 2013). Specifically, it has been proposed that, in humans, some antisense transcripts that contain non-genomically encoded polyuracil stretches and are generated using mRNAs as templates (Kapranov et al., 2010).

4. An insight into antisense functionalities

A wide milue of antisense ttranscripts have been detected and their regulatory roles have been deciphered and appreciated in various species (Table 1). One of the most interesting aspects of antisense RNAs has heralded with the experimental validation of their activity both in cis (the alleles located on the same DNA stand) as well as in trans (the alleles located on the same DNA stand). Recent works from various laboratories have enabled us to distinguish between the activity of antisense transcripts in trans and cis. The general perception beholds that the antisense transcripts mediate the effect at the site of its origin is an effect in cis. Besides antisense transcripts can interact with other loci by means of higher order chromatin structure that drives chromosomal conformations changes and thus can effectively mediate the effects in *trans* (Rinn et al., 2007; Camblong et al., 2007; Camblong et al., 2009; Berretta et al., 2008). These effects are better acknowledges in the settings that involves the investigation of chromosome conformational changes in the 3D space of the genome. It is important to note that non-coding RNAs (like HOTAIR or XIST) have shown to mediate effect by recruiting chromatin-modifying complexes (PRC2), thereby altering gene expression (Guttman and Rinn 2012).

Although the advent of RNAi have greatly simplified the loss of function approached deployed to understand the function of a gene in varied experimental settings, however, the genomic arrangement of antisense transcripts sets the empirical hurdle in dissecting the molecular function attributed to a specific cell fate without exacerbating sense expression which risks of com-

promised cellular state under investigation and hence RNAi strategy in gaining functional insights of antisense transcripts have been met with limited success (Xu et al., 2011; Camblong et al., 2009; Guttman and Rinn 2012; Guttman et al., 2009). It is important to stress that this strategy appears a potential caveat in the context of pervasive read-through transcription in the backdrop of convergent gene loci devoid of any intragenic region.

As illustrated below antisense transcripts deploys various strategies to regulate gene expression. They can intervene with transcription initiation; can form transcriptional collision in the context of cotranscriptional settings besides their interference in occluding translation machineries.

Mechanism of action	Antisense locus	Effects	Species
DNA methylation	*LUC7L*	Methylates *HBA1* promoter CpG island, which represses its expression	Humans
	Airn	Regulates *Igf2r* imprinting by DNA methylation	Mice
Chromatin modifications	*XIST* and *TSIX*	Inactivates X chromosome gene expression	Mammals
	ANRIL	Represses the tumour suppressor locus *CDKN2B–CDKN2A* by both histone H3 lysine 27 (H3K27) methylation and DNA methylation	Humans
	BDNF-AS	Represses *BDNF* by histone modification	Mammals
	HOTAIR	Silences the *HOXD* locus in *trans* by the recruitment of Polycomb proteins	Humans
	COOLAIR	Represses *FLC* sense gene by H3K4 demethylation and recruits Polycomb proteins, which increase H3K27me3 levels	Plants
	COLDAIR	Antisense to *COOLAIR*; represses *FLC* sense gene by the recruitment of Polycomb proteins	Plants
	AS to *PHO84*	Represses *PHO84* by histone deacetylation both in *cis* and in *trans*	*S. cerevisiae*
	RTL	Silences transcription of the *Ty1* retrotransposon in *trans* through chromatin modification and post-transcriptionally controls its retrotransposition	*S. cerevisiae*
Transcriptional interference	*RME2*	Represses *IME4* by transcriptional interference in *cis* and functions after transcription initiation of *IME4*	*S. cerevisiae*
Isoform variation	*ZEB2-AS*	Induces exon skipping in *ZEB2*, which produces an alternative isoform that has increased translation efficiency	Humans
Translation efficiency	AS to *Uchl1*	Increases translation efficiency of *Uchl1* using a SINEB2 domain	Mice
	SymR	Decreases translation efficiency of *SymE* by competing with binding of the 30S ribosome	Enterobacteria
RNA stability	*BACE1-AS*	Increases stability of *BACE1* by masking an microRNA-binding site	Humans
	WDR83 and *DHPS*	Increase their mutual stability by forming a duplex within their 3′ untranslated regions	Humans

Table1 Examples of functional antisense transcription across all kingdoms of life.

In order to appreciate the role of antisense transcripts in transcription initiation, it important to outline that there are various epigenetic marks like DNA methylation via genomic imprinting by de novo DNA methytransfersase like DNMT3A, DNMT3B as well as by DNA methyltransferase Maintenance-DNMT1 (and not CpG islands which serves as a hallmarks of identifying ORFs), methylation of lysine 4 and lysine 27 residues of Histone H3 and certain histone variants (like H2A.Z) that decorates the 5' end of the genes that plays diverse roles from partitioning the genome and thereby playing important role in genome integrity via the enrichment of epigenetic marks at boundary elements, enhancer region as well as the promoters. Antisense RNAs are often detected to play a crucial role in these activities either by sequestering certain chromatin modifiers or by promoting promoter competition of basal transcription factors at these sites, which are in close proximity to initiation of transcription. Some of the examples of antisense transcript mediation modulation are described below.

For long it has been a well-established fact that the paucity of an extremely crucial gene HBA1 predisposes a person to certain variation of α-thalassemia. A very elegant study demonstrated the occurrence of a trans acting antisense transcripts, LUC7L render epigenetic repression of HBA1 via promoter DNA methylation and a consequent onset of this disease (Tufarelli et al., 2003). This study provides an excellent example of antisense directed genomic imprinting. Paradoxically there are evidences that shows that antisense transcription can stimulate gene expression by protecting promoters from the onslaught of de novo methylation (Guil and Esteller 2012) through R loop formation, which involves DNA–RNA hybrids, during transcription (Ginno et al.,

2012). However the effects mediated by antisense transcripts are not restricted only to genomic imprinting that largely accounts for effects in cis but in certain setting antisense transcripts executes its function by taking the advantage of its structure besides driving genomic imprinting. Antisense transcription has also been found to affect gene expression by being vindictive to transcription (Lyle et al., 2000), as antisense transcript *Airn* (antisense to insulin-like growth factor 2 receptor (*Igf2r*)), represses *Igf2r* albeit the phenotype was also found to be conferred to a certain extent via DNA methylation as well (Latos et al., 2012).

There are also various well-characterized examples of antisense transcripts negotiating their effects through histone modification both in cis and trans. In favours of its action in cis, as outlined earlier one of the befitting example in its assurance has been illustrated from the study of mammalian X chromosome inactivation, in which the long ncRNA *XIST* that erupts from the XIC (X-chromosome inactivation centre) and propagates through the entire length of X-chromosome, sequesters Polycomb repressive complex PRC2 that trimethylates 27th lysine residue of Histone H3 (H3K27me3), and leads to chromatin compaction, which ultimately precipitates to chromosomal inactivation of X. In mice, the action of Xist can be reciprocated in cis by its antisense transcripts transcribed to its reverse complement orientation — X (inactive)-specific transcript, opposite strand (*Tsix*). Although XIST transverses through the entire length of X chromosome and spreading from its centre of origin (XIC), antisense transcripts can also convincingly do the job by exerting a local effect. To this end, *ANRIL* (antisense ncRNA in the *INK4* locus; alias *CDKN2B-AS1*), that is known to upregulated in prostate cancer, mediates re-

pression of the potent tumour suppressor locus *CDKN2B–CDKN2A*, that harbours p15 (also known as INK4B), p14 (also known as ARF or p19ARF in mice) and p16 (also known as INK4A). Specifically ANRIL recruits CBZ7 and SUZ12 in *cis*, which induces histone H3 lysine 27 methylation (H3K27me) mediated facultative heterochromatin and hence abrogates the transcriptional potential of this loci (Yu et al., 2008; Yap et al., 2010, Kotake et al.,2011). Such examples unleash the possibilities of therapeutic intervention by targeting such cis-acting repressive antisense transcripts to revert various deleterious phenotypes. To this end, a study that specifically abrogated the antisense transcript of Brain-Derived Neurotrophic Factor (*BDNF*), BDNF-AS — rescued the *BDNF* expression and as a consequence led to the betterment of the pathophysiological status (Modarresi et al., 2012). However this study did not resolve the molecular underpinnings BDNF-AS mediated BDNF perturbation. In the same lines, a seminal work from Anindya Bagchi's laboratory made a seminal discovery on the regulation of Myc by a long non-coding RNA. Myc has been implicated in most of human cancers however the mode of Myc regulation has long been elusive. This work has shown that elevated levels of MYC are associated with the concomitant induction of PVT1. Ablation of PVT1 in colon cancer cell line lead to remission owing to alleviated levels of MYC (Tseng et al., 2014), indicating PVT1 promotes translation of MYC.

Besides cis there are examples wherein antisense transcripts have been shown to mediate it effect on histone modification in *trans*. As outlined earlier, the most assuring example to this end has been the HOX transcript antisense RNA (*HOTAIR*) in mammals, antisense to homeobox C (*HOXC*) locus (Gupta et al., 2010). *HOTAIR* eludes the expression of *HOXD* locus in *trans* via the

recruitment of Polycomb group of proteins (PRC2). Besides work from our own laboratory have shown that the expression levels of a very large intergenic non-coding RNA (vlinc), VAD (Vlinc Antisense to DDAH1) that gets generated as a consequence to the partial antisense transcription against DDAH1, was demonstrated to increase at the back drop of a model of OIS using WI-38 cells and was detected to be required for the maintenance of senescence in this model. *VAD* has been shown to activate gene expression of cell cycle inhibitors by inhibiting the binding of the repressor H2A.Z to the *INK4* locus, and thus promotes cellular senescence (Lazorthes et al., 2015). A perfect analogy to aforesaid incidences can be made to the ground-breaking discovery of RNAi mediated heterochromatin formation, whereby siRNA generated by the RNAi machinery can lead to the recruitment of various players that induce and spreads the heterochromatinization at the sites of constitutive heterochromatin like, centromere, telomere and pericentric heterochromatin in *Schizosaccharomyces pombe* (Grewal and Jia 2007). Recent work from Proudfoot's laboratory also brought about a startling revelation in the aspect of trans-acting antisense transcripts. R-loops that are generally formed at 3'end (besides certain evidences pointing to its functional relevance at 5' end of the gene) and has been attributed to job efficient termination of transcription has been shown to induce antisense transcription and the generation of dsRNA that recruits RNAi machinery. As outline above, RNAi ensues formation of repressive histone H3 Lys9 dimethylation (H3K9me2) marks and formation of heterochromatin through the recognition of this mark by heterochromatin protein HP1γ (also known as CBX3) would induce or stabilize pausing and consequently efficient termination (Skourti-Stathaki et al., 2013).

Such examples of antisense transcripts mediated gene regulation at the site of transcription initiation are not restricted only in mammals. In plants, the intricate interplay between antisense transcripts and R loop appears to regulate both flowering potential as well as to adapt to cold climatic conditions in an a well orchestrated antagonistic manner. Antisense transcript that enables the plants to combat the unfavorable conditions is *COLD-ASSISTED INTRONIC NON-CODING RNA* (*COOLAIR*), which get transcribed antisense to *FLOWERING LOCUS C* (*FLC*) that regulates the flowering phenotype. In favorable condition the transcription by RNA Pol II from the promoter of COOLAIR is abated by the R loop which ensures the transcription of FLC (Fig.9) (Sun et al., 2013). However another study has shown that this process can also be regulated by another antisense transcript *COLD-INDUCED LONG ANTISENSE INTRAGENIC RNA* (*COLDAIR*; antisense to *COOLAIR)* by sequestering Polycomb proteins over the *FLC* locus, which in turn alleviated its expression (Heo and Sung 2011), thereby making a startling revelation that plant is well equipped to mediate this effect both in cis as well as in trans. But with the advent of extremely low temperature, the R-loop gets resolved leading to accumulation of COOLAIR that negative regulates FLC.

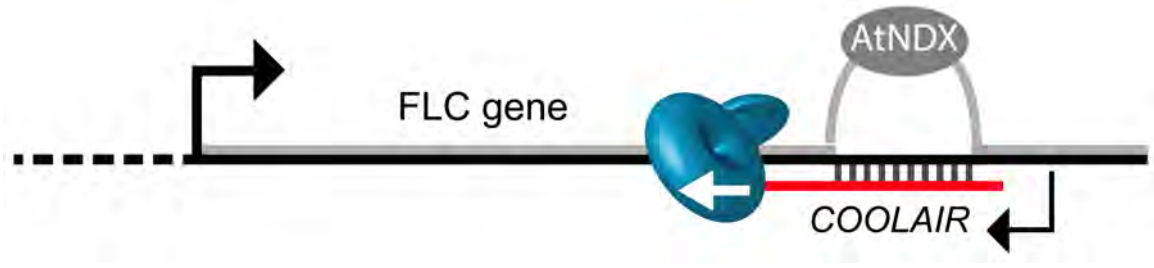

Fig.9 R loops transcriptionally regulate ncRNAs. In plants, COOLAIR antisense lncRNA controls the expression of the FLC gene. R loops form over the promoter region of COOLAIR and are stabilized by the ssDNA-binding protein AtNDX. This causes transcriptional repression of COOLAIR and, ultimately, activation of the FLC gene. (Skourti-Stathaki and Proudfoot 2014).

A very intriguing example of antisense transcript mediating its effect both in cis as well as in trans to regulate gene expression in mammals can drawn from regulation of DiHydroflate Reductase (DHFR) expression, that plays pivotal role in DNA synthesis and deficiency of this gene drives Megaloblastic Anaemia. In human, the *DHFR* locus produces an antisense transcript which suppresses *DHFR* expression as this antisense transcript overlaps the promoter and 5′ region of *DHFR*. For the proof towards the effect mediated in *cis*, it has been shown that of this antisense RNA itself block the promoter both in *cis,* while the fact that it forms a stable triple RNA–DNA helix for advocates for an effect in *trans* (Martianov et al., 2007).

Parallel to aforesaid mentioned examples, there are certain genomic settings in which the transcription of an antisense transcript occludes the pathway of transcription of an antagonistic sense transcript that culminates to a head-on collision between the two RNA Polymerases. Such genomic pandemonium especially relevant in the settings of pervasive transcription are termed as 'sitting duck' interference, which can also potentially regulate the expression of genes in *trans* (Shearwin, Callen and Egan 2005).

However the role of antisense transcripts has been shown to be involved in role well beyond turning on and off the gene expression. Strikingly besides the well-characterized role of splicing machineries, antisense transcripts have also found to aid in the generation of alternative splicing variants. For example, differentiation of mouse Embryonic Stem Cells (mESCs) to Neural Precursor Cells (NPCs) is accompanied by generation of enhancer associated antisense

transcript that leads to the generation of a shorter isoform of the associated sense transcript (Onodera et al 2012).

Although antisense transcripts draws formidable amount of attention owing to its capacity in transcriptional interference, the positive and negative effects exerted by antisense RNAs at the post-transcriptional levels, like affecting mRNA stability and translational efficiency, have started to surface. SymR, a transcript antisense to SymE that codes for an endotoxin in response to a DNA damage repair trigger, binds to the 5' end of the gene that occludes the binding site of the 30S ribosomal subunit, thereby inhibiting SymE translation (Kawano et al., 2007). Besides modulating translational efficiency, antisense transcript can also interfere in the degradation of mRNAs by its confrontation of with microRNA, the class of small regulatory RNAs that evolutionary bestowed the crucial capability of degrading mRNA. One example, in humans can be drawn from the transcripts antisense to the β-site APP-cleaving enzyme 1 gene (BACE1), which encodes β-secretase 1, an enzyme that appears to have a crucial role in driving Alzheimer's disease. The antisense transcript forms an RNA duplex with the sense mRNA (Faghini et al., 2008) that conceals the binding site for the miRNA miR-485-5p, which consequently suppresses miRNA induced decay and the consequent translational repression of BACE1 (Faghini et al., 2010). This example is indicative of antisense transcripts fine-tuning gene expression levels as miRNA sponges (Ebert and Sharp 2010).

5. RNA Polymerase II termination pathways

In order to understand the molecular underpinnings and implications of impaired transcription termination mediated generation of read-through antisense transcripts, it is important to appreciate certain fundamental principles of transcription termination.

Emerging data from studies particularly based on pervasive transcription have led us to acknowledge a rather underappreciated crucial role of transcription termination in the cell. For example, transcription termination prevents Pol II from interfering with downstream DNA elements, such as promoters of the proximal gene and instrumental in promoting recycling of polymerases (Rosonina et al., 2006; Gilmour and Fan 2008; Richard and Manley 2009). Contrary to the general perception that transcription termination is an event dedicated to end of the gene, it is highly dynamic and can occur upstream, downstream and within open reading frames (ORFs). The initial idea that termination could be modulated originated from studies conducted in prokaryotic organisms like bacteria, which revealed that bacteria deploys premature termination or attenuation to regulate clusters of amino acid biosynthesis genes (Merino and Yanofsky 2005; Naville and Gautheret 2010) a concept commonly now as “Operon Concept.” When the amino acid product encoded by these genes is present in excess, a negative feedback loop is deployed at the genomic level, whereby an RNA terminator forms within the 5′ untranslated region (UTR) of the mRNA and leads to the release of RNA polymerase before transcription of the protein-coding region. This particular example takes advantage of intrinsic RNA folding and coupled transcription and translation in bacteria, but other variations of attenuation are mediated through RNA-

binding proteins and function independently of ribosome activity (Naville and Gautheret 2010).

Compelling evidences in support of the view that sites of transcription termination also serves a the blue print of partitioning the genome has heralded with revelation that transcription is not bounded to its limits by discrete functional units such as ORFs, but rather the genome is transcribed almost to its entirety (Jacquier 2009). For genome-wide transcription to be in act with highest fidelity call for an efficient RNA termination of RNA polymerase to mitigate any sort of interference with neighbouring transcriptional units (Shearwin et al., 2005). Emerging data indicate that impaired termination of transcription casts great impact on mRNA synthesis than previously expected as it leads to inefficient splicing and elevated degradation of the RNA (West and Proudfoot 2009), and connotes with alleviated initiation of transcription and hence serves as a critical fail safe mechanism by turning off transcription with failure in termination (Mapendano et al., 2010). This indicates that how an event generally associated to the 3' end of the gene can influence Pol II processivity at the promoters. As Pol II termination is coupled with RNA 3′-end processing, the timing of Pol II release can also dictate the length of the final RNA product and thus affect the stability and ultimate functionality of nascent transcripts.

Although the release of RNA polymerase and its product are clearly important, these two independent events needs to be tightly coordinated to render efficient error-proof transcriptional rates. The remarkable speed of the Pol II elongation complex (~1–4 kb per min) (Ardehali and Lis 2009), and its processivity depend on an intricate network of interactions between polymerase proteins and nucleic acids (DNA and RNA) and base-pairing interactions be-

tween single-stranded RNA and DNA. The RNA–DNA hybrid of 8 nucleotides in length that is maintained during the elongation stage has been proposed to be the primary stability determinant of Pol II (Kireeva et al., 2000; Komissarova et al., 2002), and as such, resolving this R loop by helicase Senataxin (Sen1 in yeast) may be the pivotal event that results in efficient termination and release of nascent transcripts (FIG. 1). ˙Pol II termination can be elicited through different pathways, depending on the RNA 3′-end processing signals and termination factors that are present at the end of a gene (Lykke-Anderson and Jensen 2007; Rondon et al., 2008). Two of the best-studied pathways, the poly(A)-dependent pathway and the Sen1-dependent pathway, are as illustrated (Fig. 10) to highlight the common themes and principles involved in termination.

Better understanding of our work and its appreciation calls for outlining the key characteristics of poly(A)-dependent termination machineries from the mammalian perspective. In mammals, Poly(A) dependent termination is categorically driven by two intricately orchestrated events: polyadenylation and cleavage. Polyadenylation that involves incorporation of poly A residues to the 3’ end of the evolving nascent transcript is driven by Poly-A polymerase while the cleavage of the nascent transcripts is facilitated in a the joint venture of 2 components: Cleavage and Polyadenylation Specificity Factor (CPSF) and Cleavage stimulatory Factor (CstF). As the name implies CPSF identifies AAUAAA signal and mediates pausing of RNA Polymerase II while the later CstF cleaves the nascent transcript followed by degradation of the reminiscent RNA by 5’-3’ exoribonuclaese 2 (XRN2) that culminates with the maturation of the nascent transcripts.

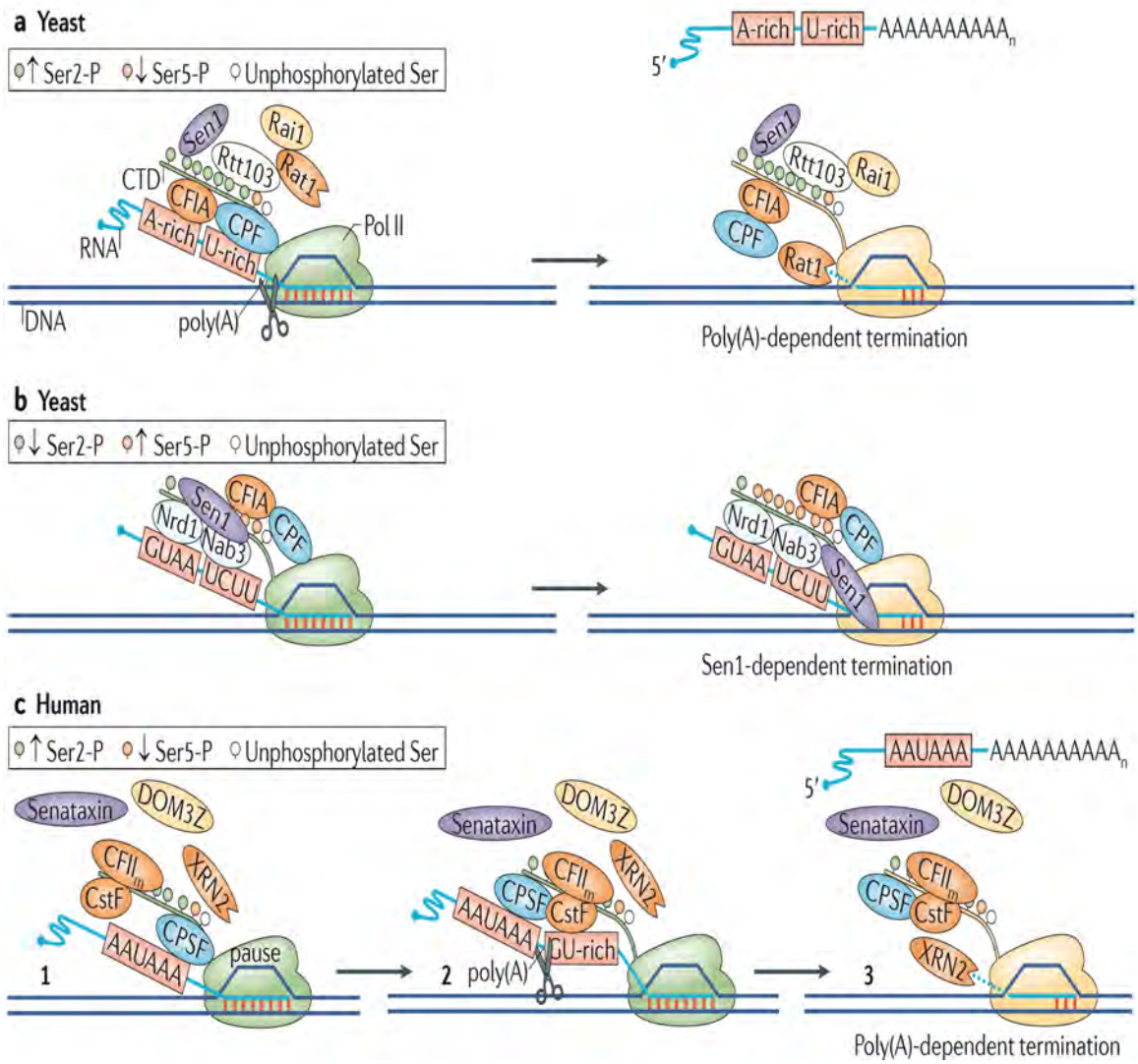

Fig.10 Factors involved in poly(A)-dependent and Sen1-dependent termination. Counterparts of termination factors in yeast and humans are shown in the same colour, and known interactions between RNA, RNA polymerase II (Pol II) and other factors are indicated by direct contacts. Pol II carboxy-terminal domain (CTD) phosphorylation dynamics are indicated as in FIG. 1b, with Ser2-P being higher than Ser5-P in regions of poly(A)-dependent termination, and the reverse pattern being observed in regions of Sen1-dependent termination. **a.** In poly(A)-dependent termination in yeast, the 5′–3′ exoribonuclease RNA-trafficking protein 1 (Rat1; XRN2 in mammals) is recruited to Pol II via proteins that interact with phosphorylated Ser2 in the CTD (such as regulator of Ty1 transposition 103 (Rtt103)) and poly(A) site RNA elements (such as the indicated A-rich and U-rich sequences). In what has been called the 'torpedo' model, Rat1 degrades the downstream RNA (dashed light blue line) that results from the 3′-end processing cleavage event (scissors), which may result in disruption of the Pol II active site hybrid. In addition to contacting the CTD, cleavage and polyadenylation factor (CPF; homologous to human cleavage and polyadenylation specificity factor (CPSF)) may also interact with the body of Pol II through its suppressor of Sua7 2 (Ssu72) subunit. Optimal association of Rat1 with chromatin requires cleavage factor IA (CFIA; homologous to human cleavage stimulatory factor (CstF)), but direct contacts with CFIA have not been reported. **b.** In Sen1-dependent termination in yeast, the mechanism that applies to most non-coding RNAs, Sen1 is recruited to Pol II via proteins that interact with Pol II Ser5-P CTD (such as Nrd1) and specific RNA ele-

ments (such as GUAA repeats). Sen1 may unwind the Pol II active site hybrid via its helicase activity. **c.** In poly(A)-dependent termination in humans, pausing of human Pol II is induced when CPSF bound to the body of Pol II recognizes the AAUAAA signal sequence that emerges in the nascent transcript (step **1**). Upon exposure of the GU-rich binding site, CstF dislodges CPSF (step **2**). Following cleavage at the poly(A) site, 5′–3′ exoribonuclease 2 (XRN2) degrades the downstream RNA product, which may displace Pol II as described above for Rat1 (step **3**). CFIIm, mammalian CFII (which contains the human homologue of yeast protein 1 of CFI (Pcf11)); DOM3Z, DOM-3 homologue Z; Nab3, nuclear polyadenylated RNA-binding 3; Rai1, Rat1-interacting 1. (Kuehner at al., 2011)

6. Generation of Pervasive Antisense RNA at convergent genes

In course of transcription, owing to impaired transcription termination, RNA Polymerase II originating from the promoter of a gene can continue to transcribe beyond the designated termination site and thereby can lead to the generation of pervasive read-through transcripts by traversing the adjacent loci located on the opposite strand of a convergent gene pair, thereby generating its antisense. As these antisense RNAs are largely associated to convergent gene pairs, they are termed Read-through Antisense RNAs. However, the accumulation of these transcripts can be deleterious, unless they are suppressed or degraded by RNA–surveillance machineries. RNAi dependent heterochromatin machinery recruits cohesion complex to mediate transcription termination specifically at the genes arranged in convergent orientation in S. Pombe (Gullerova, and Proudfoot 2008) besides the fact that RNA processing exosome is known to associate to elongating RNA Polymerase II in *Drosophila* (Andrulis et al., 2002). Furthermore it was proposed that in budding yeast that the NRD- and Rnt1-mediated fail-safe mechanisms provide a second

chance to terminate Pol II, and so minimize the accumulation of antisense transcripts (Rondon et al., 2009).

In 2009, Grewal's laboratory showed that in S. pombe, deletion of Pht1 (H2AFZ homolog in yeast), gene coding for H2A.Z, along with a RNAi component Ago2 or heterochromatin component Clr4(a Suv-39h homolog in yeast) leads to wide-spread synergistic increase of these read-through antisense transcripts that parallels to the deletion of Rrp6 (an exosome subunit, or Ex-OSC10 in mammals) alone, implicating that H2A.Z cooperates with RNAi and heterochromatin factors to suppress the generation of these read-through transcripts in a manner similar to 3'-5' exonuclease activity of the exosome (Zofall et al., 2009). This study implicated H2A.Z and heterochromatin factors in the suppression of potentially deleterious antisense RNAs (Fig.11) (Zofall et al., 2009). H2A.Z nucleosomes might directly obstruct Pol II progression, or facilitate loading of exosome or factors involved in structural organization of chromosome, which in turn might promote RNA degradation by stalling Pol II. Alternatively, H2A.Z might signal to Pol-II-associated exosome (Andrulis et al., 2002) that transcription has escaped its natural termination and therefore produced an aberrant transcript. H2A.Z and ClrC (the Rik1 subunit of which resembles the cleavage and polyadenylation factor CPSF-A7) may be components of a RNA quality control mechanism, which stimulates exosome activity by exosome cofactors (Houseley, LaCava and Tollervey 2006). Indeed, the loss of the Cid14 subunit of TRAMP, implicated in exosome stimulation (Houseley, LaCava and Tollervey 2006) causes accumulation of antisense RNAs, and the Drosophila homologue of H2A.Z mediates targeting of messenger RNA processing factors (Wagner at al., 2007). Grewal's group also

showed that loss of Mtl1 (Mtr4 like Protein) as well co-deletion of H2A.Z and Cohesin leads to elevated levels of such transcriptional read-throughs (Lee et al., 2013; Mizuguchi et al., 2014). It has also been shown that Xap5 (FAM50 homolog in yeast) acts similarly to H2A.Z in suppressing the generation of such read-through transcripts (Avner et al., 2014). A novel study demonstrates that Pol II termination can also occur via a roadblock mechanism in yeast via a transcriptional activator Reb1p, which is akin to the mechanism by which transcription of ribosomal DNA by Pol I is terminated (Colin et al., 2014), however the role of this factor in a settings of convergent gene pair remains unknown.

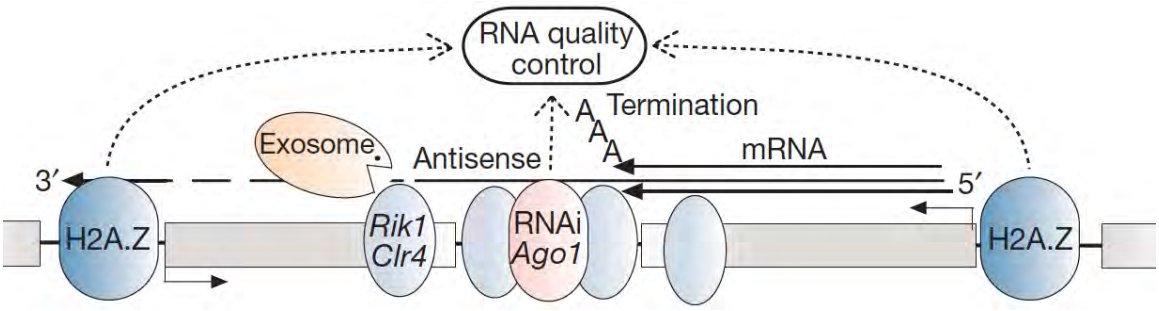

Fig.11 Model for antisense suppression at convergent genes. H2A.Z at the 5' ends of genes contributes to suppression of read-through transcripts that are degraded by exosome. Antisense suppression also requires ClrC and Ago1, which along with H2A.Z may facilitate loading of other factors to block Pol II progression and/or mediate the processing of RNAs by the exosome (Zofall et al., 2009)

Besides these factors, R-loops have also been shown to regulate the generation of such read-through antisense transcripts. Angelman syndrome (AS) is an autism-related disorder that is caused by mutations or deletions of the maternal copy of the Ube3a gene (Kishino, Lalande, Wagstaff 1997). Normally, neurons express only the maternal copy of this gene and silence the paternal

copy via the Ube3a antisense transcript. So, Ube3a mutations in the maternal copy result in a complete loss of the protein, a brain-specific ubiquitin E3 ligase. Ube3a antisense is located immediately downstream from the Snord116 gene, mutations of which cause a second disorder, Prader-Willi syndrome. The cancer drug topotecan was found to reactivate the paternal copy of Ube3a by reducing the antisense Ube3a transcript in neurons and therefore could be potentially used to treat AS (Huang et al., 2011). Even though topotecan holds promise for AS treatment, it still remains unknown how it targets specifically Ube3a and no other genes within this locus. Importantly, topotecan is an inhibitor of topoisomerase, which, as mentioned above, relaxes negative supercoiling. It is now revealed that R-loop formation plays a role in the topotecan effect (Fig.12) (Powell et al., 2013). In essence, R loops form over the G-rich Snord116 gene, which in turn causes nucleosome depletion and chromatin decondensation in the paternal allele. Under physiological conditions, Ube3a antisense transcription silences Ube3a in cis. Upon topotecan treatment, these R loops are stabilized and so accumulate (Fig.12). According to this model, this R-loop accumulation causes excessive chromatin decondensation, stalling of the transcriptional machinery, and inhibition of Ube3a antisense expression. This in turn activates paternal Ube3a expression (Powell et al., 2013).

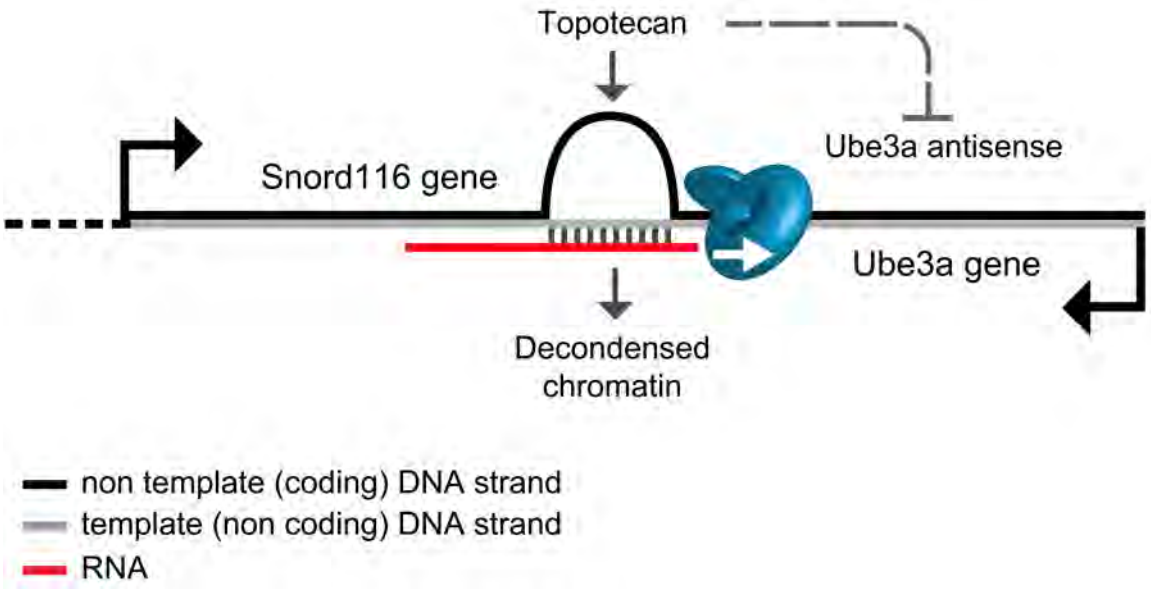

Fig.12 In human neuronal cells, topoisomerase inhibitor topotecan causes accumulation of R loops in the G-rich termination region of the Snord116 gene. This causes chromatin decondensation and blocks read-through transcription that otherwise forms the Ube3a antisense transcript. This activates the expression of the Ube3a sense transcript. Arrows indicate the direction of transcription. (Skourti-Stathaki and Proudfoot 2014).

Recently a study reported widespread accumulation of such transcripts in human upon subjection to osmotic stress induced by treatment with KCl, NaCl or sucrose. They termed these read-throughs as “downstream of gene”-containing transcripts (DoG). DoGs have been speculated a role in maintaining the integrity of the nucleus under stress (Fig.13) (Vilborg et al., 2015).

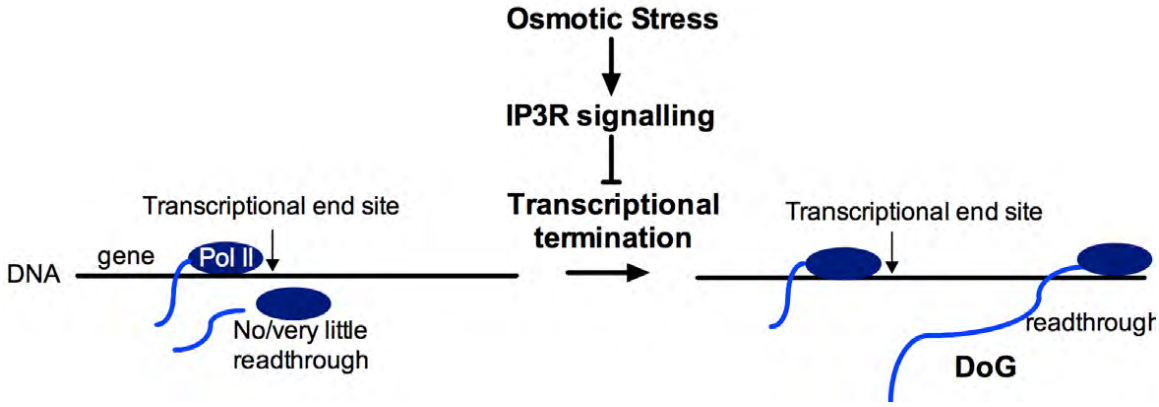

Fig.13 Inhibiting Stress-Induced DoG Induction Correlates with Aggravated Nuclear Response to Osmotic Stress. Osmotic Stress-induced transcripts generated by readthrough downstream of protein-coding genes (DoGs). DoGs are regulated by IP3 receptor signaling and remain chromatin bound. Being long (often >45 kb) and diverse (>2,000 species), DoGs contribute significantly to the human transcriptome. (Vilborg et al., 2015).

Various factors pertaining to RNAi, heterochromatin, transcription termination, polyadenylation, splicing, exosomes and other epigenetic components might have evolved to take care in suppressing the accumulation of such pervasive read-through transcripts, as they can be potentially deleterious for the cell. These read-through antisense RNAs can form RNA:DNA hybrid which can affect transcription elongation. Such structures can also block replication form and may lead to genomic instability, while others have suggested that they can open up chromatin and may thus provide access to genotoxic agents (Fig.14) (Huertas and Aguilera 2003).

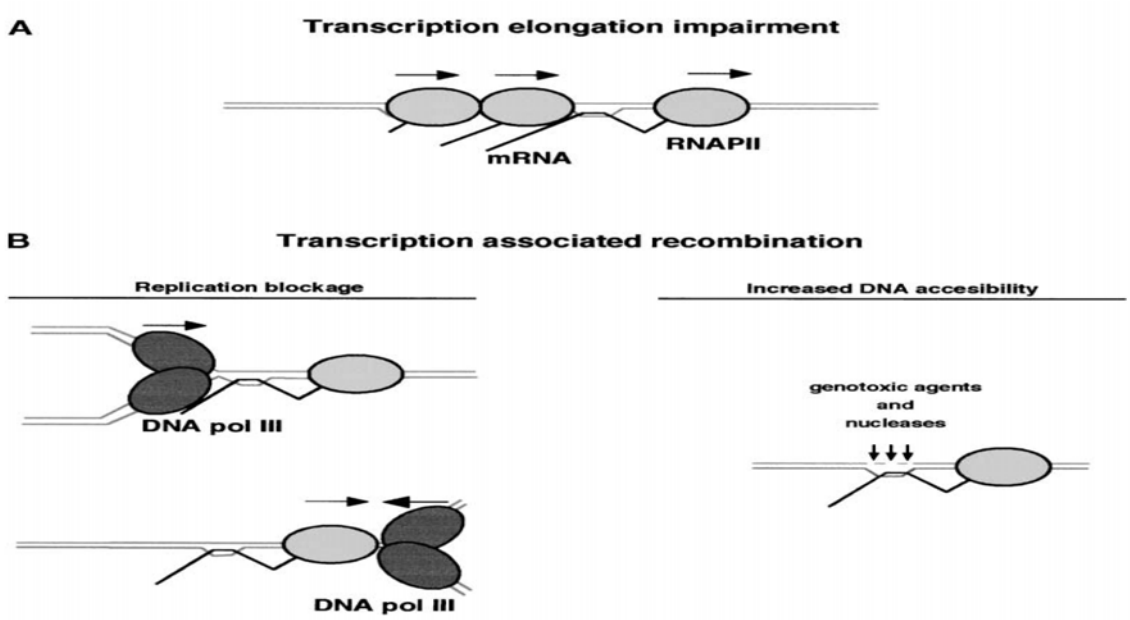

Fig.14 Models explaining how RNA can impair transcription and replication thereby can promote genomic instability. A) These non-coding transcripts if allowed to accumulate they may lead to formation of RNA:DNA hybrid which may affect transcription elongation. **B)** They may also cause blocks for replication fork may thus contribute to genomic instability. **C)** They may lead to opening-up of chromatin and allowing better access to genotoxic agents. (Figure adopted from Huertas and Aguilera 2003)

C. Implications of Non-coding RNAs in Cellular Senescence

1. Noncoding RNAs and Cardinal Tumour Suppressors

As discussed previously, p16 and p53 constitutes two sorts of archetypal mediators of cellular senescence. Roles of both small and long noncoding regulatory RNAs associated to two of the major CDKIs have started to being appreciated. Overwhelming evidences have led us to acknowledge that noncoding RNAs play a pivotal role in propagating stable cell cycle arrest while there are evidences that the cell cycle regulators can manifest many of their roles through activation of microRNAs (miRs) or Long Non-coding RNAs (lncRNAs).

a. Senescence Associated (SA) microRNAs in p53/p21 pathway:

microRNAs (miRs) belong to the class of regulatory small RNAs upon processing of pre-messenger RNAs with Dicer, Drosha, DGRC8 and Argonaute that constitute the core complex of RNA interference (RNAi) machinery.

Interestingly, the guardian of mammalian genome, p53, has been reported to be an inducer of a family of microRNA, miR-34 and has been documented to mediate activity akin of its own through this miR (Hermaking 2010). For example, p53 mediated miR-34a induction promotes senescence of endothelial cells and colon cancer cells through the disruption of E2F module (Tazawa et al., 2007). Besides miR-34a has also been shown to induce senescence, partially by preventing expression of SIRT1 (silent mating type information regu-

lator 2 homolog 1), which dismantles the senescence program in endothelial progenitor cells (Zhao and Chen 2010). Interestingly, p53 homolog ΔNp63 suppresses transcription of three microRNAs (miR-138, miR-181a, and miR-181b) documented so far that repress SIRT1, which consequently bestows ΔNp63 with the property to promote proliferation (di Val et al., 2012). Expression of another p53 dependent ncRNA that shoots up during senescence is let-7 (Wagner et al., 2008). Let-7 acts by repressing genes essential for cellular proliferation, like EZH2 and HMGA2. (Tzatsos et al., 2012; Markowski et al., 2011; Boyerinas et al., 2010; Fan et al., 2011). miR200c which is also known to be induced by p53, escalates its expression by several folds upon exposure to oxidative stress to trigger senescence via repression of a potent senescence suppressor ZEB1 (Magenta et al., 2011). From the perspective of miRs acting upstream of p53, miR885p have been reported to enhance p53 activity by suppressing the production of CDK2 and minichromosome maintenance complex component 5 (MCM5) and thus play a key role in p53 dependent senescence induction in neuroblastoma cells (Afanasyeva et al., 2011). Likewise, study conducted in HeLa and WI-38 cell lines have shown that miR-519 cripples the expression of players involved in DNA repair and calcium homeostasis thereby ensuring stress response mediated elevated p53 and p21 levels that consequently induces an efficient senescence induction (Abdelmohsen et al., 2008; Abdelmohsen et al., 2012). However there are also examples of certain obnoxious miRs like miR 25 and miR30d that act upstream of p53 and are known to suppress p53 expression by targeting the 3' UTR and hence can manifest various paradigm of p53 activity (Kumar et al., 2011).

p21 is generally known be regulated in a p53-dependent manner, although there are studies that have lead us to appreciate that expression of p21 is not always controlled by p53. In this lines various miRs with no evidence of their relationship with p53, can also regulate p21 expression. A good example of a miR that negatively regulates p21 expression can be drawn from the studies that revealed the mechanistic underpinning of Doxorubicin. This potent DNA damage inducer, is known to orchestrate senescence induction by activating p21 by lowering the levels of a cluster of miR that are constituted by miR-106b, miR-93, and miR-25, suggesting a pivotal impact of this cluster in suppressing senescence (Zhou et al., 2014). It is important to mention an interesting study that has attributed miR106a activity in obliterating p21 expression in the settings of oxidative stress (Li et al., 2009). Along similar lines, it has been shown that oncogenic Ras(G12V)- requires p21 axis to enforce senescence, however miR-106b acts up dismantling this induction while the oncogenic microRNA cluster miR-17~92 mediates its effect by disrupting the p21 mediated senescence induction and hence acts as a potential driver of oncogenic transformation (Borgdorff et al., 2010; Hong et al., 2010). Other miRs that have been shown to perturb p21 dictated senescence induction include miR-130b, miR-302a, miR-302b, miR302c, miR-302d, and miR-515-3p (Fig. 15) (Borgdorff et al., 2010).

b.SA-microRNAs in the p16 Pathway: Like above-mentioned examples of microRNAs acting upstream of p53 and p21, certain microRNAs can also regulate the expression of p16. To this end a study showed translational inhibition of p16 by miR24 (Lal et al., 2008). This observation was further supported

by another study that highlighted negative correlation between miR-24 and p16 in osteoarthritis-associated senescence, owing to reduced and elevated levels of miR24 and p16 respectively (Philipot et al., 2014). Paradoxically there are miRs (miR-26b, miR-181a, miR-210, and miR-424) that are known to promote p16 influx in the cell either through the repression of chromobox (CBX), embryonic ectoderm development (EED), enhancer of zeste homolog (EZH) and suppressor of zeste 12 (Suz12). Strikingly, depletion of p16 has been found to collapse the SA-microRNA program, indicative of a negative regulatory feedback loop at work (Overhoff et al., 2014). Another study showed that overexpression of 4 micro-RNAs that appears to be a constituent of p16/RB cascade like miR-15b, miR-24, miR-25, miR-141 mitigated p16 expression by virtue of the same target MKK4, in WI-38 cells, and the joint reduction of these microRNAs lead to accelerated p16 expression via MKK4 (Marasa et al., 2009). However miRNA can also take an indirect route towards the regulation of p16 expression by modulation the expression of p16-upstream regulator. In this line, microRNA miR-128a have been shown to promote cell senescence through its interference in the expression of BMI1, which known to be a negative regulator of p16 (Fig.15) (Venkataraman et al., 2010).

c. Senescence Associated long noncoding (SAL) RNA affecting the p53/p21 pathway: Long noncoding RNAs (lncRNAs) like small ncRNAs are transcripts that are devoid of protein-coding potential. They typically range anything between 200 bases to hundreds of kilobases.

Like the above-mentioned example in which miR act by regulating a regulator

of p16, some lincRNA deploys similar strategy in regulating gene expression. To this end it has been shown that ectopic expression of *MEG3* limits the growth of cancer cells indicating its tumour suppressive roles, whose expression is often blunted in cancer cells (Zhang et al., 2003). Mechanistically, *MEG3* prevents MDMD2 expression, a well characterized negative regulator of p53 that mediates its function by ubiquitin degradation of p53 (Zhou et al., 2012). *MEG3* has been shown to promote senescence in cervical cancer cells; (Qin et al., 2013). On the contrary, another linc RNA *7SL* whose expression gets escalated in several cancers, (Chen et al., 1997) acts by suppressing translation of *p53* via competitive triumph over RBP HuR in binding to the *p53* 3'UTR (Fig.15).

Like p53, there are certain lincRNA committed to modulation of p21 expression. One of the most promising examples of SA linc RNA belonging to p21 axis was surfaced when a study showed that Human *lncRNA-p21* that suppresses translation of β-catenin and JunB, respectively. Although its direct influence in senescence induction awaits experimental accreditation, it is transcriptionally induced by p53, gets upregulated in senescent cells, and represses translation of two proteins, β-catenin and JunB that are known to be the facilitators of cell growth (Konishi et al., 2008; Marchand et al., 2011; Ye et al., 2007). Another p53-induced lncRNA, *PINT*, interacts with polycomb repressor complex PRC2 to regulate the expression of TGF-β and p53 (Fig.15) (Marin-Bejar et al., 2013; Senturk et al., 2012; Rufini et al., 2013).

d. SAL-RNAs in the p16 Pathway: There are certain evidences where lincRNA has been shown to both negative and positively regulate the p16 ex-

pression. However in most of these effects that they impinge is indirect as they mediate this effect by modifying chromatin architecture. In favour of negative regulation of p16 by a lincRNA, it has been shown that the lncRNA *ANRIL* (alias *CDKN2B-AS1* and *p15AS*) is transcribed at a reverse complementation to the same locus as the *INK4b/ARF/INK4a* genes (Pasmant et al., 2007). *ANRIL* regulates cell cycle progression in part by recruiting CBX7 and SUZ12, a protein component of PRC1 and PRC2 complex respectively that elevates the H3K27 methylation leading to heterochromatinization and consequently to the suppresses *INK4a* and *INK4b* transcription (Yu et al., 2008; Yap et al., 2010; Kotake et al., 2011). In line to this observation, depreciated *ANRIL* expression were detected in senescent WI-38 cells whereas *ANRIL* knockdown in cancer cells display reduced proliferation (Fig.15) (Abdelmohsen et al., 2013; Kotake et al., 2011; Nie et al., 2015; Zhang et al., 2014). On the other hand, the expression levels of a very large intergenic non-coding RNA (vlinc), VAD (Vlinc Antisense to DDAH1) that gets generated as a consequence to the partial antisense transcription against DDAH1, was demonstrated to increase at the back drop of a model of OIS using WI-38 cells and was detected to be required for the maintenance of senescence in this model. *VAD* has been shown to activate gene expression of cell cycle inhibitors by inhibiting the binding of the repressor H2A.Z to the *INK4* locus, and thus promotes cellular senescence (Lazorthes et al., 2015).

2. Role of ncRNAs in various traits of senescence

a. SASP ncRNAs: As mentioned above, acquirement of a senescent phenotype (albeit not strictly a senescence signature) bestows cells with properties enable them to a wide plethora of factors associated to chronic inflammation (growth factors, ECM-degrading enzymes, and cytokines), a trait known as SASP. These inflammatory factors re-enforce senescence and various cases this positive feedback loop is mediated either by elevating alleviating microRNAs, thereby underlining a joint venture between cell non-autonomous and autonomous factors. For example, IL-1β has been shown to deplete miR-24, a potent repressor of *p16/INK4A* mRNA translation, ensuing consistent p16 activity. On the contrary, suppression of miR-146a/b elevates IRAK1 activity, which in turn activates the transcription factor NF-κB that has been shown to be the master regulator of SASP factors leading to exaggerated release of these factors (Olivieri et al., 2013). Furthermore, IκB kinases are repressed by miR-155 and miR-199a suppresses NF-κB activation (Olivieri et al., 2013). Intriguingly, IRAK1 can also enhance miR-146a/b levels, suggestive of the presence and most importantly the necessity of a negative feedback loop for fine-tuning SASP (Bhaumik et al., 2009). However there are certain microRNAs that aggravates the Senescence Messaging Secretome (SMS). To this end it has been shown that senescence driven by oxidative stress has been depicted to be associated with elevated levels of miR-183, which in turn suppresses production of the SASP factor (Li et al., 2010). Cell extrinsic microRNAs present can take the advantage of paracrine signaling a

to modulate SASP activities via cell surface receptors such as Toll-like receptors (TLRs) and hence can mimic the functionality of their ligand and thus become proficient to regulate a signaling cascade. To this end, it has been shown that let-7 and miR21 activates the inflammatory response by binding to TLR7 and TLR8 receptors respectively in mice and human (Lehmann et al., 2012; Fabbri et al., 2012).

b. ncRNAs related to Telomere: The two most commonly known ncRNAs associated to telomere are TERC (Telomerase RNA component) and TERRA (Telomeric repeat containing RNA). Both of these ncRNA plays a sort of antagonistic role in maintaining the integrity of the telomeric length besides ensuing a sustained telomerase activity. *TERC* activity assures the maintenance of length of telomere and hence shuns the onset of premature senescence and aging, as observed in *TERC*-deficient mice (Samper, Flores and Blasco 2001). Besides serving as a template for telomeric repeats TERC also serves as the scaffold to assemble protein components of the telomerase complex (Lustig 2004; Collins 2008; Greider and Blackburn 1989; Lai, Miller and Collins 2003). At a sharp contrast, family of *TERRA* ncRNAs whose length varies between 100 to less than 900nt that contain several copies of the telomere UUAGGG repeat (Poro et al., 2010) enables them to suppress telomere elongation by competitively inhibiting *TERT* (Schoeftner and Blasco 2008; Redon, Reichenbach and Lingner 2010). In the similar lines, high influx of *TERRA* ncRNAs induces premature senescence in fibroblasts through negative regulation of telomere elongation (Deng, Campbell and Lieberman 2010). However the roles played by *TERRA* are not always discouraging. On a positive note

TERRA has also been shown to be engaged in telomere protection of deprotected telomere, which is often the case in cancer and aging and thus plays a vital role in assuring genomic stability. Consistent to this, cells devoid of telomeric shelterin component, TRF2, strongly upregulates TERRA in an effort to protect telomeric ends that are susceptible to DNA damage (Porro et al., 2014; Flynn et al., 2011). This is particular important as telomeres are sensitive to this type of damage because they represent fragile sites of the genome that are difficult to replicate and carry intrinsic properties that inhibit DNA repair processes.

c. Other ncRNAs associated to Senescence Traits: Besides the ncRNAs mentioned above, several ncRNA are implicated in senescence phenotype, however in certain setting clinching evidence with their direct role in senescence phenotype still needs to be established. Although the p53 and p16 axis serve two major cardinal effectors of senescence induction, they are certainly not the only mediators of cellular senescence. Hence ncRNAs can also regulate other mediators and thus can regulate the induction of senescence. To this end, miR-29 and miR-30 repress generation of M-MYB, which drives simultaneous abolishment of proliferating and induction of senescence (Martinez and Dimaio 2011). In melanoma cells, miR- 203 can aid establishment of senescence by ablation of factors that promotes cell division (e.g. E2F3A and E2F3B) and DNA repair while miR-205 cripples E2F1 and E2F5 production, thus inducing senescence (Vernier et al., 2011; Noguchi et al., 2012; Dar et al., 2011). miR-20a have been shown act as a negative regulation of p19ARF, LRF (leukemia/lymphoma-related factor) that in turn translates to onset of

stable senescence induction (Poliseno et al., 2008). On a different note, elevated levels of miR-152 and miR- 181a in human dermal fibroblasts interfere with the translation of proteins related to cell adhesion like integrin α5 and collagen XVI which culminates to senescence establishment (Mancini et al., 2012). Strikingly there are evidences wherein multiple microRNAs cooperate in mediating a cell fate. For example, in the settings of human colorectal cancer cells, miR-186, miR-216b, miR-337- 3p, and miR-760 jointly impede the expression of the α subunit of protein kinase CKII to induce senescence (Kim et al., 2012). From the point of senescence induction owing to cell's compromised DNA repair capabilities, miR-494 reduces the levels of UV excision repair protein RAD23 homolog B (RAD23B) that precipitates senescence in lung cancer cells and diploid fibroblasts (Ohdaira et al., 2012; Comegna et al., 2014). On the other hand, the phantom class of microRNAs that prevent the generation of senescent cancer cells are termed Oncomirs. Oncomir miR-214 precludes senescence in cancer cells upon radiotherapy (Voorhoeve et al., 2006; Salim et al., 2012) and hence knockdown of microRNA-214 in therapy resistant lung cancer cells sensitizes them to radiotherapy and stimulates senescence induction (Salim et al., 2012). The lncRNA *PANDA* appears to harbor chameleon-like characteristics. In proliferating cells, *PANDA* interacts with SAFA (scaffold-attachment factor A) and PRC thereby formulating the SAFA-*PANDA*-PRC complex that acts by suppressing transcription of senescence promoting genes. However in senescent cells, *PANDA* acts by reinforcing senescence as it blocks NF-YA and E2F that enables to keep proliferative genes at check. Together, these findings indicate that *PANDA* can modulate both the triggering and impedes senescence Puvvula et al., 2014). An intriguing exam-

ple of an lncRNA that is expressed in non-senescent cells is *MALAT1* (Tripathi et al., 2013; Chandler and Peters 2013). Downregulation of *MALAT1* in young proliferating cells and in human cervical cancer cells has lead to pronounced cell cycle arrest besides reducing the tumor size, at least in part through the modulation of oncogenic transcription factor B-MYB (Tripathi et al., 2013; Abdelmohsen et al., 2013; Guo et al., 2010; Zhao et al., 2014). However the direct demonstration and well as the underlying molecular mechanistic insight of *MALAT1* counteracting senescence induction or whether the loss of it directly drives senescence induction are lacking. The lincRNA *HOTAIR* has been detected to be unregulated in senescent cells where it serves as a scaffold RNA for a substrate for E3 ubiquitin ligases thus promoting the ubiquitination and subsequent degradation of Ataxin-1 and Snurportin-1. Supportive data of positive regulatory roles of *HOTAIR* in senescence can also be drawn from the loss-of-function study conducted in a model of senescence triggered by HuR silencing wherein its suppression lead to the scarcity of senescent cell (Yoon et al., 2013).

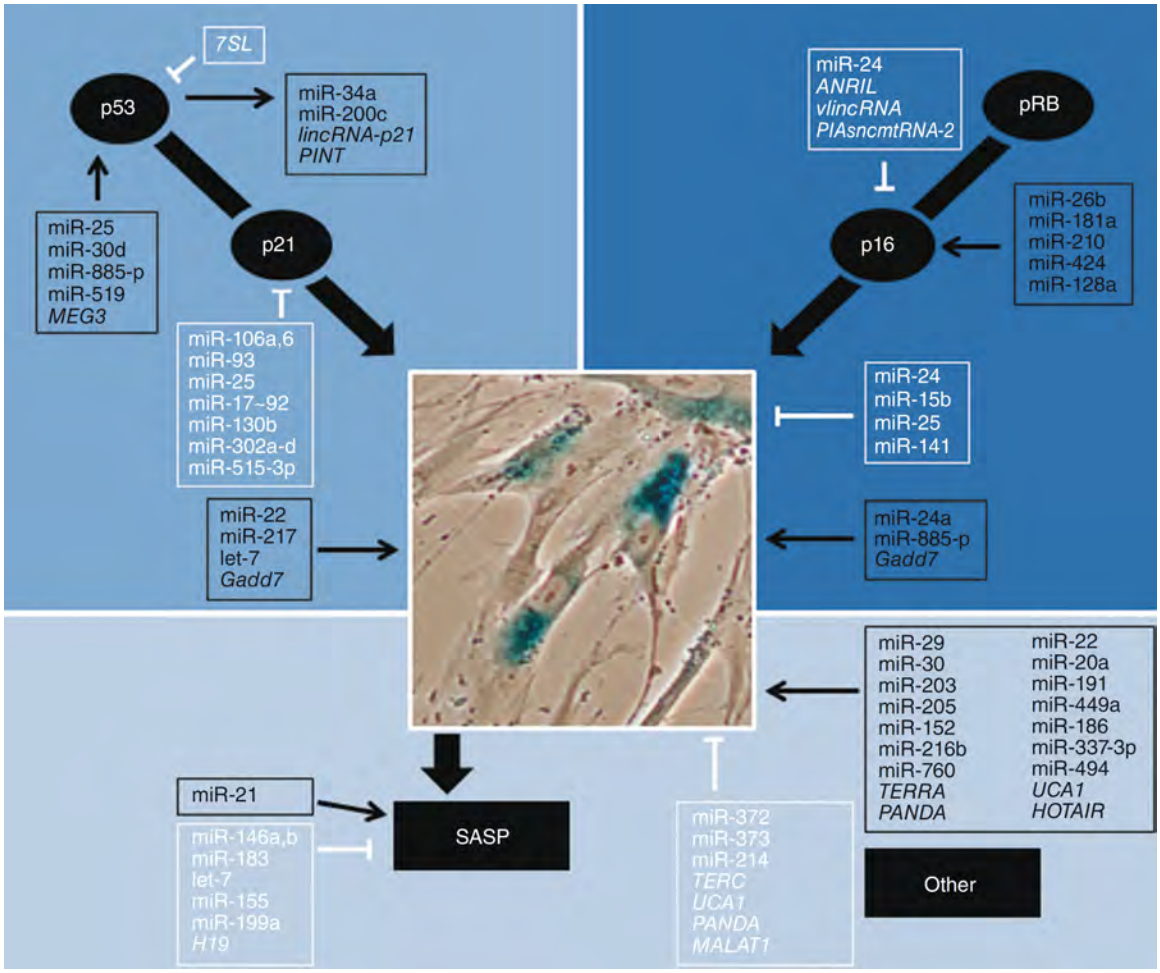

Fig.15 ncRNAs promoting and inhibiting senescence. Schematic representation of the main microRNAs and lncRNAs that promote (black) or inhibit (white) senescence phenotypes driven by p53/p21 (top left) pRB/p16 (top right), SASP and other mediators (bottom). Center, senescent fibroblasts displaying blue color indicative of SA-β-galactosidase (SA-β-gal) activity. (Abdelmohsen and Gorospe 2015).

Note: The figure in the review wrongly denotes vlinc as inhibitor of p16. It needs to be corrected to VAD (one of the vlincRNAs) as a inducer of p16 expression and hence acts as an mediator of in an in-vitro OIS model.

Part II: Results

Background, rationale and objectives of my PhD project in a nutshell

A work from Grewal's laboratory in 2009 revealed that in yeast (S.pombe) the histone variant H2A.Z suppresses antisense transcripts at convergent genes transcribed from opposite DNA strands, by favouring proper transcriptional termination. Indeed, H2AZ cooperates with heterochromatic factors to suppress these read-through antisense ncRNAs in a manner similar to the 3'-5' exonuclease subunit, Rrp6 (also known as EXOSC10 in mammals), of the RNA exosome complex (Zofall et al., 2009).

In mammals, whether such antisense transcripts (occurring by read-through transcription at convergent gene pairs) exist and how they are regulated is unknown. Interestingly, the depletion of the human H2A.Z histone variant and of the p400 ATPase, which mediates H2A.Z deposition in chromatin in mammals, is known to induce senescence (Gevry et al., 2007). We thus wondered if the regulation of particular antisense transcripts at convergent gene pairs occurs in senescence, if their regulation by H2A.Z is conserved in mammals and, if so, if a functional significance can be attributed to these transcripts.

Importantly, in a model of oncogene induced-senescence, and through the large use of chromosome or genome wide analyses of strand specific RNA expression, my PhD lab has shown that a ncRNA belonging to the vlincRNA (very long intergenic ncRNA) class is important for the maintenance of the senescent phenotype, uncovering the importance of long non coding RNA in this process (Lazorthes et al., 2015).

The general objective of my thesis is thus to investigate the involvement in cellular senescence of another class of ncRNAs, which would occur specifically at convergent gene pairs generating antisense transcripts through transcriptional read-through. More precisely, my research objectives are:

- To analyze if antisense RNAs at convergent gene pairs can be observed in senescence due to transcriptional termination defects.
- If yes, do these antisense RNAs imbibe regulatory properties.
- To determine if their regulation by H2A.Z (and others factors involved in their regulation in yeast) is conserved in humans.
- To study the function of some of such antisense transcripts in the induction and/or maintenance of senescence.

To this end we took advantage of an established *in-vitro* model of OIS developed by Carl Mann's group (Jeanblanc, M. et al., 2012), wherein WI-38 human fibroblast cell lines overexpressing hTERT harboured the following Estrogen Receptor (ER)-RAF-GFP. Upon oncogenic challenge by treatment of 4-HT(hydroxy-Tamoxifen), the activated RAF elicits a pertinent growth arrest response that mimics OIS induction *in-vivo*. Owing to an *in-vitro* model, any plausible effects that can be conferred by replicative stress attributed large to telomeric attrition has been ameliorated by the hTERT overexpressed while GFP facilitates to filter out the contextual cell heterogeneity.

Briefly, we analysed genome wide strand specific RNA-seq analysis of cells undergoing Oncogene Induced Senescence. This led us to identify numerous convergent gene loci associated with accumulation of transcripts downstream of the designated transcription termination site in senescent cells. These tran-

scripts extend to generate an antisense to the next gene located in the opposite strand of the convergent gene pair. We confirmed the RNA-seq data at two of such convergent loci. An RNAi based approach revealed that at least two of these transcripts are generated by transcriptional read-throughs. Hence we designated such pervasive transcripts as *S*enescence *T*riggered *A*ntisense *R*ead-through *T*ranscripts *(START)*. Importantly, we further found that the two STARTs for which we performed in depth studies repress the expression of the gene for which they are antisense. Finally, we demonstrate that the histone variant H2A.Z suppresses the accumulation of STARTs in proliferative cells. Since it also prevents senescence induction, this suggests that expression of START is important for cellular senescence. This observation was found to be conserved for most of the genes arranged in convergent orientation with a certain threshold of intergenic region. This has lead us to prose a model that RAF directed OIS is associated with loss of H2A.Z that leads to the wide spread accumulation of read-through transcripts owing to impaired termination control.

Transcriptional read-through as a novel mechanism generating regulatory antisense RNAs

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cellular senescence, read-through transcription, convergent genes, antisense RNAs, H2A.Z histone variant

Running title

Senescence-activated antisense read-through RNAs

Abstract

Antisense RNAs are non-coding RNAs generally produced from specific promoters. Here, by a combination of genome-wide approaches and in depth analysis at specific loci, we uncover in human cells undergoing senescence a family of antisense RNAs produced by transcriptional read-through at convergent protein-coding genes. Importantly, these antisense RNAs, that we call START RNAs, regulate the expression of their corresponding sense RNAs. Furthermore, their induction in senescent cells is due to the loss of H2A.Z-mediated repression. Our results uncover a novel mechanism of gene expression regulation, relying on the control of the expression of read-through transcripts at convergent genes.

Introduction

Senescence is characterized by a potent and irreversible cell cycle arrest (Campisi and d'Adda di Fagagna 2007). It is generally considered as a major anticancer pathway by preventing the accumulation of cells, which have lost their normal control of cellular proliferation (Braig et al. 2005; Chen et al. 2005; Xue et al. 2007; Brady et al. 2011). It is associated with the setting up of a specific genetic program. Recent findings have uncovered the importance of long non-coding RNAs (lncRNAs) as major players in the control of specific gene expression (Lee 2012). Long non-coding RNAs have been found to regulate gene expression in *cis* or in *trans*, mainly by allowing the correct structuration or targeting of chromatin modifying complexes, such as Polycomb group proteins. In addition, some non-coding RNAs can be antisense to protein-coding genes (Faghihi and Wahlestedt 2009; Khorkova et al. 2014). Such long

antisense RNAs can affect the expression of the protein encoded by the gene to which they are antisense by various mechanisms. These antisense transcripts are generally produced by the activation of promoters within or downstream of protein-coding genes. In few particular situations or loci, such as genetic mutants in yeast, they can also be produced by transcriptional read-through (loss of transcription termination) at convergent gene loci (Zofall et al. 2009; Zhang et al. 2011; Lee et al. 2013; Powell et al. 2013; Anver et al. 2014; Mizuguchi et al. 2014; Dhir et al. 2015). Here, we uncover a novel class of antisense RNAs produced throughout the genome by transcriptional read-through at protein-coding convergent genes and regulating the expression of their corresponding sense RNA in human senescent cells, therefore participating in the genomic response to oncogenic stress signaling.

Results & Discussion

In a previous manuscript, we showed the widespread occurrence of antisense transcription at 2 human chromosomes in WI38 primary fibroblasts undergoing oncogene-induced senescence (Lazorthes et al. 2015). To investigate how these antisense RNAs are produced and what could be their function, we performed and analysed strand-specific genome-wide RNA-Seq data in the same cell line (WI38 hTERT RAF1-ER) either proliferative or induced to senescence by the activation of an inducible oncogene. At some convergent gene loci, we observed the occurrence of antisense transcription activated in senescence, which could originate from a defect in normal transcription termination on the opposite genes. RNA-Seq data for two such examples are shown, the *LAMA2/ARHGAP18* and the *KIAA1919/REV3L* gene loci, with senescence-associated increase of antisense transcription at the *LAMA2* and *REV3L* genes, respectively (Fig. 1A,B). Indeed, while an increase of transcript expression occurred downstream the *ARHGAP18* or the *KIAA1919* gene, the expression of these genes

did not change in senescence, suggesting that an increase of read-through transcripts occurred in senescence at these loci. We thus focused on these two loci for in depth studies of the regulation and function of these RNAs, since *LAMA2* and *REV3L* expression were strongly repressed when their antisense RNAs were induced (Fig. 1A,B). Moreover, *LAMA2* and *REV3L* gene products are linked to senescence-associated processes or diseases. Indeed, mutation of *LAMA2* (Laminin, alpha 2), one of the subunits of merosin, a component of the extracellular matrix, causes congenital muscular dystrophy (Helbling-Leclerc et al. 1995), and REV3L (DNA polymerase zeta) is required for the maintenance of genomic integrity and cell proliferation (Lange et al. 2012).

The senescence-associated changes in the expression of protein-coding genes and of intergenic regions observed at these two loci by RNA-Seq experiments (Fig. 1B) were confirmed by random-priming reverse transcription followed by qPCR (qRT-PCR), (Fig. 1C) and by strand specific qRT-PCR for the sense and antisense transcripts (Fig. 1D).

We next tested whether the transcripts antisense to *LAMA2* and *REV3L* were produced by transcriptional read-through, that is were included in a long RNA originating from the promoter of the convergent gene (*ARHGAP18* and *KIAA1919*, respectively) and not from a distinct promoter located at close proximity to the 3' end of this gene. To this goal, we transfected senescent cells with siRNAs targeting the two ends of this putative RNA at the *ARHGAP18 / LAMA2* locus (that is the exon 1 of *ARHGAP18* or the intergenic region located between the two genes (See Fig. 2A)). Each of these two siRNAs decreased to the same extent the *ARHGAP18* pre-mRNA (measured in the last intron), the intergenic region and the transcript antisense to *LAMA2* (Fig. 2B,C), indicating that the transcript antisense to *LAMA2* is due to transcriptional read-through from the *ARHGAP18* gene. Interestingly, one of these siRNAs is a *bona fide ARHGAP18*-targeting siRNA (the one targeting exon 1 of

ARHGAP18), which as a consequence decreased *ARHGAP18* mRNA (Fig. 2A) but also the transcript antisense to *LAMA2*. Similar results were obtained using an siRNA targeting the intergenic region present between the *KIAA1919* and *REV3L* convergent genes (Fig. 2D). Taken together, these data indicate that at least on these two loci, antisense RNAs are generated by transcriptional read-through from the convergent gene. From now on, the gene from which the read-through is produced, such as *ARHGAP18* and *KIAA1919*, will be called the forward gene and the gene to which the read-through is an antisense, such as *LAMA2* and *REV3L*, will be called the reverse gene.

Antisense transcripts have been shown to regulate the expression of their corresponding sense genes (Khorkova et al. 2014). Interestingly, the expression of the *ARHGAP18* and *KIAA1919* read-through RNAs strongly increased in senescence. In parallel, the reverse genes (*LAMA2* and *REV3L*, respectively) were strongly repressed, suggesting that the two read-through RNAs could inhibit their expression. Accordingly, we found that depletion of the *ARHGAP18* read-through RNA using both siRNAs led to an increase of *LAMA2* pre-mRNA/mRNA (measured in an exon) and mRNA expression (measured in an exon-exon junction) (Fig. 2E). Strikingly, this increase was proportional to the decrease of *ARHGAP18* read-through RNA, but not of *ARHGAP18* mRNA (see data from Fig. 2B,C).

Importantly, very similar results were obtained when depleting the *KIAA1919* read-through RNA, with an increase in *REV3L* pre-mRNA and mRNA expression (Fig. 2F). Thus, these two antisense read-through RNAs possess the ability to inhibit the expression of their corresponding sense mRNAs.

As a consequence of this mechanism, the siRNA targeting exon 1 of *ARHGAP18* (a classical *ARHGAP18* siRNA) led to an increase of *LAMA2* expression (Fig. 2E) because it decreased the expression of the *LAMA2* antisense. Thus, one should be very cautious when interpreting

siRNA-based results: some effect attributed to the depletion of the protein could actually be due to the depletion of the read-through RNA, representing a new class of off-target effects. Importantly, similar effects would be obtained using other independent siRNAs, a control often used in siRNA-based experiments to rule out off-target effects.

We next investigated how these two read-through RNAs are repressed in proliferative cells. Data from yeast have uncovered several proteins involved in regulating antisense read-through accumulation at convergent gene loci, including H2A.Z and RNA-processing machineries (Zofall et al. 2009; Zhang et al. 2011; Lee et al. 2013; Anver et al. 2014; Mizuguchi et al. 2014). We found that depleting the histone variant H2A.Z using two previously validated siRNAs (Mattera et al. 2010) led to an increase in *ARHGAP18* and *KIAA1919* read-through RNAs in proliferative cells (Fig. 3A,B). These data thus indicate that H2A.Z represses transcriptional read-through RNAs at these two loci in proliferative cells. Interestingly, we found that H2A.Z expression levels strongly decreased upon senescence induction (Fig. 3C).

To analyse whether this global decrease in H2A.Z expression translates into a local decrease of H2A.Z occupancy around read-through RNAs, we performed H2A.Z ChIP-Seq experiments. As expected, we observed peaks of H2A.Z around transcription start sites, thus validating our ChIP-Seq data (Fig. 3D, see the *LAMA2* and *ARHGAP18* TSS and Supplemental Fig. S1 for metadata analysis of H2A.Z occupancy at TSS with respect to gene expression). Strikingly, we found that at the *LAMA2/ARHGAP18* locus, H2A.Z occupancy decreased in senescence throughout the region expressing the read-through RNA, whereas it was largely unchanged on the two convergent genes (*LAMA2* and *ARHGAP18*) (Fig. 3D). These data suggest that H2A.Z is located on the *LAMA2-ARHGAP18* intergenic region in proliferative cells to prevent accumulation of the read-through RNA. Altogether, our data

suggest that H2A.Z is a major factor controlling the expression of antisense read-through RNAs at convergent gene loci.

To test whether our findings could be extended, we analysed strand-specific RNA-Seq data to find all convergent gene loci on which transcriptional read-through activated (with respect to the forward gene) in senescence generates an antisense RNA. RNA polymerase II goes beyond the poly A site for a few hundred bases on all genes, a process which could interfere with the analysis we underwent. We thus restricted our analyses to loci with intergenic regions longer than 4 kb (which include the two loci we studied above). Indeed, this limit clearly separated two populations of senescence-activated read-through RNAs with respect to their regulation upon H2A.Z depletion (Supplemental Fig. S2). We therefore identified 68 convergent gene loci, which could harbour activated transcriptional read-through producing antisense RNAs (see Supplemental Fig. S3 for a schematic representation of the pipeline and Supplemental Table 1 for the list of these 68 loci).

Statistical analyses indicated that, like for *ARHGAP18* and *KIAA1919* read-through RNAs, transcriptional read-through at these 68 loci was significantly activated upon H2A.Z depletion in proliferative cells, since expression of the intergenic region was significantly activated compared to transcription of the forward gene (Fig. 4A). No such change was observed for loci harbouring antisense read-through RNAs repressed in senescence that we used as a control population. In addition, H2A.Z occupancy significantly decreased on their intergenic regions during senescence induction (Fig. 4B). Finally, the reverse genes from these 68 loci were significantly repressed in senescence (p value of the difference to 0, Wilcoxon test: $2.7*10^{-3}$) and we observed a de-correlation between changes in the expression of these genes and of their antisense transcripts (Fig. 4C), suggesting that the read-through RNAs belonging

to this population inhibit the expression of the reverse gene. Thus, Figure 4 data altogether allow us to identify a new family of antisense RNAs with features very similar to the *ARHGAP18* and *KIAA1919* read-through RNAs and characterized by the following properties:

- They are produced by senescence-induced transcriptional read-through at convergent protein-coding gene loci.
- The intergenic region between the two convergent genes is larger than 4 kb.

We call these RNAs "START RNAs" (for Senescence-Triggered Antisense Read-Through RNAs).

We report here the first observation of antisense RNAs produced by transcriptional read-through at human protein-coding convergent genes in a physiological context. These RNAs are common in senescence, representing a new family of antisense RNAs. We found that START RNAs can inhibit the expression of the gene to which they are antisense. Moreover, their expression is regulated during senescence progression. To the best of our knowledge, our data are the first demonstration of the regulation of specific gene expression through the controlled transcriptional read-though from a convergent protein-coding gene (see our model in Figure 4D). As such, we have uncovered a novel mechanism of gene expression regulation participating in the response to environmental changes.

Importantly, we found that START RNAs are repressed by H2A.Z in proliferative cells. Thus, repression of antisense read-through RNAs by the H2A.Z histone variant, previously described in *S. pombe* (Zofall et al. 2009), is thus likely conserved in human cells. The senescence-associated decrease in H2A.Z expression could therefore allow the regulation of a whole family of antisense RNAs, and, as a consequence, this could be a major mechanism

controlling the genetic program of senescence. Strikingly, depletion of H2A.Z is known to promote senescence (Gevry et al. 2007), supporting the hypothesis that START RNAs regulation could be important for senescence induction.

Although we have demonstrated its occurrence in senescence, regulation of gene expression by such a mechanism could be more common. Indeed, we identified 99 antisense RNAs with similar characteristics than START RNAs except that the transcriptional read-through is repressed in senescence. An interesting hypothesis would be that distinct sets of proteins regulate antisense read-through expression at different sets of convergent genes. In agreement with such an hypothesis, studies in yeast have uncovered other factors than H2A.Z involved in the control of transcriptional read-through at convergent genes (Zhang et al. 2011; Lee et al. 2013; Anver et al. 2014; Mizuguchi et al. 2014). Commitment into a given cell fate would as such induce a specific signature of antisense read-through RNAs that could participate in setting up the genetic program associated with this cell fate.

The mechanism of gene regulation we uncover here, without new initiation events of the transcription machinery, might be a simple and rapid way to respond to stress signals, such as oncogenic stress as shown here. In agreement with this hypothesis, osmotic stress induces a widespread read-through transcription in human cells (Vilborg et al. 2015) and in *S. pombe*, a global non-coding RNA response including antisense transcripts at convergent genes (Leong et al. 2014). Our findings thus suggest that stress-response genes or other classes of genes that need to be rapidly regulated upon environmental changes may be evolutionary selected to be convergent to other genes, allowing their rapid regulation by the mechanism we describe here. This could provide a basis for how positioning and orientation of genes within eukaryotic genomes are linked to the environment and are evolutionary conserved.

Materials and methods

Cell Culture

WI38 hTERT RAF1-ER cells were maintained in MEM supplemented with glutamine, non-essential amino acids, sodium pyruvate, penicillin– streptomycin and 10% fetal bovine serum in normoxic culture conditions (5% O_2). For induction of oncogene-induced senescence, cells were treated with 20 nM 4-HT (H7904, Sigma) for 3 days. siRNA transfection was performed using the Dharmafect 4 reagent (Dharmacon) according to the manufacturer's recommendations, except that 100 nM of siRNA was used and an equal volume of the culture medium was added 24 hours after transfection, as in Jeanblanc et al. (Jeanblanc et al. 2012). Cells were then harvested 48 hours later.

Antibodies and western blotting

GAPDH antibody (MAB 374) was purchased from Millipore. H3 (ab1791) and H2A.Z (ab4174) antibodies were purchased from Abcam.

Whole cell protein extracts were prepared using boiling buffer (1% SDS, 1 mM sodium vanadate, 10 mM Tris pH 7.4, 1% Triton, 0.5 M NaCl) supplemented with protease inbibitors (Complete, EDTA free, Roche) and phosphatase inhibitors (P5726 and P0044, Sigma) with sonication until the viscosity of the sample is reduced. Western blots were performed using standard procedures (primary antibody dilutions to 1/1,000 except for GAPDH antibody that was diluted 1/10,000).

RNA extraction, reverse transcription and qPCR

Total RNA was prepared using the MasterPure RNA Purification Kit (Epicentre Biotechnologies) supplemented with Baseline-ZERO DNAse (Epicentre) according to the

manufacturers' recommendation. RNA (200 ng) was used for each reverse transcription experiment. Strand-specific reverse transcriptions were performed at 55 °C using the Sensiscript and Omniscript enzymes (Qiagen) according to the manufacturer's recommendations. Each strand-specific reverse transcription was performed with one specific primer (LAMA2(e64)R for *LAMA2* sense, LAMA2(e64)F for *LAMA2* antisense, REV3L(INT32)R for *REV3L* sense and REV3L(INT32)F for *REV3L* antisense). In each experiment, we included a reverse transcription reaction without primer to monitor for DNA contamination. Non-strand specific reverse transcriptions were performed using random primers and Superscript III (Invitrogen) at 50 °C according to the manufacturer's recommendations. In each experiment, we included a control without the reverse transcriptase to monitor for DNA contamination. qPCR analysis was performed on a CFX96 Real-time system device (BioRad Laboratories) using the IQ SYBR Supermix (BioRad), according to the manufacturer's instructions. All samples were analysed in triplicates.

RNA-Seq

Total RNA was extracted as mentioned above and between 12 to 20 µg of RNA for each sample was submitted to BGI TECH SOLUTIONS (HONGKONG) for RNA sequencing. BGI treated the RNA by Ribozero kit to remove ribosomal RNA and sequenced by Illumina's HiSeq technology with at least 50M clean (reads after removing adaptor pollution and low quality sequence) paired-end reads per sample (lncRNA-seq). The strand-specific RNA-Seq method relied on UNG digestion of the second strand cDNA similary to strand-specific RNA-Seq performed by Parkhomchuk et al. and by the ENCODE project (Parkhomchuk et al. 2009; Djebali et al. 2012). 64,167,442 and 64,996,364 of paired-end reads were obtained for the RNA-Seq in proliferative cells and in senescent cells, respectively. 70,731,623 and

70,504,813 of paired-end reads were obtained for the RNA-Seq in siCtrl-treated cells and in siH2A.Z-treated cells, respectively.

ChIP-Seq

ChIP was performed as previously described (Lazorthes et al. 2015). 10 ng of immunoprecipitated DNA was submitted to BGI for sequencing. Samples were sequenced by Illumina's HiSeq technology with at least 40M clean single-end reads per sample. 61,088,665 and 49,960,563 of reads were obtained for the ChIP-Seq H2A.Z in proliferative cells and in senescent cells, respectively.

Statistical tests

For each list of log2ratio obtained as described in the Supplemental methods (per couple of datasets and per part of the read-through pairs (forward gene, intergenic region, reverse gene and antisense part of the read-through)), we applied the statistical test of Shapiro to determine whether the list of ratios is normally distributed (p-value > 0.05) or not normally distributed (p-value < 0.05). To compare 2 lists, if at least one of the lists is not normally distributed we applied the Mann-Whitney-Wilcoxon test, otherwise we applied the Student t test (Student t-test if variances from the two lists are homogenous, Welch t test if not). In both case if the p-value < 0.05 the mean of the 2 lists of ratios are significantly different. When we compared two different parts (forward gene, intergenic region, reverse gene and antisense part of the read-through) for the same list of read-through pairs, we applied the paired version of these tests. To test whether the mean of a list of ratios is significantly different from 0, we applied these same tests (Mann-Whitney test if the list of ratio is not normally distributed, otherwise Student test) with a theoretical distribution of mean equal to 0. In both cases, if the p-value < 0.05, the mean of the list of ratios is significantly different from 0.

siRNA and primers

siRNAs and primers were purchased from Eurogentec. The sequences are described in Supplemental Table 2.

Bioinformatic analysis

Boxplots, metadata and analysis of activated read-throughs using RNA-Seq senescence and proliferation are described in the Supplemental methods.

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Legends to Figures

Figure 1: Examples of two convergent gene loci with putative antisense transcripts generated by transcriptional read-through

(A) WI38 hTERT RAF1-ER cells, which are immortalized by hTERT expression and contain an inducible RAF1 oncogene fused to an estrogen receptor (ER), were induced or not to enter senescence by 4-hydroxy-tamoxifen (4-HT) addition for 3 days. Total RNA was extracted and subjected to strand-specific RNA-Seq experiments. RNA-Seq data are shown for the *LAMA2/ARHGAP18* and *KIAA1919/REV3L* convergent gene loci. Transcript variants from Ref Seq, visualized in UCSC browser, are also indicated. The two regions indicated by dotted arrows correspond to putative read-through RNAs whose expression increases in senescence. *(B)* The total number of reads from strand-specific RNA-Seq data in the indicated genomic regions in proliferative and senescent cells were calculated for the two loci. The log2 of the Sen/Prolif value is plotted. The chromosome strand of the analysed region is annotated. The values for the gene from which the read-through originates, the intergenic region of the read-through, the read-through entire domain, the antisense (AS) part of the read-through (AS part to *LAMA2* or *REV3L*) and the gene (sense, *LAMA2* or *REV3L*) to which the read-through is

antisense are shown. *(C)* Same as in (A), except that total RNA was subjected to random qRT-PCR using the indicated primers: e1 for exon 1, e64-e65 for the exon64-exon65 junction. Data are normalised to *GAPDH* mRNA expression. The mean and standard deviation from 3 independent experiments are shown. *(D)* Same as in (A), except that total RNA was subjected to strand-specific qRT-PCR and analysed using the indicated primers. Data were normalised to *GAPDH* mRNA expression. The mean and standard deviation from 3 independent experiments are shown.

Figure 2: *LAMA2* and *REV3L* antisense transcripts are generated by transcriptional read-though and are regulatory antisense RNAs

(A) Schematic representation of the *LAMA2/ARHGAP18* and *KIAA1919/REV*3L loci with the location of siRNAs (orange) and PCR primers (purple). *(B)* Senescent WI38 hTERT RAF1-ER cells were transfected using an siRNA targeting *ARHGAP18* exon 1 (ARHG e1) or control (Ctrl). 72 hours after transfection, total RNA was extracted and subjected to random qRT-PCR using the indicated primers (left) or analysed by strand-specific qRT-PCR to monitor the expression of the region antisense to *LAMA2* (right). Data are normalised to *GAPDH* mRNA expression. The mean and standard deviation from 3 independent experiments are shown. *(C)* Same as in (B), except that cells were transfected using an siRNA targeting the read-through region (ARHG R-th). *(D)* Senescent WI38 hTERT RAF1-ER cells were transfected using an siRNA targeting *KIAA1919* read-through region (KIAA R-th). 72 hours after transfection, total RNA was extracted and subjected to random qRT-PCR using the indicated primers (left) or analysed by strand-specific qRT-PCR to monitor the expression of the region antisense to *REV3L* (right). Data are normalised to *GAPDH* mRNA expression. The mean and standard deviation from 3 independent experiments are shown. *(E)* Same as in (B) and (C), except that *LAMA2* (pre-mRNA/mRNA) sense expression was monitored by

strand-specific qRT-PCR in an exon (left panel) and the spliced *LAMA2* mRNA expression was assessed by random qRT-PCR (right panel). *(F)* Same as in (D), except that *REV3L* (pre-mRNA) sense expression was monitored by strand-specific qRT-PCR in an intron (left panel) and the spliced REV3L mRNA was assessed by random qRT-PCR (right panel).

Figure 3: *ARHGAP18* and *KIAA1919* read-through RNAs are repressed by H2A.Z in proliferative cells

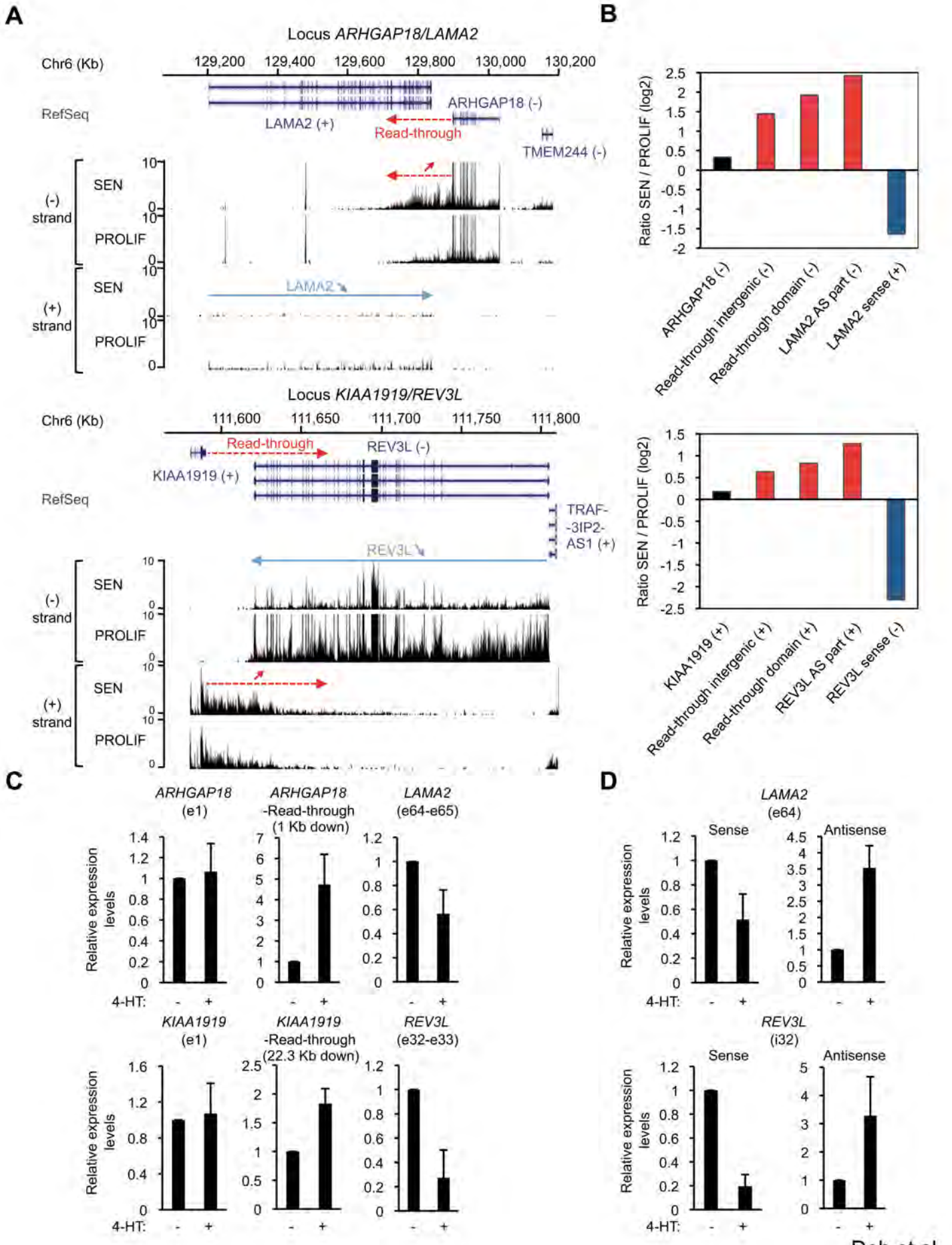
(A) Proliferative WI38 hTERT RAF1-ER cells were transfected using the indicated siRNAs. 72 hours following transfection, total RNA was prepared and subjected to random qRT-PCR using the indicated primers. Data are normalised to *GAPDH* mRNA expression. The mean and standard deviation from 3 independent experiments are shown. *(B)* Same as in (A), except that the H2A.Z#2 siRNA was used. *(C)* WI38 hTERT RAF1-ER cells were induced or not to enter senescence by 4-HT addition for 3 or 6 days, as indicated. Total cell extracts were analysed by western blot using the indicated antibodies. *(D)* WI38 hTERT RAF1-ER cells were induced or not to enter senescence by 4-HT addition for 3 days and subjected to a ChIP-Seq experiment using H2A.Z antibodies. ChIP-Seq data showing H2A.Z enrichment at the *LAMA2/ARHGAP18* locus in proliferative (bottom) and senescent (top) cells are shown.

Figure 4: The properties of *ARHGAP18* and *KIAA1919* read-through RNAs can be generalized

(A) Proliferative WI38 hTERT RAF1-ER cells were transfected using the H2A.Z#1 or the Ctrl#1 siRNA. 72 hours following transfection, total RNAs were prepared and then depleted of ribosomal RNA and sequenced. For the 68 activated read-through RNAs generating an antisense RNA at convergent gene pairs with intergenic region > 4 kb, the log 2 of the variation upon H2A.Z depletion (log2(siH2A.Z/siCtrl)) of the expression of the forward gene

(the gene from which the read-through RNA originates) and of the intergenic region were computed. The boxplots show the median, the 1st and 3rd quantiles and the highest and lowest values (excluding outliers) of the two populations. Note the significant increase (p value = $1.8 * 10^{-3}$, paired Wilcoxon test) of the expression of the intergenic regions compared to the forward genes upon H2A.Z depletion. On the right, the same analysis is shown for the 99 loci with repressed read-through RNAs (ns: not significant). *(B)* Proliferative and senescent WI38 hTERT RAF1-ER cells were subjected to an H2A.Z ChIP-Seq analysis. Boxplots showing the log2 of the variation in senescence (log2(sen/prolif)) of H2A.Z occupancy at the indicated regions are shown after standardization. Boxplot for the TSS of the 25% most expressed genes (TSS high) is shown as control. For the 68 activated read-through RNAs producing antisense RNAs at convergent gene pairs with intergenic region > 4 kb, boxplots of the log2(sen/prolif) of H2A.Z occupancy are shown for the forward gene TSS and the intergenic regions. Note that H2A.Z occupancy significantly decreased in senescence on the forward gene TSS and on the intergenic regions (p values of the difference to 0 being $3.3*10^{-6}$ (Wilcoxon test) and $2.0*10^{-8}$ (Student t test), respectively). It also decreased significantly more on the intergenic regions of the activated read-through RNAs (p value = 0.0064, Wilcoxon test) than on the TSS of the forward genes. *(C)* WI38 hTERT RAF1-ER cells were induced or not to enter senescence by 4-HT addition for 3 days. Total RNA was extracted and subjected to strand-specific RNA-Seq experiments. For the 68 activated read-through RNAs producing antisense RNAs at convergent gene pairs with intergenic region > 4 kb, we removed the loci for which the reverse gene is not expressed either in senescence or in proliferation condition, resulting in 58 loci. The log 2 of the variation in senescence (log2(sen/prolif)) of the expression of the antisense parts of the read-through RNAs and of the reverse genes (the genes to which the read-throughs are antisense) were computed and represented as a boxplot. Note the significant decrease (p value = $1.09 * 10^{-5}$, paired

Wilcoxon test) of the expression of the reverse genes compared to their antisense parts of the read-through RNAs. *(D)* Our working model of START RNA regulation and function. In senescence, loss of H2A.Z in the intergenic region between two convergent genes triggers an increase of read-through transcripts from one gene of the convergent gene pair (gene B or also called forward gene in this study), making an antisense to the other gene of the convergent gene pair (gene A or also called reverse gene in this study). START RNAs repress the convergent genes to which they are antisense.



Deb et al., Figure 1

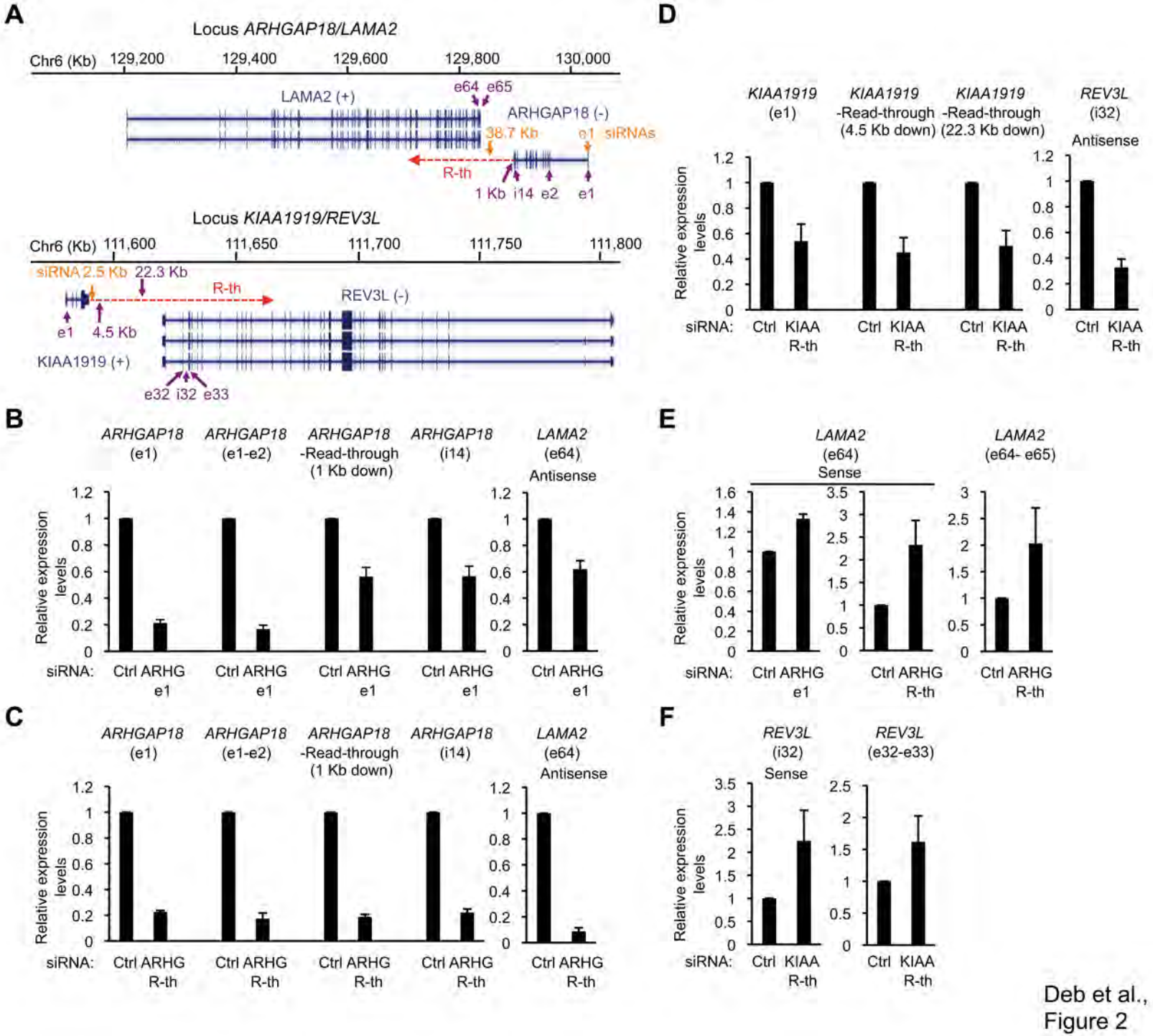


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Figure 2

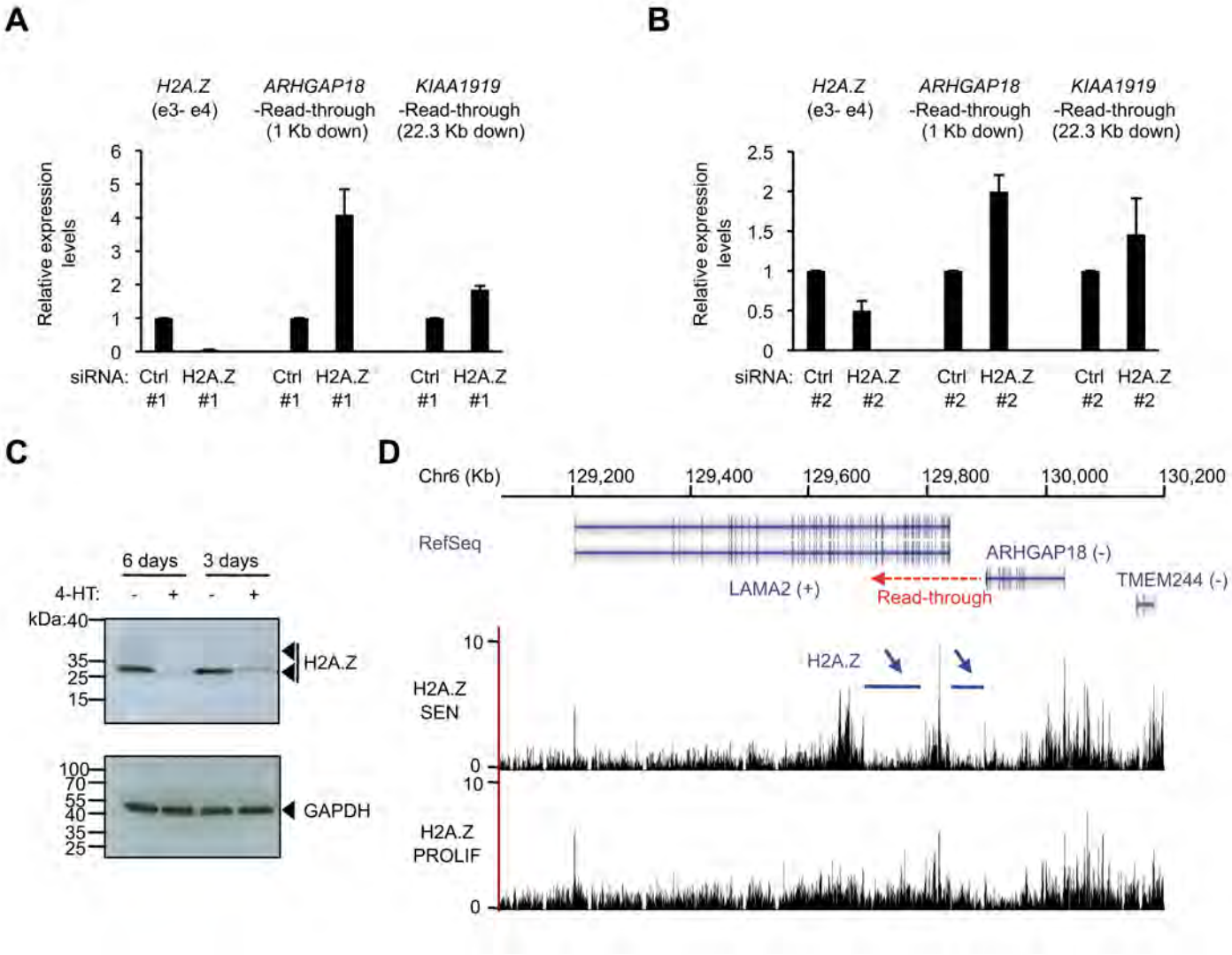

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Figure 3

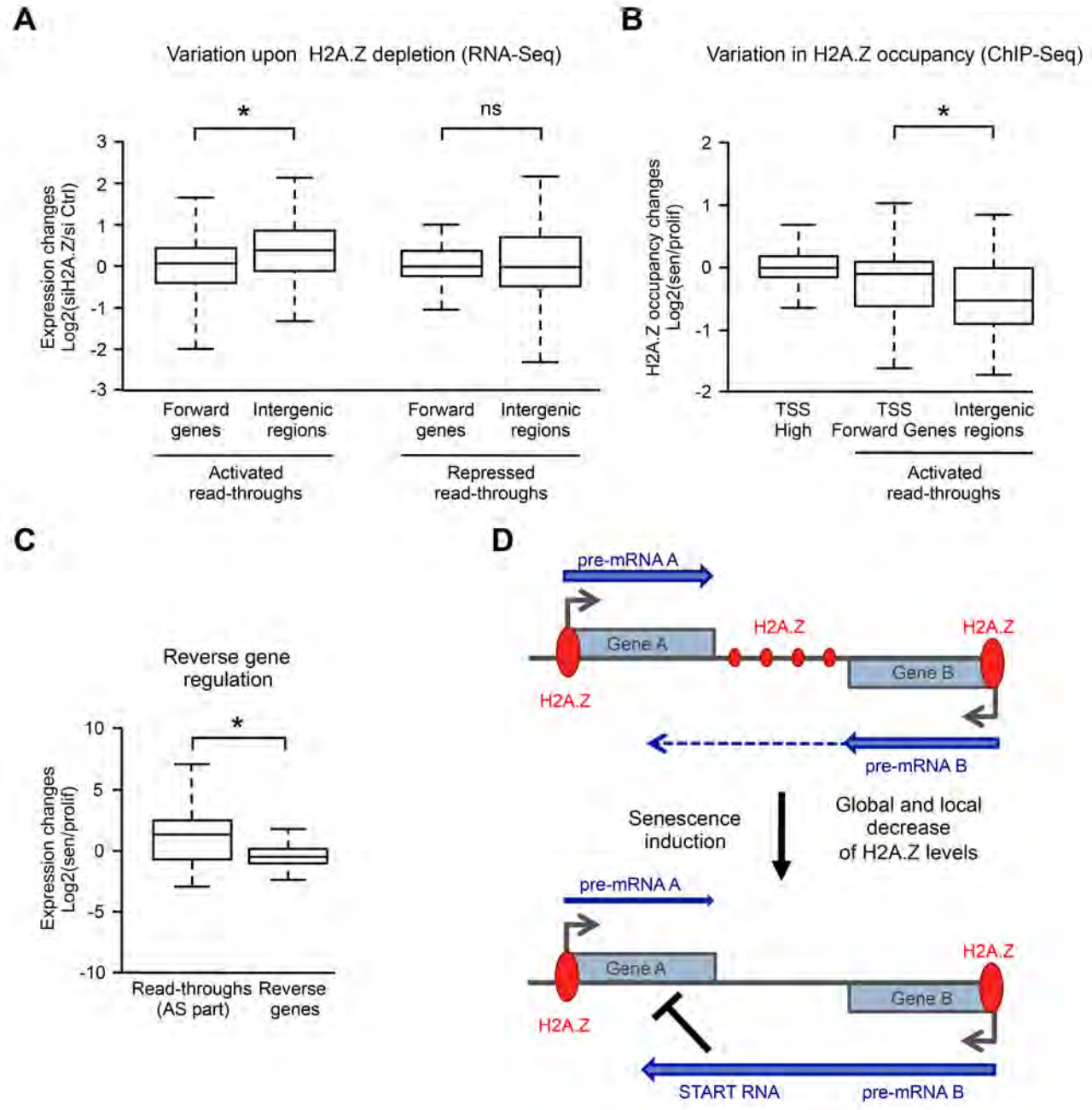

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Figure 4

Deb et *al.* Supplemental methods

High-throughput sequencing and bioinformatic analysis

ChIP-Seq and RNA-Seq datasets (FASTQ files) were mapped to the human genome build (hg19). We aligned the single-end reads of the 2 H2A.Z ChIP-Seq (senescence and proliferation) data via Burrows-Wheeler transformation (*bwa*) version 0.6.2-r126 and we aligned paired-end reads of the 4 strand-specific RNA-Seq (senescence, proliferation, siH2AZ and siCtrl) data using Spliced Transcripts Alignment to a Reference (*STAR*) version 2.4.0. For these alignments all parameters are kept as default.

After the alignment, we applied on all aligned datasets several steps using *samtools* software: we converted aligned file from Sequence Alignments/Map (sam) format into the Binary Alignment/Map (bam) format which stores the same data in a compressed, indexed, binary form. We sorted data by position on the genome, removed PCR duplicates (i.e. reads mapped at the exact same position on the genome) and created an index of each bam file (bai format). We then converted these cleaned files in wiggle files using *R* via the *rtracklayer bioconductor package*. We obtained the following numbers of aligned reads: 37,405,144 (paired-end, RNA-Seq in proliferation), 35,253,773 (paired-end, RNA-Seq in senescence), 55,952,673 (paired-end, siCtrl RNA-Seq), 56,299,579 (paired-end, siH2A.Z RNA-Seq), 40,730,057 (H2A.Z ChIP-Seq in senescence) and 51,574,290 (H2A.Z ChIP-Seq in proliferation). We applied on each dataset a normalization by these total numbers of aligned reads.

The ChIP-Seq and RNA-Seq data were visualized and explored using the Integrative Genome Browser (IGB) or the UCSC genome browser.

Identification of activated read-throughs using RNA-Seq data from senescence and proliferation

1) Finding transcripts regulated in senescence

This analysis is schematized in the Supplemental Fig. 3. We first applied a succession of steps in order to determine all transcribed domains regulated in senescence existing in the two datasets (RNA-Seq in proliferation and in senescence). First, for each dataset we divided the entire genome into intervals of 200 bp. For each interval with a density (i.e., total number of aligned reads per base on the interval /length of the interval) lower than 1, we set the density at 1 by manually setting the total number of aligned reads per base at 200. We did such a correction in order to avoid dividing by zero or a number close to zero, which could distort our computation of the log2 ratio Senescence/Proliferation. After this step, we computed, for each interval, the log2 ratio of the sum of the number of reads aligned per base in Senescence over the sum of the number of reads aligned per base in Proliferation. We computed this ratio for both strands (+) and (-). Because we were looking for domains regulated in Senescence, we kept intervals with an absolute log2 ratio higher than 1. For each strand, we then merged intervals with ratios of the same sign when they were closer than 5 kb. We re-computed the log2 ratio Senescence over Proliferation for these new domains and removed domains shorter than 1 kb to restrict the analysis to long transcribed regions. We next merged again domains with ratios of the same sign when they were closer than 5 kb. We re-computed the log2 ratio Senescence over Proliferation for these final domains and we selected domains with an absolute log2 ratio higher than 0.5. This final list of domains represented transcripts regulated in senescence and was crossed with the list of convergent genes in step 3.

2) Finding convergent gene loci

We downloaded from the UCSC table browser the database hg19 (Feb. 2009 GRCh37/hg19), group Genes and Gene Prediction, track RefSeq Genes, table RefFlat containing all natural mRNAs and non-coding RNAs. For each gene (unique gene name), if the gene mapped at one location on the genome with only one variant, we kept for this gene one line with the coordinates of the gene. If the gene mapped at different places in the genome, we kept one line per position and counted the gene as many times as it mapped in different locations. If the gene mapped at one location but with more than one variant, we kept one line for the locus of the gene, the coordinates starting at the first start of the variants and ending at the last end of the variants. From this database, we removed HIST genes and we selected convergent genes separated by less than 10 Mbp. We ended with a list of convergent gene loci that we used for step 3.

3) Identifying START RNAs

To select antisense read-throughs, we kept pairs of convergent genes when there was at least one domain present 500bp after one of the 2 genes and overlapping on the other gene in the antisense orientation. When read through occurred on both strands, we kept genes twice, one for each strand. Then, we cleaned our selection by removing read-throughs when the convergent genes overlapped and when at least one of the genes was a MIR or a LOC gene. We ended with 592 convergent gene pairs with a read-through from one of the convergent genes generating an antisense to the other gene. We then selected activated read-throughs with respect to the forward gene, that is when the subtraction between the log2 ratio (number reads senescence / number reads proliferation) on the antisense part of the read-through and the log2 ratio (number reads senescence / number reads proliferation) on the forward gene was higher than 0. Finally, we removed read-throughs when the intergenic region between the

2 convergent genes was less than 4 kb, ending with 68 senescence-triggered antisense read-through RNAs (START RNAs).

Boxplots and metadata

Figures and statistical test were done using R.

We computed the average number of reads per base pair on +/-1kb around the TSS of the forward genes and on the intergenic regions in the H2A.Z ChIP-Seq in senescence and in proliferation datasets. We next computed the log2ratio (mean number of reads per base pair in senescence + 0.01 / mean number of reads per base pair in proliferation + 0.01). To standardize ChIP-Seq signal levels between senescence and proliferative datasets, we computed the log2ratio (mean number of reads per base pair in senescence +/-1kb around the TSS for the 25% most expressed genes (the 5342 genes that were present in the highest expressed classes of both proliferation and senescence sets of data from Fig. S1) / mean number of reads per base pair in proliferation +/-1kb around the TSS for the 25% most expressed genes) and we subtracted that factor to the log2ratios obtained in senescence dataset. We made the boxplots without outliers of the standardized ratios. Ratios were considered outliers if they were lower than 1st quantile – 1,5x(3rd quantile – 1st quantile) or higher than 3rd quantile + 1.5x(3rd quantile – 1st quantile).

We also computed the average number of reads per base pair on the forward genes and on the intergenic regions in the siH2AZ and siCtrl RNA-Seq datasets, as well as on the antisense parts of the read-throughs and on the reverse genes in the senescence and proliferation RNA-Seq datasets. Then we computed the log2ratio (mean number of reads per base pair in siH2AZ + 0.01 / mean number of reads per base pair in siCtrl + 0.01) and the log2ratio (mean number of reads per base pair in senescence + 0.01 / mean number of reads per base pair in

proliferation + 0.01)). For each category of read-throughs, we made boxplots without outliers of these ratios.

For metadata analysis of H2A.Z enrichment at +/-1kb around the TSS, we first divided the genes in four equal classes based on their expression in the RNA-Seq datasets in senescence and in proliferation. Genes smaller than 1 kb were removed. For each base in the region +/-1kb around the TSS, we computed the average number of reads for all the genes in the different classes (low, medium low, medium high and high) for both senescence and proliferation ChIP-Seq datasets.

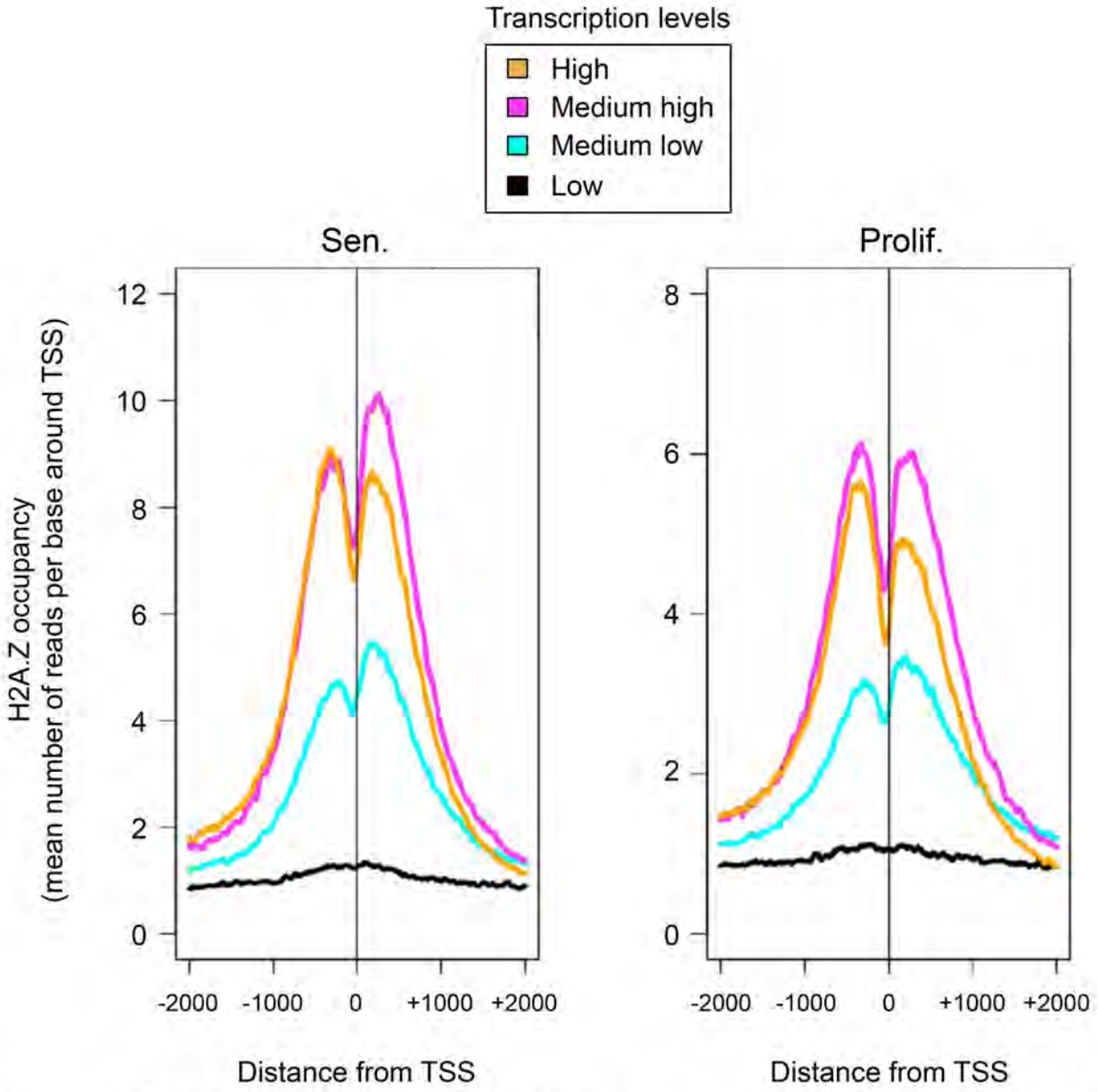


Figure S1: Metadata analysis of H2A.Z occupancy at TSS with respect to gene expression

Proliferative and senescent WI38 hTERT RAF1-ER cells were subjected to a ChIP-Seq experiment using H2A.Z antibodies. Genes were sorted in four identical classes with respect to the mean of the RNA-Seq signal on the gene body. Metadata analyses of H2A.Z occupancy around TSS are shown for the four classes of genes (black/blue/pink/orange from the less expressed to the more expressed) using data obtained from senescent (left) and proliferative (right) cells. Proliferative and senescent H2A.Z ChIP-Seq datasets for Figure 4 analysis were standardized according to the difference of the 25% most expressed genes signals. Note that the ChIP-Seq data show an increase of H2A.Z occupancy with respect to the expression levels, in a similar way to what has been previously published (Barski et al. 2007).

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. 2007. High-resolution profiling of histone methylations in the human genome. *Cell* **129**: 823-837.

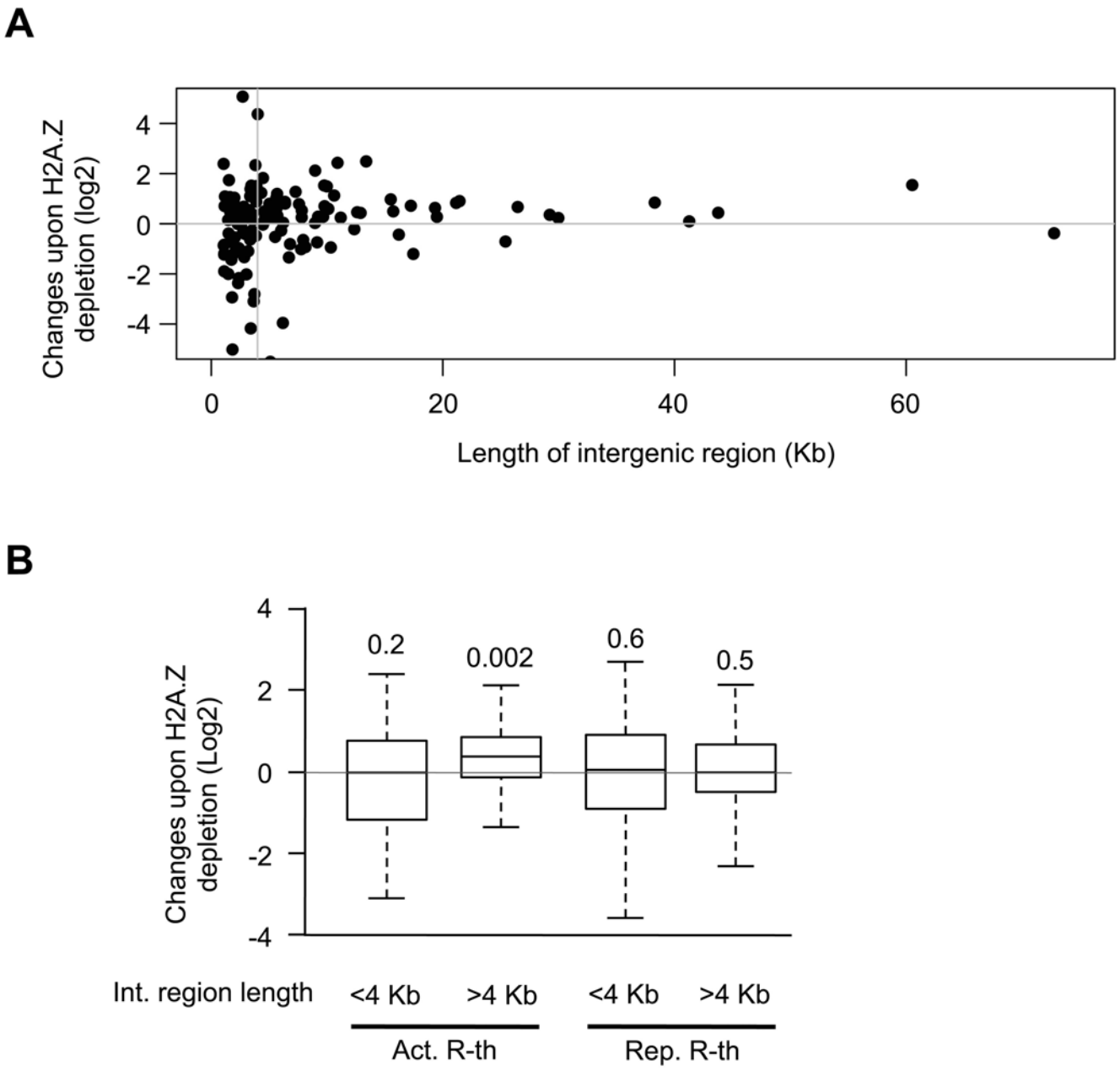


Figure S2: The 4 Kb-limit of the intergenic region separates two populations of activated antisense read-through RNAs

A) Proliferative WI38 hTERT RAF1-ER were transfected using the H2A.Z #1 or the Ctrl #1 siRNA. 48 hours following transfection, total RNAs were prepared and then depleted of rRNAs and sequenced. We computed the expression change of the intergenic region upon H2A.Z depletion (siH2A.Z/siCtrl) for each activated read-through with an intergenic region larger than 1 Kb. The dot plot shows this value plotted against the size of the intergenic regions for each activated read-through RNA. A vertical lane at 4 Kb of the intergenic region is also plotted. B) Activated antisense read-through RNAs (Act. R-th) were separated in two populations with respect to the length of the intergenic region, as indicated. The log 2 of the variation upon H2A.Z depletion were computed for each locus. The box plots show the median, the 1st and 3rd quantiles and the highest and lowest values (excluding outliers) of the two populations. On the right, the same analysis is shown for the loci with repressed read-through RNAs. The p-values of the difference to 0 (Wilcoxon test) are indicated above each box plot.

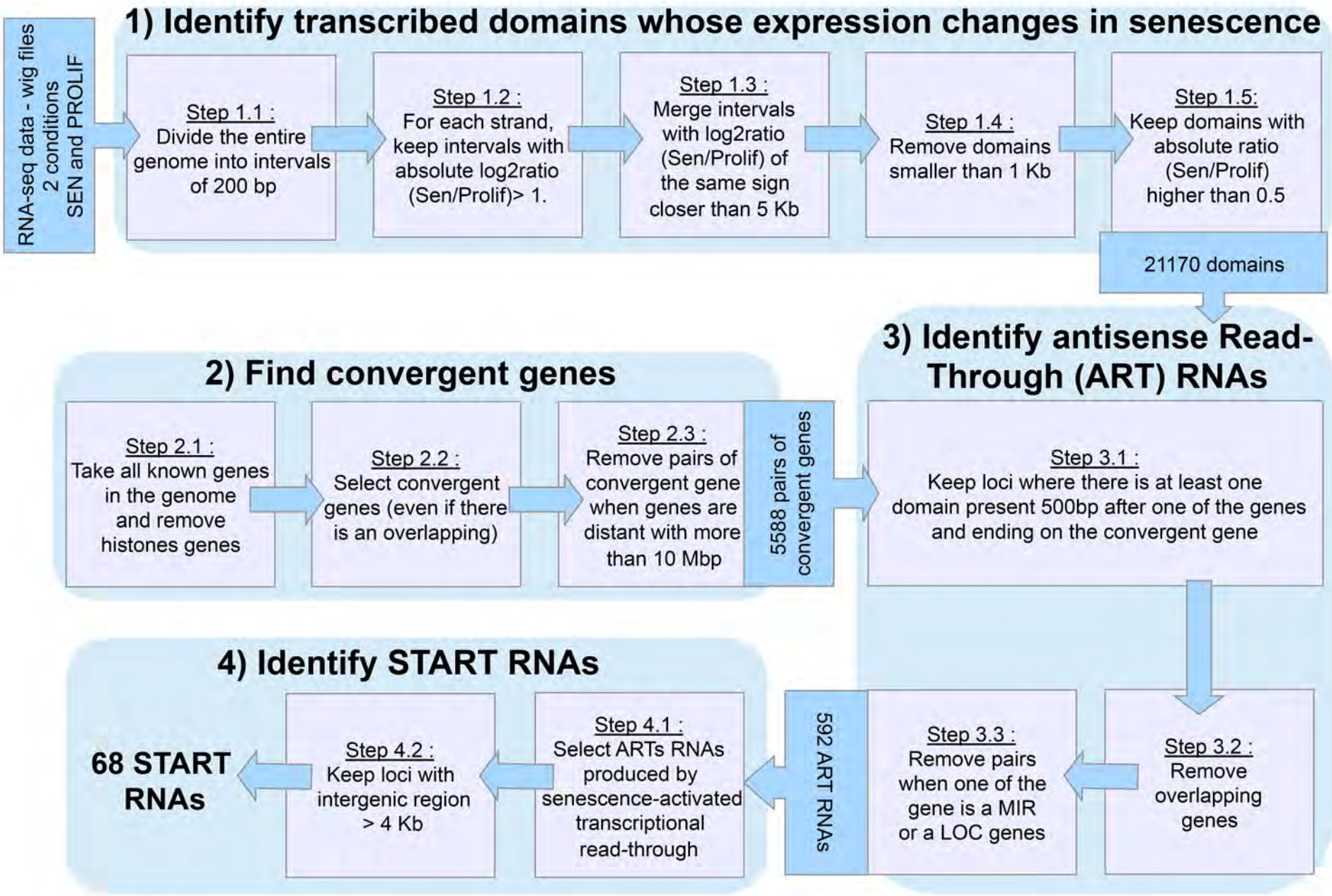


Figure S3: Schematic representation of the pipeline leading to START characterization

Deb et al.

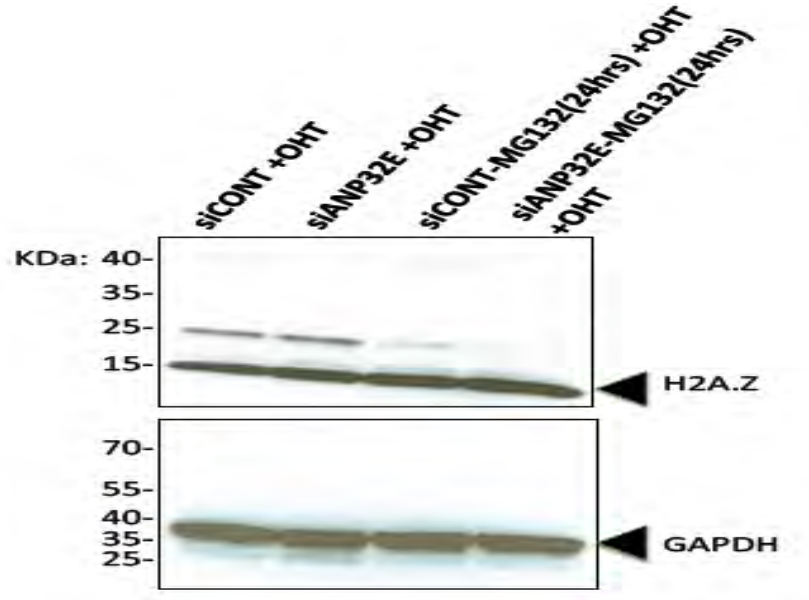
Additional data

Once we confirmed that induction of cellular senescence is associated with loss of H2A.Z and that this feature connotes accumulation of *STARTs*, we wanted to delineate the underlying mechanism of H2A.Z loss upon senescence induction and if perturbing the mehansim can affect the generation of *STARTs.*

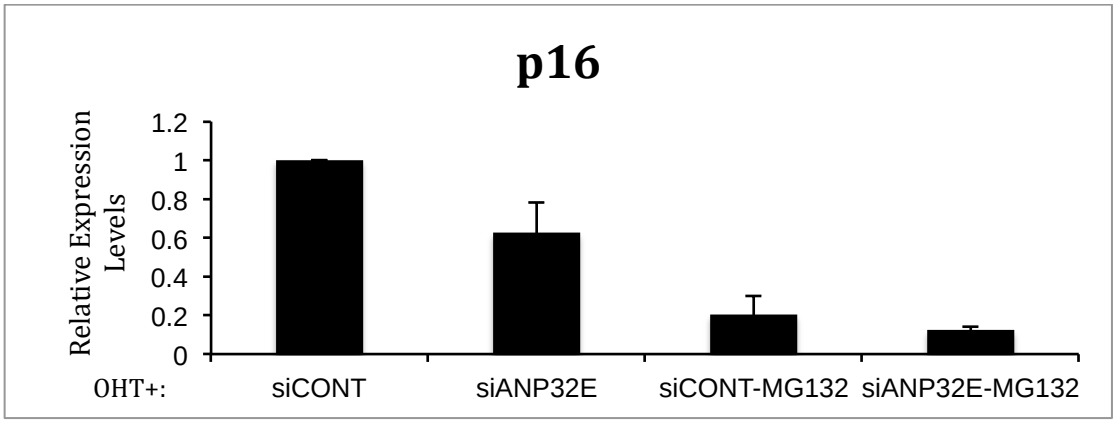
As outlined earlier (in the introduction) senescent cells exhibit SAPD (Senescence Associated Protein Degradtion) a phenotype mediated by UPP (Ubiquitin Proteasome Pathway). Additionally it has also been shown that ANP32E is a histone chaperone that removes H2A.Z from chromatin (Obri et al., 2014). Hence we reasoned that H2A.Z probably undergoes proteasomal degradation in cells that senesce besides the possibility of elevated ANP32E levels driven H2A.Z elimination upon senescence induction cannot be ruled out. To this end, I treated proliferative cells individually with MG132, an ubiquitin-proteasome inhibitor and with siRNA against ANP32E an then induced senscence. Although each of the treatment indeed led to an appreciable bypass of senscent mediated H2A.Z loss, however the combinations of treatments lead to a profound H2A.Z retainment (Fig.16a). This suggests that ANP32E and ubiquitin proteasome degradation mediates loss of H2A.Z upon induction of cellular senescence. Transcriptome analysis of these cells using primers designed at 1kb down stream of ARHGAP18 (intergenic region between ARHGAP18 and LAMA2) surprisingly revealed loss of ARHGAP18 associated *START* that paralleled the decrease in p16 expression levels (Fig.16b). These results suggest that perturbing the mechansim that drives

loss of H2A.Z in cells undergoing senescence is sufficient to impair senescence induction and concomitantly suppress senescence associated read-through transcripts.

a)



b)



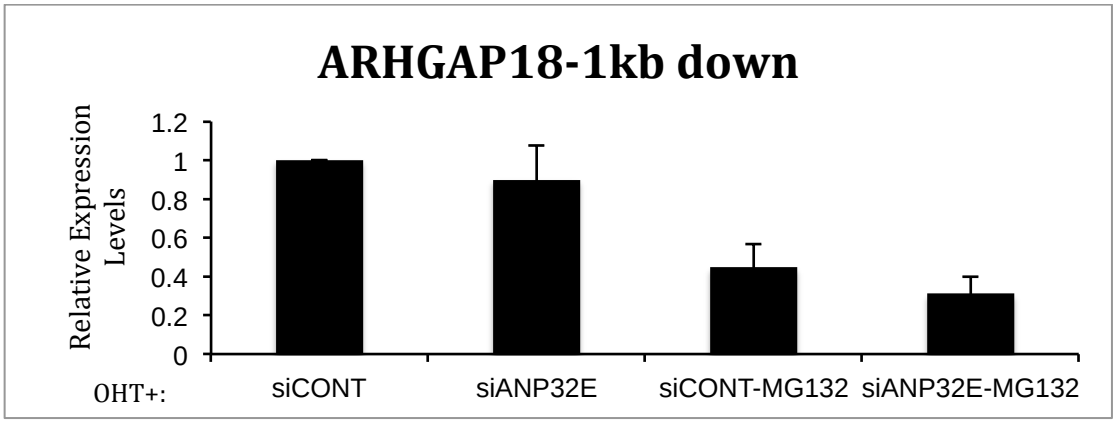


Fig.16 a) Simultaneous depletion of ANP32E and MG132 treatment followed by growth arrest induction leads to rescue of H2A.Z protein levels and b) parallels concomitant suppression of read-through transcripts and CDKi.

Part III: Discussion

Discussion on various facets of my PhD project

This GWAS based on a model of OIS takes advantage strand specific RNA-seq approach that lead to the identification of plethora of putative convergent gene loci with in-competent transcription termination based on validation of 2 such loci. This study has shown for the first time how a histone variant H2A.Z, plays an integral role of RNA surveillance machinery and thereby safeguards the genome from the wide spread accumulation of antisense transcripts in mammals. These antisense transcripts are unique in a way that they get generated due to impaired termination of transcription of a gene located in a convergent orientation to another gene on the opposite strand. As these impaired termination mediated generation of antisense RNAs are largely found to be associated to convergent genes, they are generally termed as read-through transcripts. These antisense RNAs are due to impaired termination, and are thus termed START (Senescence Triggered Antisense Read-through Transcripts). However there are certain caveats to this study that needs to be acknowledged for the further evolution of this field of convergent loci associated antisense RNAs towards its implementation in translational research and ultimately for therapeutics.

A putative senescence landscape

OIS refers to a specific phenomenon wherein cells enter a state of irrevocable cell cycle arrest in response to diverse oncogenic insults, thereby representing as one of the major fail-safe mechanisms that counteract tumorigenesis. Various intricate works from different laboratories investigating cellular senes-

cence *in-vivo* have shown that upon establishment of senescence, the 0cells continue to resists oncogenic insults that attempts to force cell cycle re-entry even upon depletion of potent Cyclin Dependent Kinase inhibitors (CDKi) like p16 and p53. Given the fact that cellular senescence is inherently an *in-vivo* physiological phenomena, and its reversibility have not been proven in *in-vivo* settings, it is but critical that any *in-vitro* models that are developed to study various facets of cellular senescence should adhere to this gold-standard criteria of irreversibility. *In-vitro* OIS model used in this study takes the advantage of Tamoxifen mediated activation of oncogene RAF that facilitates the induction of a state of a cell cycle arrest that mimics a senescent cell. To rule-out the plausible interference of replication stress in mediating this arrest, hTERT has been overexpressed. Hence this model should not be considered as physiological state per se but a model that mimics a physiological state. Moreover the fact that the loss of p21 and p16 has been reported to reverse this OIS sheds considerable doubt on its claim in attaining the state of cellular senescence (Jeanblanc, M. et al., 2012). Whether this model represents a genuine senescence model or just reflects a growth arrested non-senescent population- a sort of quasi-steady growth arrested state, remains uncertain. Alternatively it is possible that these cells might have entered a sort of dormant growth arrested ("quiescent like") state and re-enters cell cycle upon conducive cellular environment. In this regard a recent review of Sharpless and colleagues (Sharpless and Sherr 2015) states "Perhaps the field should resist the convenience of what may be an inadequate catch-all definition and restrict the term senescence (as we have) to depicting states of irreversible stress-induced growth arrest' and further suggests "whenever possible, sub-

stitution of more accurate descriptors to define the phenotypes under study would prove far less confusing. Specific terms like cell cycle arrest, secretory activity, paracrine signaling, chromatin remodeling and others are more illuminating. As for associated molecular events, the detection of levels of $p16^{INK4A}$ and $p21^{CIP1}$, states of RB phosphorylation and telomere length, DNA-damage foci and secretion of specific cytokines are indisputable experimental findings that do not require extensive qualification. Rather than applying the term senescence to what are clearly complex and possibly distinct cellular states, we should aim for greater clarity by using judicious choices of more explicit terms. The senescence field would profit from greater precision, given the importance of, and widespread interest in, the many underlying biological phenomena and their implications."

Such in-vtro models of OIS can indisputably be best described as oncogene-induced pseudo-senescence or oncogene induced durable growth-arrested model, which nevertheless does not underestimate the potential of the outcome of this work. Rather this work might encourage conducting similar investigation in the settings of *in-vivo* senescence that can probably unleash its potential for translation to therapeutic intervention.

The surveillance machineries at work

Strand specific RNA seq data followed by its validation by strand specific qPCR revealed that in proliferative cells convergent gene loci are accompanied by certain levels read-through transcripts which gets exaggerated in *in-vitro* OIS model used in this study. This suggests that these convergent loci which are devoid of stringent canonical transcription termination. This raise the question what is/are the factors involved that makes these loci susceptible to such transcriptional read-though, or in other words what are factors renders these loci prone to compromised stringency of transcription termination?

The inclination of such growth arrested cells in being refractory of decorating their DNA with certain epigenetic modifications committed to 3' end of the gene cannot be ruled out. Alternatively intergenic H2A.Z might represent positioned nucleosome and hence act as an essential factor in nucleosome positiong and thus as a barrier to such invasive transcripts and conditions associated with its loss might trigger the formation of NFR (Nucleosome Free Region) thereby facilitating the generation of such transcriptional read-throughs.

Unlike in yeast where H2A.Z alone is incompetent to suppress antisense transcripts (Zofall et al. 2009) this study demonstrates that in mammals the loss of H2A.Z is sufficient in elevating the accumulation of read-through transcripts (Model 1 and 2), however not all convergent gene loci were found to associated with such transcription read-throughs. To this end it appears that H2A.Z is not totally incumbent in regulation of read-through transcripts as these convergent gene loci inherently displays basal level of leaky read-through transcription (Model 1a and 2a). This might suggest that H2A.Z might play a dis-

tinct non-canonical role of transcription termination selectively at these loci (in lieu of stringent canonical termination machinery installed at other convergent loci), thus rendering these selective loci error prone to transcription termination, which further gets exacerbated upon deletion of H2A.Z alone. Alternatively this can also be due to the heterogeneity of cells (e.g. existence of growth-arrested cells or cellular states with depreciated levels of H2A.Z) in the proliferating cell population.

Earlier studies have shown that cohesion mediates transcription termination by recruiting heterochromatin machinery (Gullerova and Proudfoot 2008) besides emerging studies indicate that Rad21 facilitates long-range interaction (Mizuguchi et al., 2014). So is H2A.Z mediates control on transcription termination through cohesion dependent long-range interaction thereby acting as a genome-indexing mechanism in suppressing the accumulation of antisense transcripts for genes with impaired transcription termination control?

Consistent with previous findings in yeast, our preliminary data indicated that depletion of 3'-5' exonuclease activity of exosome (EXOSC10) leads to accumulation of such read-through antisense transcripts in a manner similar to H2A.Z (Zofall et al., 2009) and also at all convergent loci that are found to affected upon loss of H2A.Z. Alternatively as work from John Lis lab have shown that exosome associates with elongating Pol II (Andrulis et al., 2002), it is tempting to imagine that H2A.Z can act as a genome-indexing switch in a situation where once the Pol II have gone past its designated termination site and as a consequence invaded the next door convergent gene, H2AZ located at the promoter of that invaded gene might activate exosome to take care of this invasive transcript (Hypothesis b). Alternatively a higher order chromatin

structuring might promote (plausibly by Cohesin) a long-range interaction between the H2A.Z (located at the promoter of the read-through originating gene or local direct interaction of intergenic H2A.Z) and EXOSC10 might facilitate the surveillance of these error prone termination mediated read-through generation (Conjecture a&b respectively). If this is indeed the case then it might suggest that loss of EXOSC10 might enhace the stability of these read-through transcripts (from getting degraded by by 3'-5' exonuclease activity of Exosome) thereby facilitating the accumulation (Model 3). However the possibility of H2A.Z enrichment at the intergenic region serving as a potential roadblock to the POLII progression (Model 2) and hence serving, as a genome-indexing factor against the accumulation of such invasive deleterious erroneous antisense transcripts and or by activating Exosome cannot be ruled out (Conjecture b). Hence this works provides a hint of differential role played by H2A.Z at different sites of the genome. H2A.Z generally known to be enriched at the promoter is associated in promoting transcription whereas intergenic H2A.Z might represent surveillance machinery against the generation of such pervasive transcripts.

It is also encouraging to hypothesize that the circuitry of H2A.Z, Cohesin, Exosomes, RNAi and heterochromatin factors might orchestrate to restrict the generation of these antisense at the convergent genes that are more vulnerable to impaired transcription. However whether all these factors are present even at these convergent loci and if yes, whether there are similarly operational demands further investigations (Model 2).

A key finding of this work is that the induction of the growth arrest has been found to be associated to the depletion of H2A.Z levels across bodies of the

gene and in the intergenic regions turned out to be a common feature of the convergent genes that are associated elevated accumulation of read-through transcripts. As long as the emergence of such novel category of antisense RNAs are concerned it is perhaps suggestive of H2A.Z enrichment represents a transcriptional surveillance machinery and with emerging data in yeast indicating that mRNA splicing and polyadenylation machineries (TRAMP and MTREC complexes respectively) also plays a vital role in restricting the accumulation of such transcriptional read-through, it can be very provocative to speculate that H2A.Z localized at the gene bodies and at intergenic regions might play role in association with splicing and polyadenylation components to ensure proper processing of the pre-mRNAs, however this warrants an experimental validation. If this turns out to be true then the most rational question is to ask how does H2A.Z enrichment at the gene bodies and at the intergenic regions becomes dispensable in other convergent gene pairs that are associated with elevated read-through transcripts but do not correlate with change in H2A.Z levels or in other words what is the machinery and the mechanism at play that drives to bypass this fragile surveillance by H2A.Z for the accumulation of unspliced read-through transcripts? Furthermore what are the players and mechanisms that govern the fate of other convergent gene loci that were not associated with accumulation of read-through transcripts either upon induction of the growth arrest or loss of H2A.Z and EXOSC10?

Cause & Effect

We also tried to delineate the mechanism of H2A.Z loss in the *in-vitro* OIS model used in this study and if intervening with the mechanism that directs loss of H2A.Z can significantly foil the generation of read-through transcripts. We found that by dismantling the mechanism that alleviates H2A.Z in the *in-vitro* OIS model used in this study not only retains H2A.Z but also suppress the generation of read-through transcripts at the convergent gene loci of AR-HAGP18 and LAMA2. Although these events paralleled p16 repression, Cell Proliferation Assay needs to be conducted to check if these interventions by-pass the induction of the growth arrest and not just a mere suppression of p16 levels as compared to its control counterpart. Besides this strategy can simultaneously upregulate certain mitogenic factors or other factors unrelated to H2A.Z that promotes cell division. Hence the observed phenotype cannot be attributed to solely to H2A.Z.

However these observations failed to uncouple the two phenomena and hence the key question of whether the read-through transcripts per se are important for the stable growth arrest or vice versa remains unresolved. One of the plausible ways of uncoupling the two phenotypes will be to deploy CRISPR-Cas9 strategy to specifically target these read-throughs and then check if that allows the cell to re-enter the phase of cell division or escape senescence induction.

Nevertheless these results acknowledges the fact that there are factors that stand at the regulatory cross roads of mediating proper transcription termina-

tion thereby suppressing the generation of antisense transcripts and cyclin dependent kinase inhibitors (CDKi).

The players at the cross-roads of the 2 phenotypes

In a quest to find factors besides H2A.Z that simultaneously regulates the induction of read-through transcripts and senescence associated features, we investigated factors that reported to facilitate the generation of read-through transcripts in yeast. Preliminary data suggests that depletion of H2A.Z, EXOSC10 and Dicer individually lead to elevated levels of read-through transcripts and p16 expression (as a read-out for growth arrested state). However we also found factors that uncoupled this regulation. Preliminary data revealed that depletion of factor like FAM50A either individual or in combination with depletion of H2A.Z lead to prompt and robust induction of growth arrest but often failed to connote the accumulation of read-through transcripts whereas depletion of G9a a H3K9 methyltransfease, lead to elevated accumulation of such read-through transcripts without any effect on p16 (data not shown). However the latter finding might also indicate that G9a acts downstream of p16, although there is no conclusive evidence in favour of this assumption.

On the other hand this evidence also elicits intriguing question on the aspect of the *in-vitro* OIS model used in this study as well. If loss of H2A.Z at the gene bodies is associated with induction of growth arrest, it is intriguing to ask if there is a component dedicated in the eradication of H2A.Z from gene bod-

ies alone or there is a division of labour among the negative regulators of H2A.Z specific chaperons like p400 (a SWI/SNF DNA dependent ATPase) and SRCAP (Snf2 Related CBP Activator Protein) with that of a recently discovered evictor ANP32E? Alternatively from the epigenetic perspective it is worthwhile to think if certain histone modifications like that of H3K56ac described in yeast, can drive promiscuous H2A.Z eviction primarily from the gene bodies plausibly due to altered specificity of the H2A.Z chaperons? Such findings will be beneficial from the therapeutic aspects by exploiting these components in achieving potential interface in abrogating tumour growth.

Mechanistic insight of the associated growth arrest

A key aspect of this study that remain unresolved is whether there are certain key proliferation promoting genes or genes involved as positive regulator of DNA damage response or facilitator of DNA repair that gets disrupted by these antisense transcripts Alternatively cumulative effects on various genes that are abrogated by the genome-wide accumulation of various antisense transcripts could drive this growth arrest? Furthermore, although this study provides evidence in the regulatory roles of the antisense RNAs at the mRNA level, the effect of these antisense RNAs at the protein level or a precise phenotypic change as a consequence of altered gene expression remains unresolved. Specifically this study shows that the read-through antisense transcripts abrodages the gene expression of next (sense) gene located on the opposite strand. Specifically this study shows that the read-through antisense

transcripts abrodages gene expression of next (sense) gene located on the opposite strand. Moreover, conditions associated to the STARTs, i.e. both upon induction of growth arrest as well as on depletion of H2A.Z, we detected a profound suppression of REV3L and LAMA2, although the effect at the post transcrip-tion level has not been shown. However, repression of these two genes by their corresponding STARTs cannot be conferred the title of “pro-growth ar-rest machinery” as depletion of these two STARTs, albeit individual-ly, failed to evoke reversion of cellular growth (data not shown).

Alternatively to decipher if these read-through transcripts are the direct con-sequence of the growth arrest and are indispensible to maintain this state or independent of it, one can revert this growth arrest by simultaneous depletion of p21 and p16 and check the status of these read-throughs. If the read-throughs can still be detected, this can suggest that they are insufficient in maintaining the growth-arrested cells, but this can also imply that that these read-through transcript acts upstream of p16 or p21 in inducing cell cycle ar-rest. However they might act as a “memory marker” for cells that have revert-ed back from a once-upon-a-time growth arrested state. Understanding if the-se read-through transcripts either upstream or downstream of this growth-arrested state might appear to be imperative in acknowledging its potential for therapeutic interventions.

The proof of read-through harboured startling revelations

One of the caveats in ameliorating the negative effect of these read-throughs transcripts is to perturb these antisense transcripts without affecting the expression of the read-through-originating gene. As these antisense RNA originates from the same promoter as that of the read-through-originating gene which ironically qualifies them of being true transcription read-throughs and refutes the possibility of being originated from the cryptic promoter elements, siRNA mediated depletion of these antisense transcripts by targeting the intergenic (read-through) region also leads to depletion in the mRNA read-through originating gene. To this end, as an alternative to selectively targetting the read-through for degradation (as proposed earlier) one can use CRISPR-CAS9 to introduce a stringent Transcription Termination Site (TTS).

Alternatively RNAi mediated depletion of the read-through-originating gene suppressed the expression of the read-through antisense transcripts that connoted the rescue in expression of the antisense gene located on the opposite strand. This later aspect unravel one of the key finding of this study that calls for revisiting the siRNA experiments that conducted so far on the genes with a convergent oriented partner-gene located on the opposite strand. For example let us consider a study that involved loss of function study of a gene “X” in order to gain insights to the functionality of the gene “X” and this gene is located in a convergent with another gene “y” located on the opposite strand. If this convergent gene loci that is comprised of “X” and “Y” with the gene “X” is associated to impaired transcription termination then there can be a similar accumulation of read-through transcripts that may transverse across these

two genes and further makes antisense to gene ”Y”. *Prima-facie* of such circumstances phenotype deciphered from loss of gene “X” cannot be attributed only to silenced “X” but may also due to relief in expression levels of “y” owing to the parallel lose of antisense read-through transcripts that was emanating from “X”. This is indeed one of the most interesting outcomes of this study and hopefully this aspect will be taken into accountability in the interpretation of preceding loss of function-related studies.

The fact that these read-through antisense transcripts are functional in human has been recently shown in physiological background in two independent studies (Powell et al., 2013; Vilborg et al., 2015), however the fact the histone variant acts as a guardian and thus an integral part of RNA surveillance machinery against the generation of these functional read-through transcripts is the novel aspect of this study.

Therapeutic angle

The outcome of this study can manifest itself into various clinical implications. If a further study carried out on the backdrop of physiological OIS can reveal that such read-through transcripts are indispensible for the maintenance or the survival of the senescent cancerous cells then these novel category of antisense transcripts can turn out to be a real nuisance to the cells. Indeed they might stand at the crucial junction where these senescent cells can drive fulminant growth and metastasize the adjacent cancer cells. Primafacie of such

menace, suppressing these transcriptional read-throughs comes at a cost of suppressing the expression of the gene from which the read-through transcript in question originates. Such effect can augment the complexity in dealing with these read-throughs at the therapeutic level. The only viable option in that case will be to drug the read-through only if the gene from where it originates have dispensible functionalty in a given physiological setting, as otherwise these read-through can pose a potential bone of contention. Alternatively if these read-through transcripts are found to drive senescence then the factors that specifically suppress these antisense RNAs can be blocked and thus can be clinically exploited in senescence driven therapeutic interventions. However discerning the tissue specificity can be a riddle in this aspect.

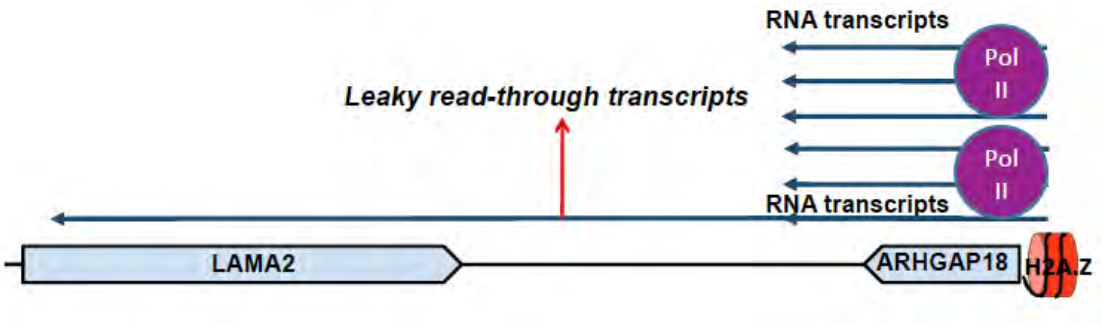
The Holy Grail in the mystery of aging?

Overwhelming studies conducted over the last decade have strongly indicated that aging represents the biggest risk factor for majority of human diseases. Aging is associated with progressive decline in tissue homeostasis and compromised regenerative capabilities and to an appreciable extent this is due depreciated levels of functional stem cell pool (Sharpless and Depinho 2007). Seminal work published by Norman Sharpless's laboratory in 2006 (Krishnamurthy et al., 2006) showed for the first time that elevated p16 expression with aging counteracts regenerative potential of pancreatic cells. However mere elevated p16 levels cannot unequivocally qualify for the evidence of se-

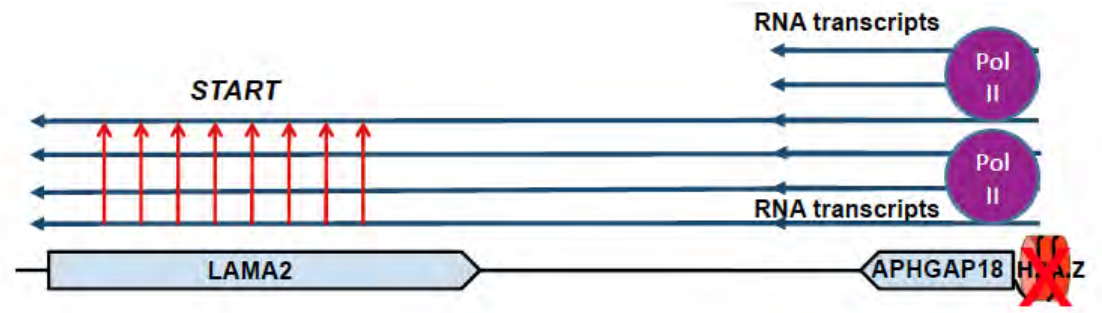
nescent cells, but rather a molecular marker of aging (Krishnamurthy et al., 2004). As the accumulation of STARTs connotes elevated p16 expression, there appears to be a formidable chance of these aberrant transcripts to get accumulated with age. Besides with emerging evidence suggesting H3K36me3 specific HMT suppresses the emergence of such pervasive transcripts in the settings of renal carcinoma (Grosso et al., 2015), it is provocative to investigate for plausible induction and regulatory roles of these transcripts in aging mouse models and cognate human tissues. This might cast a considerable light on the underlying mechanism by which the loss of H3K36me3 can impede longevity besides the suppression of aberrant transcripts originating from cryptic promoter elements that has been demonstrated to be detrimental for the replicative lifespan in yeast (Sen et al., 2015) through the identification of genes that can be potentially perturbed by such pervasive regulatory antisense transcripts and thus might act as potential perpetrators of decline of tissue homeostasis upon aging. In that case elevated levels of aging associated read-through transcripts can serve as a direct read-out of stoichiometry between drivers of organismal aging and facilitators of such pervasive transcription.

Models deciphered from the PhD project

a

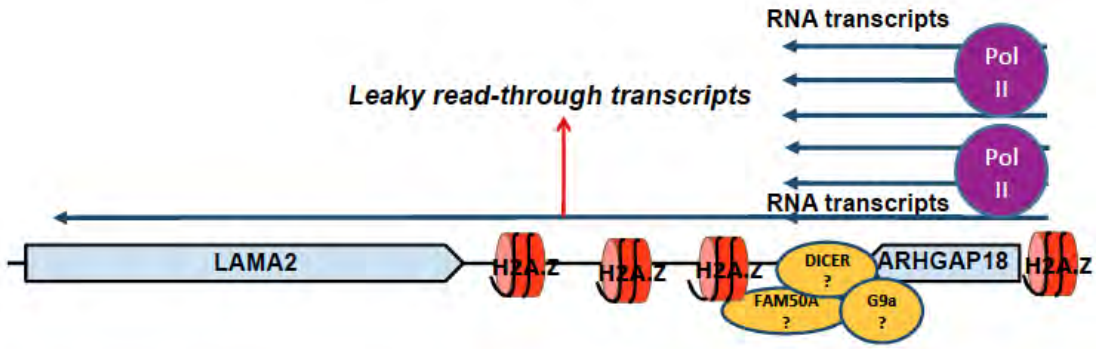


b

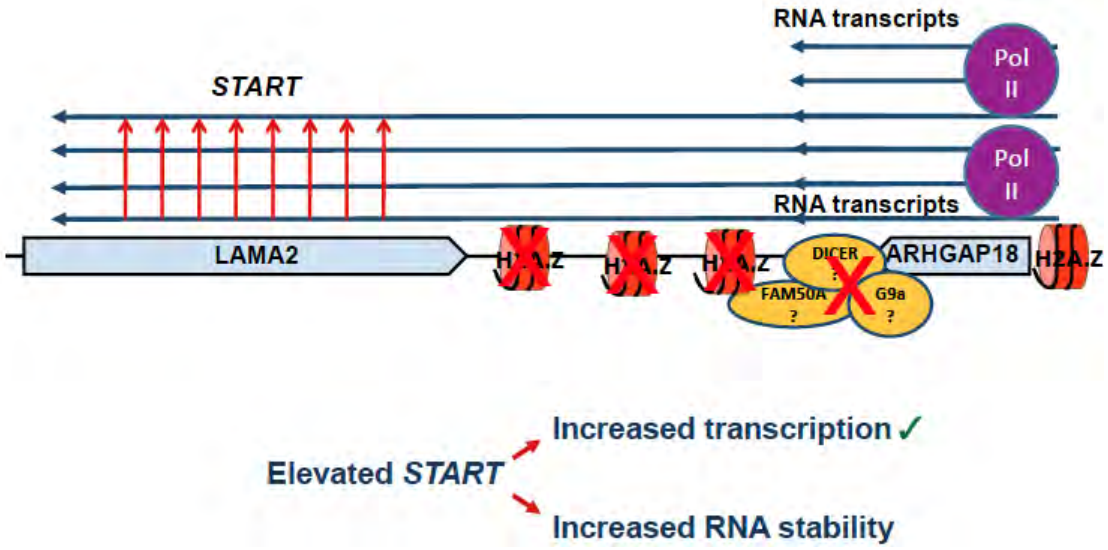


Model 1: Validation of the mechanism functional in yeast in suppressing antisense transcriptions in mammals using WI-38, a human fibroblast cell line. a) The figure represents a locus of convergent gene pair susceptable to generation of transcriptional read-throughs. **b)** Accompanying the loss of H2A.Z, which can either mediated by proteasomnal degradation or through specific chaperone or alternatively upon siRNA treatment, there is an accumulation of read-through antisense transcripts owing to impaired transcription termination.

a

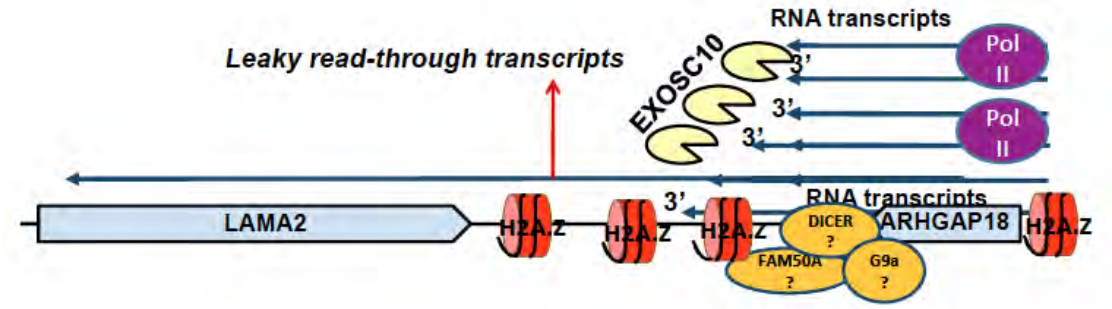


b.

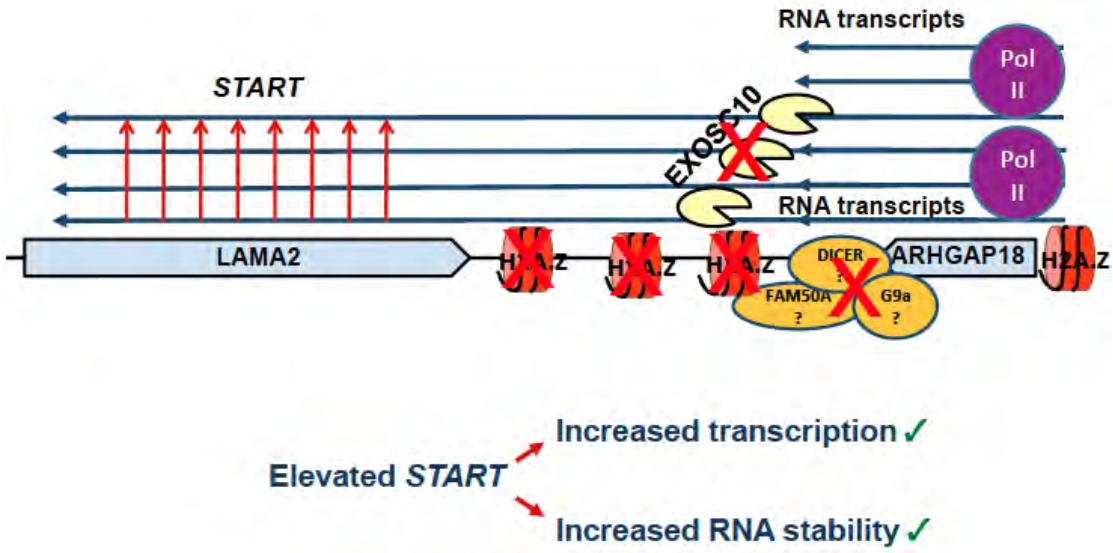


Model 2: Intergenic H2A.Z serves as a potential roadblock to transcriptional read-throughs. a) ChIP-seq identified enrichment of H2A.Z at a convergent gene pair. **b)** Connotation in loss of intergenic H2A.Z and recapitulation in the accumulation of read-through transcripts suggests that this owing to elevated transcription. Besides preliminary data also suggest that loss of other factors (Dicer, G9a and FAM50A) that are mostly known to associate to the TTS can also generate similar read-through transcripts.

a.

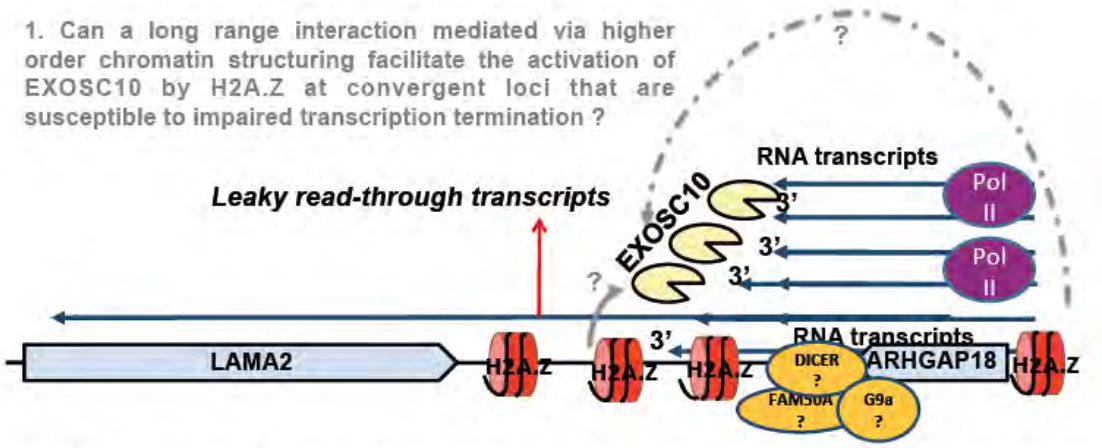


b.



Model 3. 3'-5' Exonuclease activity of Exosome can also participate in the surveillance against read-through transcripts. a) Preliminary data suggests that EXOSC10 may also also participate in regulating the emrgence of read-through antisense transcripts. **b)** Besides the loss of H2A.Z (intergenic), Dicer, G9a and FAM50A, preliminary data reveals that depletion of EXOSC10 can also lead to the accumulation of antisense transcripts. This might suggest that loss opf EXOSC10 increases the stability of read-through transcripts (by preventing its degradation by the exonuclease activity of EXOSC10).

Conjecture



a. ***Impaired long range interaction between H2A.Z at the promoter and EXOSC10 or other components at 3'end of the gene that might facilitate its recruitment owing to loss of exclusive factors that promotes higher order chromatin structuring mediated transcription termination at selective convergent loci might explain the mechanism that assists the generation of such pervasive transcription.***

a) ***Loss of intergenic H2A.Z might manifest in a failure to instruct EXOSC10 to execute its function on the RNA transcript transcribed by RNA Polymerase II that has missed its designated termination site can also be a potential possibility in demonstrating the mechanism of generation of read-through transcripts.***

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Supplementary Table 1: list of the 68 loci harbouring STARTs

	Chr	START Strand	Gene Forward Name	Gene Reverse Name
1	chr1	+	BTBD19	PTCH2
2	chr1	+	SYT11	RIT1
3	chr1	+	GPR137B	ERO1B
4	chr2	+	CLIP4	ALK
5	chr3	+	OXNAD1	RFTN1
6	chr3	+	NIT2	TOMM70A
7	chr4	+	SAP30	SCRG1
8	chr5	+	SLF1	MCTP1
9	chr6	+	KIAA1919	REV3L
10	chr6	+	SYNJ2-IT1	SERAC1
11	chr7	+	PTPN12	APTR
12	chr8	+	SH2D4A	CSGALNACT1
13	chr8	+	WISP1	NDRG1
14	chr9	+	UHRF2	GLDC
15	chr9	+	TEK	LINC00032
16	chr9	+	PGM5	TMEM252
17	chr9	+	OR1N2	OR1L8
18	chr9	+	OR1Q1	OR1B1
19	chr11	+	ZNF215	ZNF214
20	chr11	+	SEC14L1P1	ALKBH3-AS1
21	chr12	+	SMIM10L1	TAS2R42
22	chr12	+	BORCS5	DUSP16
23	chr12	+	MDM2	CPM
24	chr12	+	NUDT4P1	UBE2N
25	chr14	+	MIPOL1	FOXA1
26	chr14	+	LRR1	RPL36AL
27	chr14	+	YLPM1	PROX2
28	chr15	+	NR2E3	MYO9A
29	chr16	+	SNX29P1	NPIPB3
30	chr16	+	ZNF771	DCTPP1
31	chr17	+	MILR1	POLG2
32	chr18	+	ZNF271P	ZNF24
33	chr19	+	ZNF586	ZNF552
34	chr20	+	MCTS2P	HM13-AS1
35	chr20	+	STK4	KCNS1
36	chr1	-	SERBP1	IL12RB2
37	chr1	-	ACP6	BCL9
38	chr1	-	IVNS1ABP	SWT1
39	chr1	-	WDR26	CNIH4
40	chr2	-	TMEM127	STARD7-AS1
41	chr2	-	NEMP2	MFSD6
42	chr3	-	MGLL	ABTB1
43	chr3	-	PIK3CB	FAIM
44	chr4	-	LIN54	THAP9
45	chr5	-	SMIM15	NDUFAF2
46	chr5	-	RAPGEF6	CDC42SE2
47	chr5	-	UBTD2	EFCAB9
48	chr6	-	FAM162B	KPNA5
49	chr6	-	ARHGAP18	LAMA2
50	chr7	-	ZNF394	ZNF789
51	chr9	-	DDX58	ACO1
52	chr9	-	LPAR1	MUSK
53	chr10	-	ITGB1	CCDC7
54	chr11	-	RRAS2	SPON1
55	chr11	-	INTS4	AAMDC
56	chr11	-	CHORDC1	NAALAD2
57	chr11	-	NPAT	ACAT1
58	chr12	-	CSRP2	ZDHHC17
59	chr14	-	CNIH1	CDKN3
60	chr14	-	ADAM20P1	ADAM21
61	chr15	-	SPG21	ANKDD1A
62	chr15	-	UACA	SALRNA2
63	chr17	-	NXN	RNMTL1
64	chr17	-	FAM222B	TRAF4
65	chr17	-	ARL16	CCDC137
66	chr19	-	ZNF792	ZNF30
67	chr19	-	RTN2	FOSB
68	chrX	-	C1GALT1C1	MCTS1

Summary

Cellular senescence represents one of the major fail-safe mechanisms that counteracts tumour development is defined as a state of irreversible cell cycle arrest as a consequence of stress response such as oncogenic challenge. Such cells undergoing Oncogene-induced Senescence (OIS) display profound alternation in their epigenome as their chromatin are largely decorated with prominent drivers of constitutive heterochromatin.

Antisense RNA-mediated gene regulation has been attributed to play diverse roles in mediating various cellular processes and cell fates *per-se*. In yeast, histone variant H2A.Z cooperates with RNAi and heterochromatin machinery to regulate antisense transcription at convergent gene loci which can otherwise generate pervasive read-through transcripts owing to improper transcription termination.

In mammals, whether such antisense transcripts (occurring by read-through transcription at convergent gene pairs) exist and how they are regulated remains unknown. Interestingly, the depletion of the human H2A.Z histone variant has been reported to induce cellular senescence. We thus wondered if the regulation of particular antisense transcripts at convergent gene pairs occurs in senescence, if their regulation by H2A.Z is conserved in mammals and, if so, if a functional significance can be attributed to these transcripts. To this end we took advantage of a well-established in-vitro OIS model

Briefly, we analysed genome wide strand specific RNA-seq analysis of cells undergoing Oncogene Induced Senescence. This led us to identify numerous convergent gene loci associated with accumulation of transcripts downstream of the designated transcription termination site in senescent cells, and extending to generate an antisense to the next gene located in the opposite strand of the convergent gene pair. We confirmed the RNA-seq data at two of such convergent loci. An RNAi based approach revealed that at least two of these transcripts are generated by transcriptional read-throughs. Hence we designated such pervasive transcripts as *S*enescence *T*riggered *A*ntisense *R*ead-through *T*ranscripts *(START)*. Importantly, we further found that the two *STARTs* for which we performed in depth studies repress the expression of the gene for which they are antisense. Finally, we demonstrate that the histone variant H2A.Z suppresses the accumulation of *STARTs* in proliferative cells. Since it also prevents senescence induction, this suggests that expression of *START* is important for cellular senescence. This has lead us to propose a model that human cells undergoing OIS are associated with loss of H2A.Z that leads to the wide spread accumulation of read-through transcripts owing to impaired termination control.