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Mechanistic insights into age-associated chromosome mis-segregation and senescence

Monika Barroso Vilares

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SEDE ADMINISTRATIVA **INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR**

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Mechanistic insights into age-associated chromosome mis-segregation and senescence

Tese de Candidatura ao grau de Doutor em Biologia Molecular e Celular

Programa Doutoral da Universidade do Porto (Instituto de Ciências Biomédicas Abel Salazar e Faculdade de Ciências)

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“Sê todo em cada coisa. Põe quanto és, No mínimo que fazes.”

“Be whole in everything. Put all you are, Into the smallest thing you do.”

– Ricardo Reis, in "*Odes*" (Fernando Pessoa)

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Declaration

The author hereby declares that she fully authored the here-presented thesis and that it was never used or presented elsewhere in the past. The author further declares that she has provided a major contribution to the design, technical execution, interpretation of the results and manuscript preparation of all work presented within this thesis, which resulted in the following published articles:

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"The butterfly counts not months but moments, and has time enough"
– Rabindranath Tagore, Nobel Prize in Literature (1913)

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Abstract

Aneuploidy, an abnormal number of chromosomes, is often found in tumors and has also been found to accumulate with aging. Extensive research helped to establish from the molecular to the organismal level what characterizes the aging process. Cellular senescence, one of the common denominators of aging and which refers to the permanent cell cycle arrest of cells with unrepairable damage, has been shown to accumulate and contribute to the time-dependent deterioration of tissues and organisms with age. Recent studies reveal a senescence-inducing potential of chromosome dosage imbalances and show that the accrual of cytoplasmic DNA driven by faulty DNA replication and/or chromosome segregation is essential for the secretory phenotype and non-cell autonomous effects of senescent cells. In this work we provide mechanistic insights into how loss of genome stability with age or constitutional trisomy of chromosome 21 contributes to cellular senescence development during aging and Down Syndrome (DS), respectively. Particularly, we show that the increased numbers of stable kinetochore–microtubule (k-MT) attachments established in elderly cells are less efficiently corrected and that the resulting chromosome imbalances contribute to their cellular senescence phenotype. In addition, we disclose a small molecule that antagonizes the cellular senescence phenotype of aged cells by augmenting kinesin-13 MT-depolymerizing activity. Importantly, we found that this small molecule also counteracts genome instability (GIN) and cellular senescence in cells with trisomy 21. DS patient-derived fibroblast samples reveal increased levels of cellular senescence that do scale with advancing age and correlate with a higher incidence of DNA damage and chromosomal instability, when compared with their healthy diploid counterparts. Overall, we show that loss of genome stability may contribute to age-related phenotypes by fueling cellular senescence and propose pharmacological inhibition of GIN as a potential strategy to extend healthspan and be pre-clinically validated in the future.

Keywords: aging; down syndrome; aneuploidy; genome instability; senescence.

Resumo

A aneuploidia, um número anormal de cromossomas, é recorrente em tumores e parece acumular com o envelhecimento. Investigação nesta área permitiu que se determinassem, desde o nível molecular ao nível do organismo, as alterações que caracterizam o processo do envelhecimento. Um denominador comum é a senescência celular, um estado no qual as células são forçadas a entrar quando sofrem danos irreparáveis e que parece acumular e contribuir para a degeneração de tecidos e órgãos com a idade. Estudos recentes mostraram ainda que alterações no número de cromossomas podem causar a entrada da célula em senescência, e que a acumulação de ADN no citoplasma em resultado de erros na replicação e/ou segregação de cromossomas é essencial para o secretoma e efeito parácrino das células senescentes. O presente trabalho contribui para a compreensão mecanística de como a perda de estabilidade genómica com a idade ou trissomia do cromossoma 21 leva ao desenvolvimento de senescência celular em contexto de envelhecimento e Síndrome de Down, respetivamente. Em particular, demonstramos que células envelhecidas estabelecem um maior número de interações estáveis entre cinetócoros e microtúbulos, as quais corrigem com menor eficiência, e que as alterações no número de cromossomas resultantes contribuem para o processo de senescência. Além disso, revelamos um composto que ao induzir a atividade despolimerizadora de microtúbulos das cinesinas-13 foi capaz de diminuir a senescência em células envelhecidas. Curiosamente, o mesmo composto demonstrou ser capaz de contrariar a instabilidade genómica e a senescência celular em contexto de trissomia 21. Em comparação com controlos diploides saudáveis, fibroblastos provenientes de pacientes com Síndrome de Down têm níveis superiores de senescência celular, os quais não só aumentam com a idade, mas também correlacionam com uma maior incidência de danos no ADN e instabilidade cromossómica. Assim sendo, é possível que a perda de estabilidade genómica contribua para os fenótipos associados ao envelhecimento através da senescência celular. Por conseguinte, propomos que a inibição farmacológica da instabilidade genómica venha a ser validada pré-clinicamente no futuro como uma potencial abordagem que visa promover o envelhecimento saudável.

Palavras-chave: envelhecimento; síndrome de Down; aneuploidia; instabilidade genómica; senescência.

Index

Abbreviations

ACA	anti-centromere antibody
APC/C	anaphase promoting complex/cyclome
ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia and Rad3-related
BER	base-excision repair
BN	binucleated
CCF	cytoplasmic chromatin fragment
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
C/EBPb	CCAAT/enhancer-binding protein beta
CENP	centromeric protein
CEP	chromosome enumeration probe
cGAMP	2′3′-cyclic-GMP-AMP
cGAS	cyclic GMP-AMP synthase
chr	chromosome
CIN	chromosome instability
CR	caloric restriction
CRM	caloric restriction mimetics
CytoD	cytochalasin D
DAPI	4',6'-diamino-2-fenil-indol
DDR	DNA damage response
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DS	Down syndrome
DSB	double-strand break
dsDNA	double-stranded deoxyribonucleic acid
ESR	environmental stress response
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FISH	fluorescence *in situ* hybridization
FMD	fasting-mimicking diet
FoxM1	forkhead box protein M1
FoxO4	forkhead box protein O4
GATA4	GATA binding protein 4

GFP	green fluorescent protein
GIN	genomic instability
H	diploid fibroblasts from healthy donors
HDF	human dermal fibroblasts
HGPS	Hutchinson-Gilford progeria syndrome
HR	homologous recombination
HRP	horseradish peroxidase
IFN	type I interferon
IL	interleukin
iPSC	induced pluripotent stem cells
IR	ionizing radiation
Kif2C	kinesin family member 2C
k-MT	kinetochore-microtubule
KT	kinetochore
MCAK	mitotic centromere-associated kinesin
MCC	mitotic checkpoint complex
MCP	monocyte chemoattractant protein
MEM	minimal essential medium Eagle–Earle
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinases
MMR	mismatch repair
MN	micronuclei
mTOR	mammalian target of rapamycin
MVA	mosaic variegated aneuploidy
MS	mis-segregation
MT	microtubule
NE	nuclear envelope
NEB	nuclear envelope breakdown
NER	nucleotide-excision repair
NF-κB	factor nuclear kappa B
NHEJ	non-homologous end joining
OSKM	Oct4, Sox2, Klf4 and c-Myc (known as Yamanaka factors)
PBS	phosphate-buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
Rb	retinoblastoma

RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
SAC	spindle assembly checkpoint
SAHF	senescence-associated heterochromatin foci
SASP	senescence-associated secretory phenotype
SA-β-gal	senescence-associated β-galactosidase activity
siRNA	small interfering RNA
ssDNA	single-stranded deoxyribonucleic acid
STAT1	signal transducer and activator of transcription 1
STING	stimulator of interferon genes
STLC	S-trityl-L-cysteine
T21	fibroblasts with trisomy 21 derived from Down Syndrome patients
TBS	tris-buffered saline
UV	ultraviolet
VEGF	vascular endothelial growth factor

Chapter – I

General Introduction and Research Aims

Parts of the following chapter were published in:

Barroso-Vilares, M & Logarinho, E (2019). *Chromosomal instability and pro-inflammatory response in aging*, Mechanisms of Ageing and Development, 182: 111118. https://doi.org/10.1016/j.mad.2019.111118

I.1. The cell cycle: a brief overview

Cell division is the mainstay of growth and development in all living organisms. It encompasses a series of controlled and orchestrated events collectively referred to as cell cycle (or cell-division cycle), which goal is to duplicate and split all nuclear and cytoplasmic contents to form two identical daughter cells. It essentially divides into two main stages: interphase and mitosis (Fig I.1). While interphase represents the 'growing phase' of the parental cell, the purpose of mitosis (or M-phase) is to equally split the duplicated genetic material between the daughter cells. In either phase, control and repair mechanisms are in place to mitigate eventual failures during replication and/or segregation of the genome which would inevitably translate into harmful karyotypic alterations, including changes at both DNA sequence (*i.e,* mutations) and chromosome (*i.e,* aneuploidies or polyploidies) levels. This section aims to provide a simplistic overview of cell division, which will be essential to then explore the causes and consequences of genome stability loss.

I.1.1. The cell cycle control system

Faithful progression through the cell cycle lies on specific switch-like transitions, of which the first is prior to DNA replication, and then the other two coinciding with the entry and exit of mitosis. For this, a plethora of regulatory pathways are in place to control the order and timing of the transitions, while surveilling that key events of the cell cycle such as DNA replication and chromosome segregation are completed with the maximum fidelity possible (Elledge, 1996). At the basis of this cell cycle control system is the stage-specific cooperation between cyclin-dependent kinases (CDKs) and cyclin subunits (reviewed in Morgan, 1997). In addition, specific checkpoints have to be satisfied for a flawless cell cycle progression (Fig I.1). A checkpoint is a surveillance pathway that is constitutively active to monitor if all pre-requisites have been properly satisfied at a specific stage of the cell cycle before allowing the cell to proceed to the next one (Hartwell & Weinert, 1989; Rieder, 2011). The first is known as 'restriction point' and acts in G1 where it safeguards that cells have adequate nutritional conditions and a proper size before being allowed to replicate their DNA (Pardee, 1974). Next, the DNA replication checkpoint ascertains whether DNA duplication was successful and ensures that cells enter mitosis only in the absence of DNA damage (Xu *et al*, 2002). Finally, during mitosis the spindle assembly checkpoint will halt mitotic exit until all chromosomes arrange in a plate upon being properly attached to microtubules (MTs) from opposite spindle poles (Musacchio & Salmon, 2007). Thus, the highly sophisticated nature of the cell cycle lies on the interconnection and functionality of

these regulatory and surveillance pathways, which allows cells to faultlessly perpetuate their contents each time they divide.

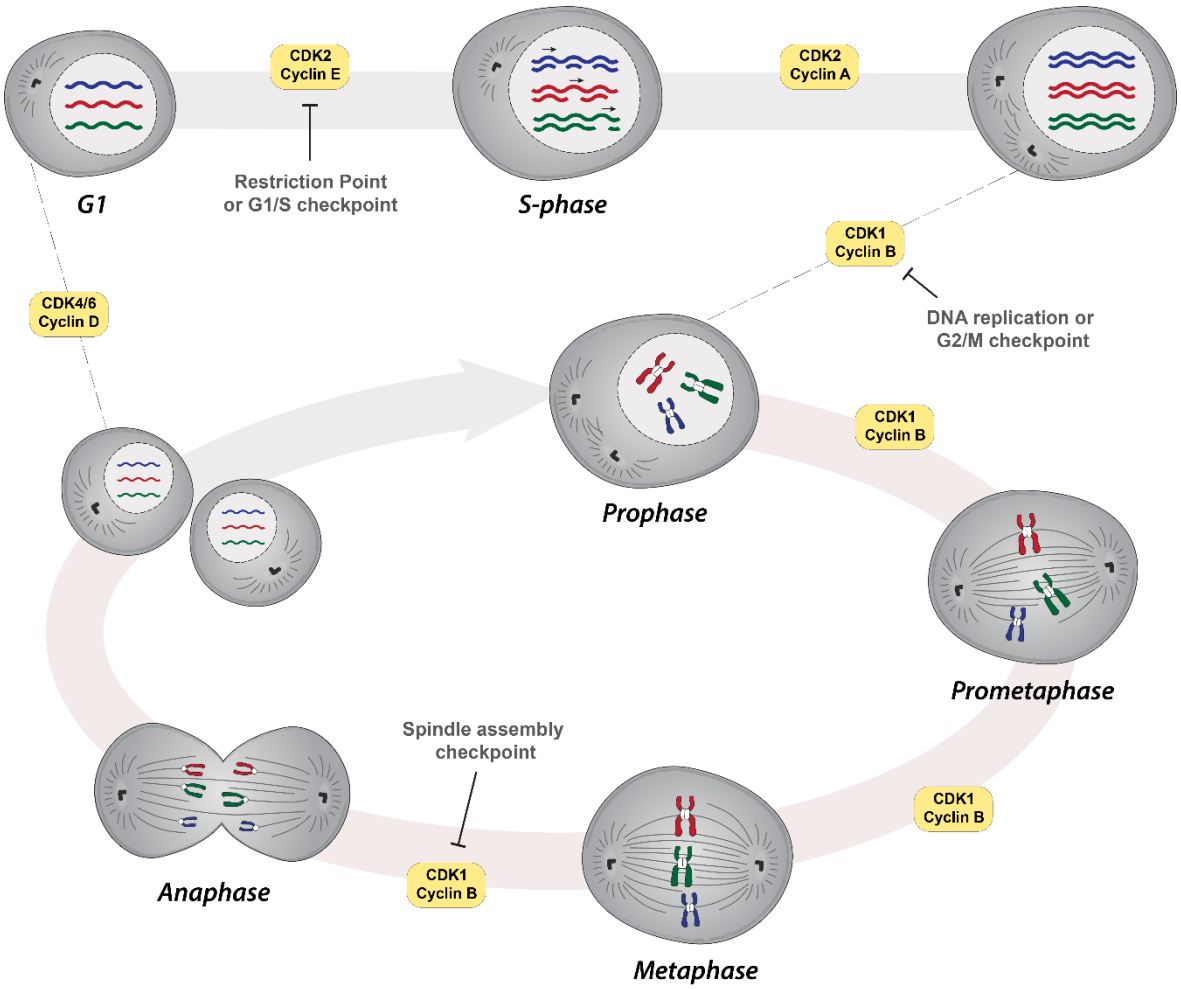

Figure I.1. Illustration of the cell cycle stages and its control system. Cell division can be split into two main stages: interphase (top) and mitosis (bottom). During interphase the parental cell devotes time to duplicate all its contents (*e.g,* DNA, organelles), the accuracy of which relies on the coordinated interaction between CDK2 and cyclin E/A and is surveilled by two distinct checkpoints (in G1 and G2/M). During mitosis, all efforts are channeled towards faithful chromosome segregation, where levels of CDK1 and cyclin B1 are kept high until anaphase onset. Mitotic exit (metaphase-to-anaphase transition) is allowed following bi-orientation of all chromosomes, which turns off the spindle assembly checkpoint and sets free the degradation of cyclin B1. At the end of mitosis, chromatin decondenses and the NE reassembles (telophase), which prepares the parental cell to split its cytoplasm during cytokinesis.

I.1.2. Interphase

The very first stage of the cell cycle is termed Interphase and, as the name suggests, it is the period between mitotic divisions that is dedicated to the duplication of all the components of the cell. This cell cycle stage comprises three main events: S-phase and two gap phases (G1 and G2) (Fig I.1). Cells entering G1 phase come from either a previous

mitotic division, or alternatively from a quiescent state known as the G0 phase. G0 is a temporary physiological state into which cells escape in the presence of inadequate 'environmental conditions' (*e.g,* limited nutrient supply) or due to cues inherent to their developmental program. Eventually, extracellular mitogenic signals will push cells back into the replicative cycle (O'Farrell, 2011; Terzi *et al*, 2016). Essentially, G1 phase represents the period of time where the cells get 'prepped' for the genome replication process: reach a critical cell size, amass enough nutrients to withstand the remaining phases and ensure replication origin licensing (Limas & Cook, 2019; McIntosh & Blow, 2012; Nevis *et al*, 2009; Pardee, 1974). The stage that follows – S-phase – is a particularly critical period for genome stability maintenance (*i.e,* faithful duplication and transmission of the whole genome content to daughter cells), marked by the initiation and completion of DNA replication. It presents a significant challenge to the cell, given the size and inherent complexity of genomes. Successful passage through S-phase heavily depends on appropriate replication origin licensing in G1 and the intertwined action and control of DNA replication checkpoint and DNA repair mechanisms (extensively reviewed in Takeda & Dutta, 2005) (described in more detail below). Once the checkpoint mechanisms allow transition, the cells enter G2 phase, the last subphase of interphase (Vermeulen *et al*, 2003). During this period cells further grow and actively synthesize proteins to prepare entry into mitosis or M-phase.

DNA replication and DNA damage repair

DNA replication takes mostly place during the S-phase, and depends on a strict regulation that ensures the whole genome becomes faithfully replicated only once during cell division (Alberts, 2003; Hochegger *et al*, 2008). A large number of non-randomly distributed replication origins throughout the genome become properly licensed in G1, so once the cell reaches all requirements to proceed into S-phase, the controlled firing of those origins ensures timely replication of the whole genome in all its length and complexity. This is the ideal scenario. However, the DNA replication process can experience complications, such as origin licensing defects, or cells can become exposed to endogenous or exogenous conditions that compromise proper progression and stability of replication forks (*e.g,* nicks, gaps, breaks). Such conditions are referred to as 'DNA replication stress', will originate incomplete DNA replication intermediates and jeopardize timely DNA synthesis (reviewed in Ovejero *et al*, 2020). In the presence of damage, cells activate checkpoint mechanisms to slow down DNA synthesis. These mechanisms tightly balance DNA replication and DNA repair to provide cells with further time to fix DNA damage preventing by this way situations of genome instability (GIN) (Aguilera & Garcia-Muse, 2013). Two main checkpoint mechanisms are in place: ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia and Rad3-related) (extensively reviewed in Abraham, 2001; Awasthi *et al*,

2015). The ATM and ATR kinases, as well as their best-known downstream target checkpoint kinases 1 and 2 (Chk1 and Chk2), are recruited to defective replication forks or sites of DNA damage where they upregulate specific cellular responses such as cell cycle arrest, DNA replication inhibition, and DNA repair (Abraham, 2001; Awasthi *et al,* 2015). The recruitment of ATM or ATR occurs through specific co-factors and depends on the type of DNA lesions. While ATM is primarily activated in response to DNA double-strand breaks (DSBs) (Paull, 2015), ATR responds to stalled replication forks and single-stranded DNA (ssDNA) alterations (Cimprich & Cortez, 2008). The main pathways that function to repair different types of DNA lesions are homologous recombination (HR) and non-homologous end joining (NHEJ), but mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) are also in place (Fleck & Nielsen, 2004). HR, as the name suggests, uses homologous genetic material (generally found on the sister chromatid) to repair DSBs. Conversely, NHEJ is a highly stochastic repair mechanism in which the break ends of DSBs are directly rejoined by ligation without any homologous template (both recently reviewed in Han & Huang, 2019; Scully *et al*, 2019). MMR, NER and BER all function by excision of bases or lesions which re-allows the cell to correctly synthesize the DNA sequence (Fleck & Nielsen, 2004). DNA damage can be found in any stage of the cell cycle, either as a consequence of endogenous (*e.g,* reactive oxygen species or ROS) or exogenous (*e.g,* UV light, IR) stressors, or as a result of DNA replication stress as abovementioned. While NHEJ is the predominantly engaged mechanism in repair of DSBs formed during G1, DSBs that occur during S/G2 phases are mostly repaired by HR as sister chromatids are likely closer to each other. Next, insertion, deletion, or mis-incorporation of bases that can result from flaws in the DNA replication process are taken care of by MMR. Finally, NER is the primary pathway used to remove bulky DNA lesions such as those caused by exogenous stressors including UV light or mutagens (see Branzei & Foiani, 2008). Consequently, the orchestrated coordination of DNA replication with DNA repair under the surveillance of checkpoint mechanisms during interphase is key to ensure that daughter cells inherit an exact copy of the parental cell's genome at the end of mitosis.

I.1.3. Mitosis

The term mitosis was proposed by the German biologist Walther Flemming almost 140 years ago to describe the ordered process by which the threadlike structured chromatin (today known as chromosomes) are partitioned to originate two genetically identical daughter cells (Flemming, 1882).

The five phases of mitosis

Based largely on the behavior and organization of chromosomes observed under the microscope, the process of mitosis is divided into five distinct phases (Walczak *et al*, 2010) (Fig I.1). At prophase, the first stage of mitosis, chromosomes begin to condense, and centrosomes gradually move to opposite sides of the nucleus, which allows the initiation of mitotic spindle assembly (Inoue & Salmon, 1995). The mitotic spindle is a large intracellular structure assembled by intrinsically dynamic hollow cylinders of heterodimers of globular α-tubulin and β-tubulin molecules (also known as microtubules) that alternate between phases of polymerization (growth) and spontaneous depolymerization (shrinkage) (Mitchison & Kirschner, 1984; Prosser & Pelletier, 2017). As MTs are nucleated by the centrosomes, interaction between MTs and chromosomes is facilitated following the dissolution of the nuclear envelope (or nuclear envelope breakdown – NEB), an event that points out the onset of prometaphase. During this second stage of mitosis, the spindle reaches its maximum organization and chromosomes gradually become moved toward the center of the cell, a process known as congression (Kops *et al*, 2010). When all chromosomes form a plate at the cell equator in which the chromatids of each chromosome are interacting with MTs from opposing poles via their kinetochores (specialized proteinaceous structures that assemble over the centromeric DNA), the cell has entered the third stage of mitosis – metaphase. Here, the cell has surveillance/correction machineries which efforts are channeled towards the correct biorientation of all chromosomes. Only after the very last kinetochore becomes properly attached to MTs, the cell is allowed undergo anaphase (Rieder *et al*, 1994). During this fourth stage of mitosis, sister chromatids split apart and begin to synchronously move towards the poles (anaphase A). The addition of tubulin subunits to the plus ends of the polar microtubules, together with the activity of motor proteins that facilitate these MTs to slide on each other, allows the spindle poles to move farther apart from each other (anaphase B) (Walczak *et al,* 2010). When the chromosomes start reaching the poles they gather and form a mass, an event that marks the entry into the fifth and final stage of mitosis – telophase. As the mitotic spindle breaks down, the nuclear envelope (NE) reassembles and the chromosomes decondense (Walczak *et al,* 2010). Following nuclear partition, cytokinesis then splits the cytoplasm of the parental cell. This is safeguarded by the shrinkage of an actomyosin contractile ring, a proteinaceous structure that arranges around the equator of the cell just underneath the plasma membrane of the cell (Glotzer, 2005). The result of this tightly controlled five-stage process is the formation of two identical daughter cells.

The spindle assembly checkpoint and kinetochore-microtubule dynamics

For the segregation of the duplicated chromosomes to be faithful during mitosis, all sister kinetochores must establish proper stable end-on attachments with MTs from opposite poles (amphitelic kinetochore-microtubule or k-MT interactions) (Fig I.2). The spindle assembly checkpoint (SAC) is in charge to surveil this condition and prevents cells from undergoing anaphase until it is met. Entry into anaphase primarily lies on the proteolytic cleavage of mitotic substrates by the anaphase promoting complex/cyclome (APC/C), a large multi-subunit E3 ubiquitin ligase activated by CDC20. Cyclin B1, essential for the maintenance of the mitotic state, as well as securin, involved in the regulation of sister chromatid cohesion, are amongst the mitotic substrates cleaved by APC/C. While the degradation of securin releases separase to cleave cohesin and allow chromosomes to be segregated, degradation of cyclin B1 results in the inactivation of CDK1 to proceed with mitotic exit (extensively reviewed in Sivakumar & Gorbsky, 2015). Consequently, the SAC prevents anaphase onset by sequestering the APC/C activator CDC20 in a complex known as the mitotic checkpoint complex (MCC). This complex assembles on unattached kinetochores and is composed by the proteins MAD2, BUBR1, BUB3 and CDC20 (Foley & Kapoor, 2013; Lara-Gonzalez *et al*, 2021; Musacchio & Salmon, 2007; Rieder *et al*, 1995). With CDC20 trapped, the mentioned chain of events is not set in motion, which provides the cell with time to properly bi-orientate chromosomes between the separated spindle poles. Only when all kinetochores are stably end-on attached to MTs, checkpoint satisfaction ultimately allows the cell to proceed to anaphase (Lara-Gonzalez *et al,* 2021; Musacchio & Salmon, 2007; Rieder *et al,* 1994).

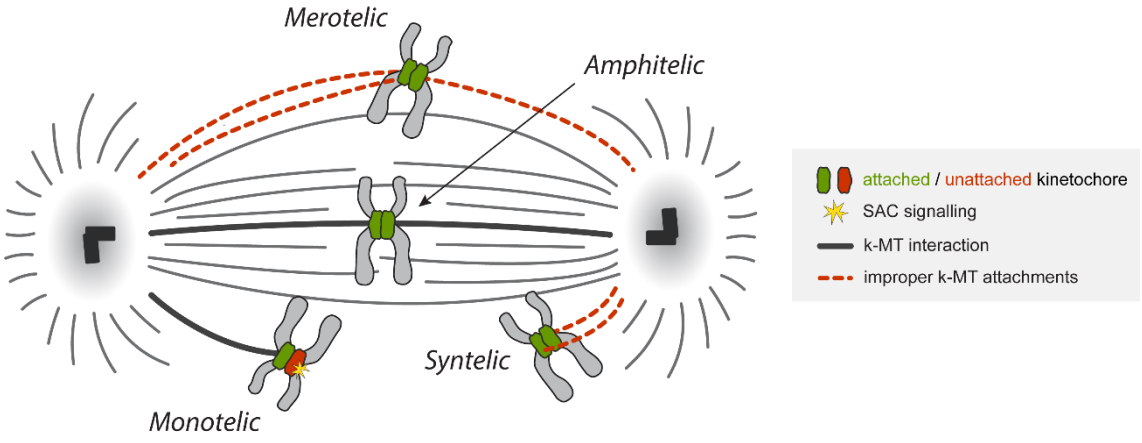

Figure I.2. Schematic representation of how kinetochores can initially interact with microtubules. The metaphase-to-anaphase transition surveilled by the SAC requires all kinetochores to establish stable end-on attachments with MTs from opposite spindle poles (amphitelic configuration). However, the stochastic nature of prometaphase occasionally leads to the generation of other non-optimal interactions – syntely and merotely – which rely on MT dynamics to generate monotelic attachments (only one sister chromatid attached to MTs) giving the cell by this way another chance to establish a proper interaction.

Syntelic attachments (both sister kinetochores bound to MTs emanating from the same spindle pole) or merotelic attachments (single kinetochore interacting with MTs emanating from opposite spindle poles) are particular k-MT interactions poorly sensed by the SAC (Fig I.2), that have to be corrected prior to anaphase onset in order to avoid chromosome mis-segregation. Such attachments occur because of the stochastic nature of prometaphase, where constant growth and shrinkage of MTs is necessary for the search and capture of kinetochores to achieve bi-orientation (Tanaka *et al*, 2005). As a result, the cell relies on the tight regulation of k-MT dynamics. This balance ensures that there will be enough stability for a k-MT to form and avoid prolonged mitotic arrest, while an adequate level of instability will allow correction of erroneous k-MTs to prevent chromosomes from mis-segregating (see Bakhoum & Compton, 2012). A major player participating in the regulation of this balance is Aurora B, a kinase that phosphorylates specific targets (including MT depolymerizing kinesin-13 proteins) involved in the disassembly of improper k-MT interactions (Bakhoum *et al*, 2009; Lampson *et al*, 2004). This returns chromosomes to a mono-oriented state (with an unattached kinetochore) (Fig I.2), which gives the cell the opportunity to establish an amphitelic k-MT attachment (Rieder & Maiato, 2004). Consequently, the combined action of both networks (SAC and k-MT dynamics) during mitosis is crucial to prevent the generation of cells with abnormal numbers of chromosomes (also known as aneuploidy).

I.2. Aneuploidy: causes and consequences

The term aneuploidy describes an unbalanced karyotype or state in which the cells no longer have a chromosome number that is a whole multiple of the organism's normal haploid complement. Deviations can range from gains or losses of entire chromosomes (numerical) to changes in chromosome portions (structural), and can be present in all cells (constitutional) or only partially in somatic cells (acquired) of an organism (Jackson-Cook, 2011) (Fig I.3). Aneuploidy has been reported at very low frequencies in healthy tissues (Knouse *et al*, 2014), which underscores the functionality and high efficacy of the protection tiers the cells have in place to prevent or halt the propagation of damage. Conversely, high frequencies of aneuploidy are recurrent in diseased states. For example, aneuploidy has been linked to neurodegeneration, infertility, cancer and aging (extensively reviewed in Harton & Tempest, 2012; Nagaoka *et al*, 2012; Naylor & van Deursen, 2016; Ricke & van Deursen, 2013; Siegel & Amon, 2012; Weaver & Cleveland, 2006). As a result, increasing research efforts have allowed to enlighten the basic biology of aneuploidy, going from the causes up to the consequences of this genomic state, with the goal to better understand the role of its presence in pathological conditions and seek for potential aneuploidy-centered therapeutic approaches. The purpose of this section is thus to summarize the state-of-the-art in the field of aneuploidy, whilst highlighting recent studies focused on aneuploidy in cancer and aging.

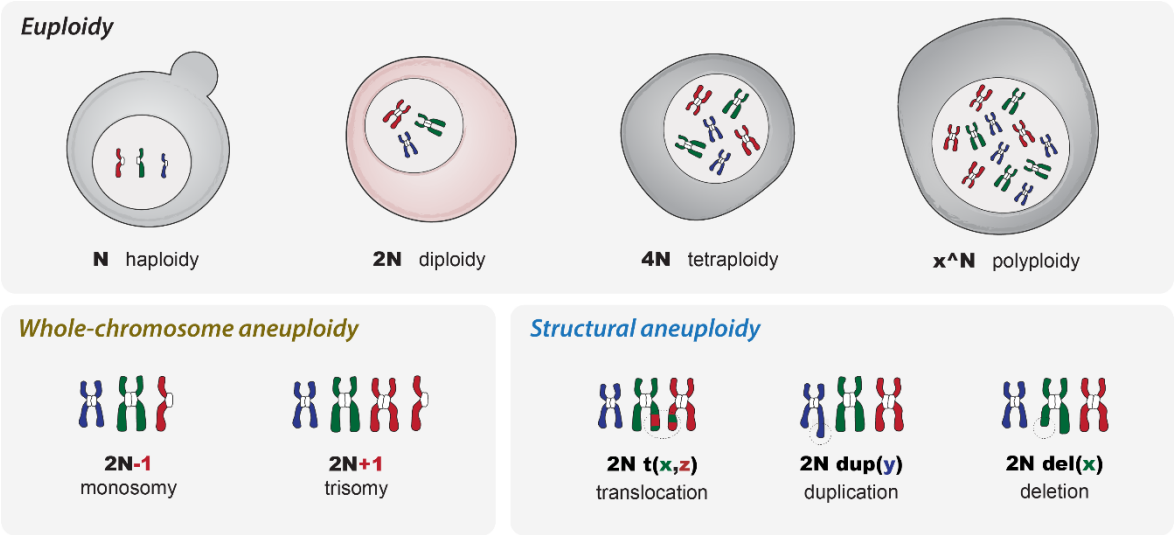

Figure I.3. Euploidy vs. aneuploidy. Euploidy (top) refers to a balanced genomic state which, depending on the species, can be haploid (N), diploid (2N) or polyploid (4N; or x^N). Conversely, aneuploidy (bottom) refers to an unbalanced genomic state that deviates from the multiple of the haploid complement, including gains (2N+1) or losses (2N-1) of entire chromosomes (left; whole-chromosome aneuploidy), as well as imbalances of chromosome portions (right; structural aneuploidy).

I.2.1. Routes to aneuploidy

While structural karyotypic defects are generally a result of stress and unresolved errors during DNA replication, whole-chromosome imbalances are the cost of a faulty passage through mitosis (Compton, 2011; Holland & Cleveland, 2012; Thompson *et al*, 2010) (Fig I.4). Faithful chromosome partition requires a suitable amount of time for MTs to organize into a bipolar mitotic spindle and properly interact with all chromosomes at kinetochores. The mitotic checkpoint is in place to surveil the execution of these events (Musacchio & Salmon, 2007). Thus, defective SAC signaling will allow premature progression into anaphase, which dramatically increases the chances of mis-segregation events and subsequent generation of aneuploid cells (Fig I.4A). Also, sister kinetochores must interact with MTs from opposite spindle poles (amphitely). Merotelic k-MT interactions are not sensed by the mitotic checkpoint but are, in most cases, corrected prior to anaphase onset as a result of the tightly balanced stability of k-MTs (Cimini *et al*, 2004; Cimini *et al*, 2003). However, dysregulation of k-MT dynamics allows improper attachments to persist into anaphase, generating aneuploid cells following mis-segregation of both sister chromatids towards the same pole or the exclusion of the lagging chromatid that is left behind in the spindle midzone from the main nuclei (Cimini *et al*, 2002; Cimini *et al*, 2001) (Fig I.4B). The likelihood of such improper attachments increases in the presence of supernumerary centrosomes (Fig I.4C), which is a frequent phenomenon in cancer cells and generally a result of cell division failure, cell fusion, centrosome over-duplication or loss of spindle-pole integrity (Maiato & Logarinho, 2014; Nigg, 2002). Lastly, deficits in the cohesin-dependent physical connection that opposes the pulling forces generated upon k-MT interaction have also been pointed out as a source of whole-chromosome aneuploidy (Fig I.4D), as untimely sister chromatid separation can lead to the asymmetric distribution of the sister chromatids by the daughter cells (Peters & Nishiyama, 2012; Tanaka *et al*, 2000).

I.2.2. Impact on cellular and organismal fitness

Aneuploidy has been systematically given a negative connotation, which becomes evident from the well-established association between aneuploidy and an abnormal development. Many contributions have been made over the years sustaining this link, from Theodor Boveri's pioneering studies in sea urchin embryos up to observations made in plants, *D. melanogaster*, budding and fission yeast, *C. elegans*, mouse and human trisomy 21 patients. Overall, aneuploidy was found to be lethal early in development and, in case it is tolerated, the imbalances result in substantial developmental defects. Viability and

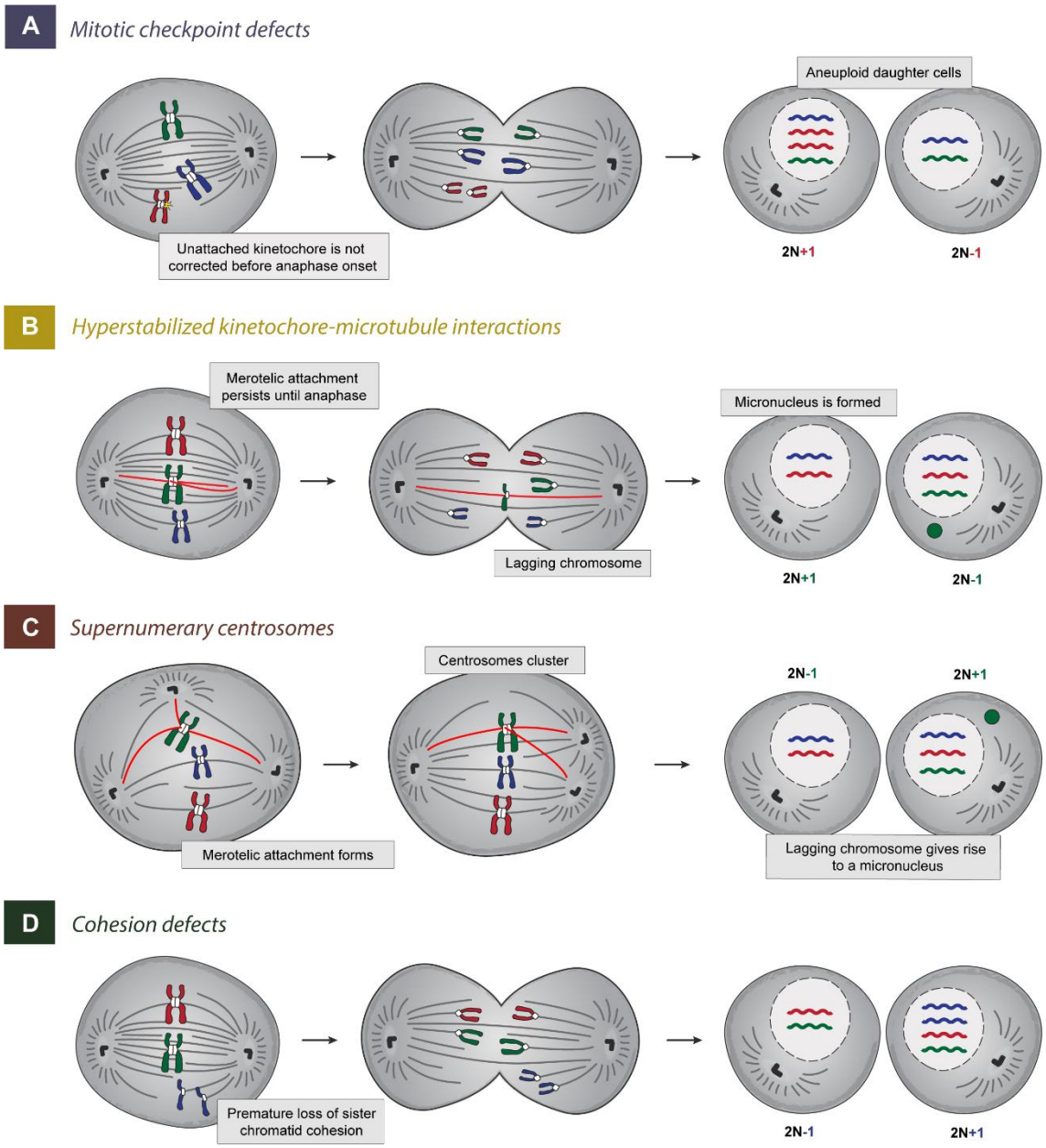

Figure I.4. Mechanisms of aneuploidy. The gain or loss of entire chromosomes can result from (**A**) defects in spindle assembly checkpoint signaling, (**B**) the establishment of hyperstable k-MT attachments leading to increased frequency of merotelic interactions, (**C**) assembly of multipolar mitotic spindles due to supernumerary centrosomes, or (**D**) premature loss of sister chromatid link as a result of cohesion defects.

severity of the consequences on organismal fitness seem to be governed by the coding sequence load of the affected chromosome and it has been appreciated that the loss is far less well tolerated than the gain of genetic material (extensively reviewed in Siegel & Amon, 2012; Torres *et al*, 2008).

Regarding the consequences on individual cells, they only started to be elucidated with the help of newly established yeast strains and mammalian cell lines that carry either defined or randomly induced aneuploidies (Stingele *et al*, 2012; Torres *et al*, 2007; Upender *et al*, 2004; Williams *et al*, 2008). Studies that compared these different cellular models of

aneuploidy with their euploid counterparts revealed a typical scale-up phenomenon in the gene expression and proteomic profiles of the cells (recently reviewed in Kojima & Cimini, 2019). Basically, there is not only a proportional impact on the expression of the genes encoded on the extra chromosome (Ben-David *et al*, 2014; Torres *et al,* 2007; Williams *et al,* 2008), but also on a set of genes mapping to other chromosomes (Dephoure *et al*, 2014; Hwang *et al*, 2021; Stingele *et al,* 2012; Upender *et al,* 2004). This scale-up then extends to the protein level, except for kinases or protein subunits part of multi-protein complexes, which are kept at stoichiometric levels (Dephoure *et al,* 2014; Hwang *et al,* 2021; Liu *et al*, 2017; McShane *et al*, 2016; Pavelka *et al*, 2010; Stingele *et al,* 2012). Importantly, the simple presence of non-expressed extra DNA was not able to induce an aneuploidy-equivalent phenotype (Torres *et al,* 2007), which suggests that the stress inflicted on specific biological pathways may render the phenotypes transversal to different aneuploidies, rather than being unique to specific chromosomal imbalances. Indeed, analyses on aneuploid cells from diverse organisms revealed an environmental stress response (ESR) signature (Sheltzer *et al*, 2012; Torres *et al,* 2007), and currently it is recognized that there is a set of specific and conserved phenotypes transversal to aneuploid cells (reviewed in Chunduri & Storchova, 2019; Zhu *et al*, 2018) (Fig I.5). Specifically, aneuploid cells have defective proliferative capacity (Beach *et al*, 2017; Niwa *et al*, 2006; Pavelka *et al,* 2010; Pfau *et al*, 2016; Torres *et al,* 2007; Williams *et al,* 2008) and proteostasis (Dodgson *et al*, 2016b; Donnelly *et al*, 2014; Ohashi *et al*, 2015; Oromendia *et al*, 2012; Santaguida *et al*, 2015; Tang *et al*, 2011; Torres *et al,* 2007), and undergo changes in metabolism (Hwang *et al*, 2017; Tang *et al,* 2011; Tang *et al*, 2017; Torres *et al,* 2007; Williams *et al,* 2008), protein trafficking and membrane integrity (Dodgson *et al*, 2016a; Hwang *et al*, 2019).

Finally, and important for the here-presented thesis, chromosomal imbalances were found to affect also both duplication and segregation of the genetic material, thus increasing the tendency of genome alterations over cell divisions (or genomic instability) (reviewed in Nicholson & Cimini, 2015; Passerini & Storchova, 2016). Both yeast and human cellular models of aneuploidy show slowed or stalled replication fork progression (Blank *et al*, 2015; Lamm *et al*, 2016; Passerini *et al*, 2016; Santaguida *et al*, 2017; Sheltzer *et al*, 2011). As a result, incidence of DNA breaks and damage becomes increased in aneuploid cells (Blank *et al,* 2015; Fabarius *et al*, 2003; Kost-Alimova *et al*, 2004). Besides, replication stress or entry into mitosis in the presence of unresolved DNA damage was also found to induce chromosome mis-segregation (Bakhoum *et al*, 2014; Burrell *et al*, 2013; Lamm *et al,* 2016; Santaguida *et al,* 2017), suggesting that DNA replication defects might also foster chromosomal instability (CIN). Although CIN is often mistakenly used to refer to aneuploidy, CIN characterizes persistent whole-chromosome segregation errors over several cell

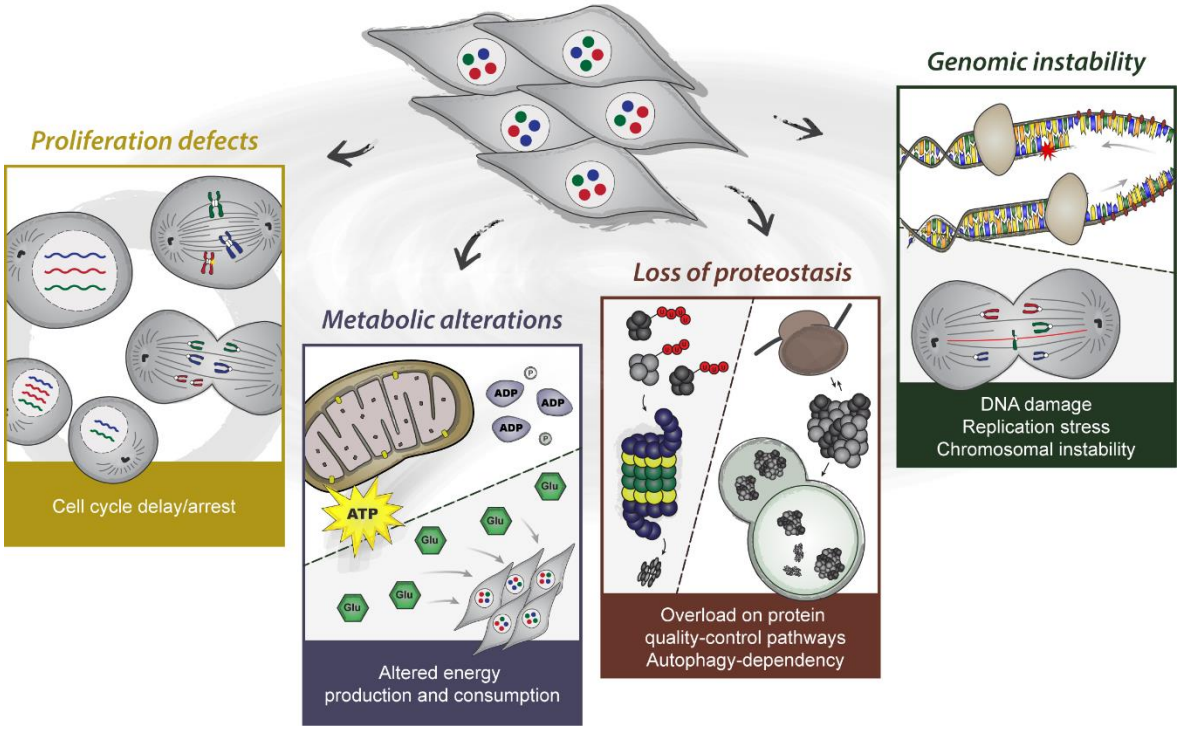

Figure I.5. Consequences of aneuploidy. The gain or loss of chromosomes inflicts severe distress on cells, originating the so-called pan-aneuploidy phenotypes. Amongst them are slowed proliferation, loss of proteostasis, metabolic stress and genomic instability. Glu - Glucose; U - Ubiquitin.

generations (rate), that then lead to the accumulation of cells with aneuploid karyotypes (state) (Geigl *et al*, 2008; Schukken & Foijer, 2018). Observations taken from cancer cell lines and yeast strains bearing defined or random aneuploidies, and cells derived from patients with constitutional aneuploidy suggest, indeed, that the chromosomal imbalances decrease chromosome segregation fidelity (Duesberg *et al*, 1998; Hintzen *et al*, 2021; Lengauer *et al*, 1997; Nicholson *et al*, 2015; Passerini *et al,* 2016; Valind *et al*, 2013; Zhu *et al*, 2012; our unpublished data). Most events of chromosome mis-segregation arise from persistent merotelic k-MT attachments, which manifest as lagging chromosomes in anaphase and generate micronuclei (MN) after being excluded from daughter cell nuclei (Cimini *et al,* 2001; Thompson & Compton, 2008). This is particularly relevant since MN have defective nuclear envelope assembly (Liu *et al*, 2018; Maass *et al*, 2018), and improper recruitment of DNA replication and repair machineries (Crasta *et al*, 2012), rendering them particularly susceptible to rupture and massive DNA damage (known as chromothripsis) (Hatch *et al*, 2013; Ly & Cleveland, 2017; Stephens *et al*, 2011; Zhang *et al*, 2015). Also, evidence shows that chromosomes trapped in micronuclei fail to establish a proper kinetochore and are thus liable to segregation errors in the next cycle (Soto *et al*, 2018). The impact of aneuploidy on genome stability seems thus to be a vicious circle: replication stress increases DNA damage and reduces chromosome segregation fidelity, which negatively impacts the fitness of the cell, generating amongst others additional DNA

damage and chromosome segregation defects in the next cell cycle (see Bakhoum *et al*, 2017; Fenech *et al*, 2020; Soto *et al*, 2019).

I.2.3. Aneuploidy in cancer and aging

As mentioned before, in opposition to its rare presence in normal tissues, abnormal numbers of chromosomes are relatively prevalent in human tumors and also found in aged tissues. Even though over the past years research already shed some light onto the causes and consequences of aneuploidy in cancer and aging, the connection between aneuploidy and these diseased states remains elusive.

Cancer is a multi-step somatic evolutionary process that lies on out competition of less fit cells by highly proliferative ones (Hanahan & Weinberg, 2000, 2011; Hanahan, 2022). Higher fitness results from continuous genetic shuffle and acquisition of alterations that confer adaption to several challenges faced by cells within the tumor microenvironment (*e.g,* endogenous anti-cancer barriers, nutrient deprivation, hypoxia or chemotherapeutic treatment). So, with all troubles that come with the aneuploid state, it is intriguing how cancer cells deal with and can take advantage from this state (Sheltzer & Amon, 2011). It has been observed that aneuploidy impacts genome stability (Nicholson & Cimini, 2015; Passerini & Storchova, 2016) and that this can set in motion further karyotype heterogeneity (Soto *et al,* 2019). Also, evidence shows that the aneuploidy-induced stress response and the selection of certain karyotypes can confer phenotypic plasticity that allows single-cell organisms to cope with stressful environments (Chen *et al*, 2012; Millet *et al*, 2015; Pavelka *et al,* 2010; Rutledge *et al*, 2016; Selmecki *et al*, 2006). Thus, aneuploidy likely generates a genetic and phenotypic ‘chaos’ that, after being tolerated, can be exploited for evolution and adaptation (discussed in Durrbaum & Storchova, 2016; Giam & Rancati, 2015; Lukow & Sheltzer, 2021) (Fig I.6A). For example, transient loss of chromosome stability allowed for selection of cell populations with recurrent aneuploidies which developed resistance against anti-cancer therapies (Ippolito *et al*, 2021; Lukow *et al*, 2021; Replogle *et al*, 2020). However, and based on most recent evidence, aneuploidy should not be regarded to as a universal driver of cancer development. The correlation between aneuploidy and cancer is complex (extensively reviewed in Vasudevan *et al*, 2021). Not all aneuploidies uniformly drive malignancy, and the impact of certain aneuploidies on tumor progression highly depends on the genetic and environmental *milieu* (Sheltzer *et al*, 2017; Vasudevan *et al*, 2020). Essentially, ‘jackpot’ combinations have to take place. In fact, karyotypic heterogeneity seems to evolve from random chromosome gains in early phases to recurrent gains of a few chromosomes in established tumors, as elegantly shown by two recent studies in mice

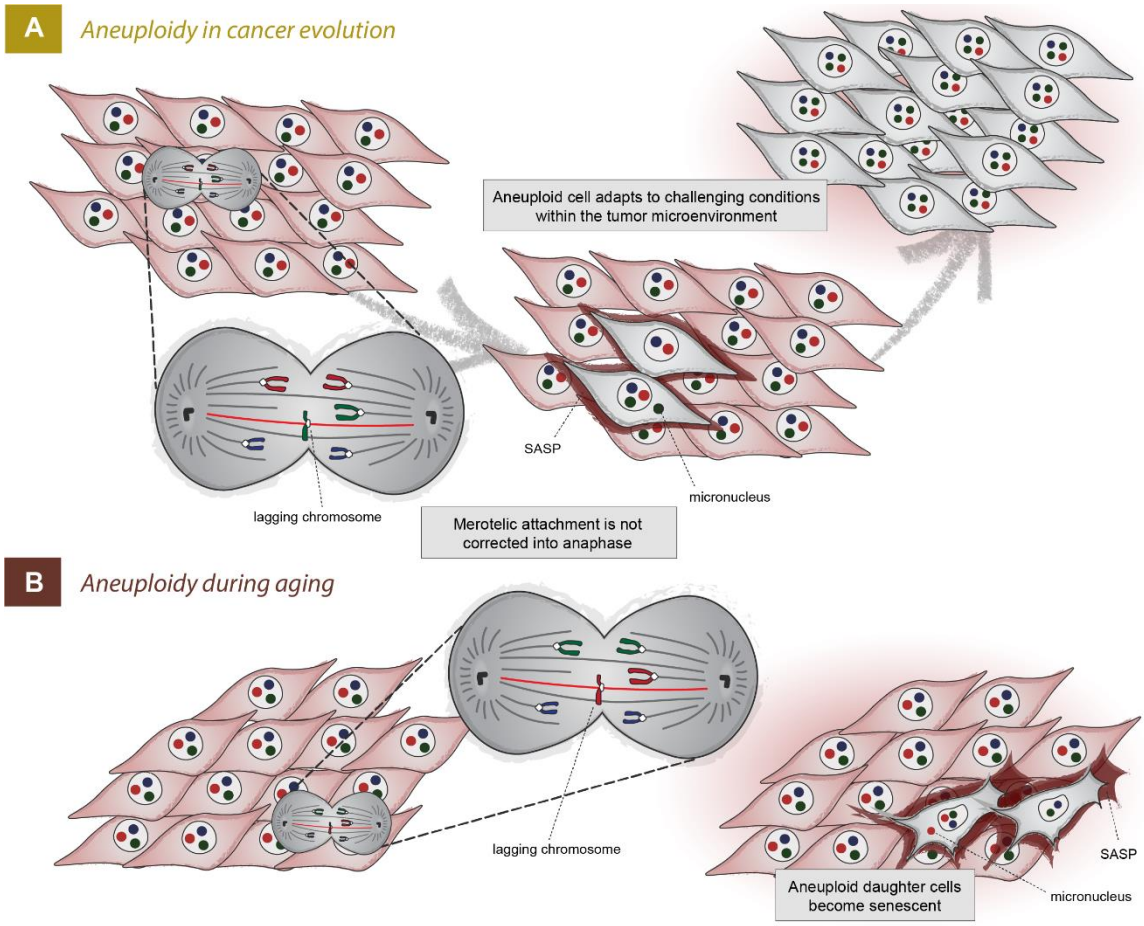

Figure I.6. Aneuploidy in disease. (**A**) In the context of cancer, generation of aneuploidy and further chromosomal instability can foster adaptability via karyotypic heterogeneity within the cell population. In the presence of defined environmental conditions, certain karyotypes/phenotypes (depicted here as a specific aneuploid cell) within the population will adapt, survive and outcompete less fit cells promoting cancer evolution. On the other hand, (**B**) along with age cells lose the ability to faithfully propagate their chromosomal content by the progeny. The generation of aneuploid daughter cells as a result of the mitotic dysfunction that occurs with age will aid the development of a full-blown senescence phenotype. Non-cell autonomous effects of aneuploid senescent cells will compromise tissue homeostasis by influencing the neighbor cells and tissue microenvironment, ultimately contributing to the aging process. SASP, Senescence-Associated Secretory Phenotype.

(Shoshani *et al*, 2021; Trakala *et al*, 2021). Thus, when put in proper context, certain gene expression imbalances will positively impact the tumorigenic process. Besides changes in expression, it has been recognized that chromosome dosage imbalances can result in cellular senescence (Andriani *et al*, 2016; Giam *et al*, 2020; Joy *et al*, 2021; Lentini *et al*, 2012; Macedo *et al*, 2018), which itself can profoundly impact tumorigenesis via the non-cell-autonomous effects of this cellular state (discussed in more detail in the next section and in '*Senescence in health and disease*'). For example, a correlation between aneuploidy and upregulation of senescence-associated secretory phenotype (SASP) factors was found at the invasive tumor front in patient samples of invasive ductal breast carcinoma (He *et al*,

2018). Thus, albeit SASP is known to signal for immune-mediated clearance of aneuploid senescent cells (Santaguida *et al,* 2017; Wang *et al*, 2021), the pro-inflammatory phenotype can foster tumorigenesis in a paracrine-dependent manner where the aneuploid senescent cells eventually become bystanders and can even be cleared out (He *et al,* 2018). The bottom line is that aneuploidy should never be regarded to as exclusively tumor-promoting or tumor-suppressive, and that the outcome of aneuploidy on the tumorigenic process is highly context-dependent (*i.e,* tissue identity, presence of tolerating-mutations, identity of duplicated regions, tumor stage and/or immune system). However, once ideal genetic and environmental conditions are met, and the negative effects of aneuploidy are outbalanced by the generation of an advantageous *milieu*, chromosome imbalances can turn out beneficial for cancer evolution and that is probably why aneuploidy is highly prevalent in human tumors (extensively reviewed in Ben-David & Amon, 2020; Zhou *et al*, 2020).

In opposition to cancer, aging defines a biological process characterized by the gradual decline in proliferative potential and coincides with the accumulation of extensive molecular damage, which dictates severe alterations in cellular physiology and ultimately compromises physiological functions (Lopez-Otin *et al*, 2013). Thus, conversely to the paradoxical presence of aneuploidy in cancer, it is not surprising that aging and genomic imbalances were already associated decades ago (Jacobs & Court, 1966). Indeed, a link between the hallmarks of the aging process and loss of chromosome segregation fidelity during cell division has been suggested (Macedo *et al*, 2017). Accordingly, an age-associated loss of X and Y chromosomes in females and males is observed (Jacobs *et al*, 1963; Pierre & Hoagland, 1972; Stone & Sandberg, 1995), and chromosome errors were found in the aging mouse brain (Faggioli *et al*, 2012), aged oocytes (Nagaoka *et al,* 2012), peripheral blood lymphocytes (Mukherjee *et al*, 1996) and skin fibroblasts from elderly individuals (Mukherjee & Thomas, 1997). Also, an analysis comprising panels of fibroblast and lymphocyte cultures from young and old age groups unveiled age-related expression changes in several genes involved in both the cell cycle and proliferation, including genes implicated in mitotic spindle assembly and centromere/kinetochore function. Notably, such changes correlated with increased levels of aneuploidy (Geigl *et al*, 2004). All these findings suggests that, in addition to a possible negative impact of the aging hallmarks on the mitotic machinery, there may be an inherent decline of mitotic function with age which likely dictates loss of chromosome segregation fidelity and may eventually contribute to the aging process. In fact, studies with SAC-impaired mice models uncovered a link between compromised checkpoint function and premature onset of aging phenotypes (Baker *et al*, 2013; Baker *et al*, 2004; Baker *et al*, 2006). It turned out that premature onset of aging phenotypes in mice with mitotic checkpoint gene defects is linked to cellular senescence, as life-long removal of senescent ($p16^{Ink4a}$-positive) cells delayed tissue dysfunction and extended healthspan

(Baker *et al*, 2011) (see also '*Senescence in aging and age-related pathologies'*). This fits with evidence from cellular models demonstrating that aneuploidy and CIN do drive cellular senescence (Andriani *et al,* 2016; Giam *et al,* 2020; Joy *et al,* 2021; Lentini *et al,* 2012; Santaguida *et al,* 2017). In addition to the SAC-defective mouse models, the link between mitotic dysfunction, CIN and senescence likely also applies to the pediatric condition known as Mosaic variegated aneuploidy syndrome (MVA), for which patients have diminished steady state levels of the mitotic checkpoint protein BubR1 as a result of a mutation (Suijkerbuijk *et al*, 2010), and do experience chromosome mis-segregation alongside with progeroid features (Hanks *et al*, 2004; Matsuura *et al*, 2006). By putting all these pieces of evidence together, it is reasonable to positively correlate loss of mitotic function and senescence during aging. Yet, clear evidence for such gradual mitotic dysfunction in dividing cells during normative aging and its association to cellular senescence was only recently shown by a study from our laboratory. Human dermal fibroblasts derived from neonatal to octogenarian individuals revealed a transcriptional 'shutdown' with age correlating with the repression of the transcription factor Forkhead box M1 (FoxM1) that drives expression of most G2-M genes. Importantly, increased aneuploidy and senescence in aged cells could be modulated upon improvement of mitotic fitness through the expression of a constitutively active form of the repressed transcription factor (Macedo *et al,* 2018). Thus, even though mechanistical insights are still largely missing, these findings suggest that aneuploidy is causally linked with cellular senescence during aging which, as discussed later during this thesis, may implicate aneuploidy accumulation in the pathophysiology of the aging process (Fig I.6B).

I.3. Cellular senescence: a friend or a foe?

Cells do have multiple layers of defense including, as aforementioned, several quality-check stages and repair mechanisms to prevent or minimize accumulation of damage such as karyotypic alterations. In case these mechanisms are not successful, precluding cells from further cycling is the only means to limit the propagation of damage and by this way avoid transition into a tumorigenic state (transformation). This is achieved by pushing cells to undergo death (apoptosis) or enter a non-proliferating state (senescence). This section will focus on cellular senescence, the purpose of which extends far beyond being a mere 'break' of damage propagation. In fact, as described later, senescence plays an important role in tissue remodeling during development or after injury and has been implicated in the aging and cancer processes.

I.3.1. Cellular senescence at a glance

Senescence defines the state in which cells irreversibly arrest in the cell cycle in response to sustained stress- and damage-inducing stimuli (reviewed in Hernandez-Segura *et al*, 2018; Rodier & Campisi, 2011; Salama *et al*, 2014). It was originally coined by Hayflick and Moorhead, which observed this response in primary human cells in culture that reached the endpoint of their replicative lifespan (Hayflick & Moorhead, 1961). However, there are now other well-established senescence instigators that can act on most division-competent cells (Campisi & d'Adda di Fagagna, 2007; Shay & Roninson, 2004), namely loss of genomic stability, oxidative stress, mitochondrial dysfunction, oncogene activation, epigenetic deregulation and paracrine signaling (Hernandez-Segura *et al,* 2018). In the presence of such stimuli, besides the already mentioned growth arrest, senescent cells will undergo a series of characteristic changes, including metabolic hyperactivity (Dorr *et al*, 2013) and morphological alterations that can be used to discern them (described in detail below) (Fig I.7). Curiously, cumulative evidence suggests that the senescent state can extend to post-mitotic cells such as adipocytes (Minamino *et al*, 2009), neurons (Jurk *et al*, 2012; Oubaha *et al*, 2016), osteocytes (Farr *et al*, 2016; Farr *et al*, 2017) or hepatocytes (da Silva *et al*, 2019), which seemingly retain cellular machineries able to engage programs and exhibit phenotypes of senescence when in the presence of stressors (extensively reviewed in Sapieha & Mallette, 2018). Unless otherwise stated, cellular senescence will be used here-after to refer to proliferative/mitotic cell populations (*e.g,* fibroblasts, epithelial and endothelial cells).

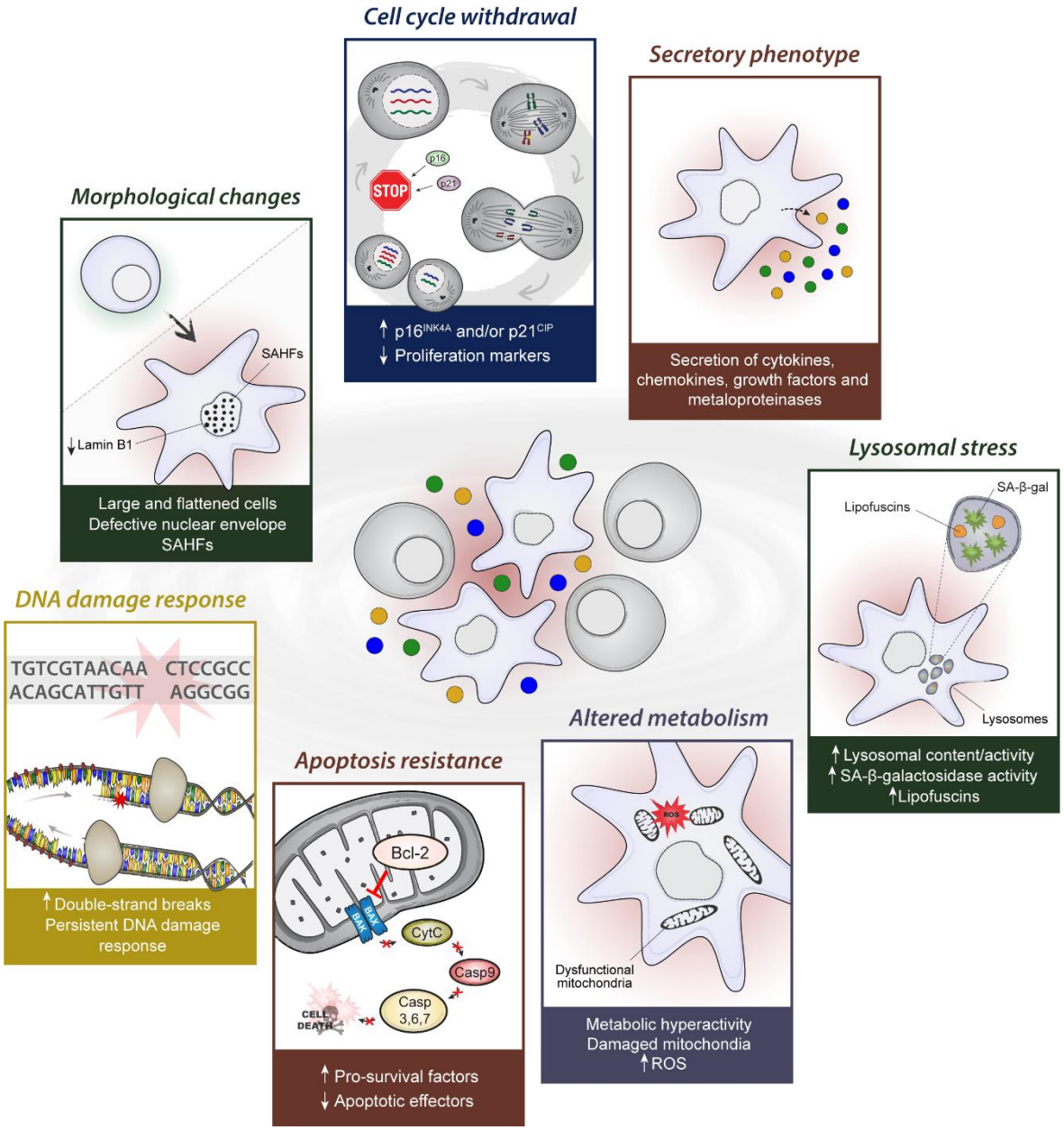

Figure I.7. **Cellular senescence**. Following exposure to certain stresses (*i.e,* GIN, oxidative stress, mitochondrial dysfunction, oncogene activation, epigenetic deregulation and/or paracrine signaling) cells enter into a non-dividing state called senescence. In addition to cell cycle withdrawal, such cells become larger and flattened, exhibit persistent DNA damage response (DDR), senescence-associated heterochromatin foci (SAHFs), changes in nuclear envelope composition, lysosomal stress and altered metabolic needs. Also, senescent cells develop resistance to cell death (or apoptosis) and present a peculiar secretome comprising a variety of soluble and insoluble factors (also known as senescence-associated secretory phenotype or SASP).

Cell-cycle arrest

Amongst the phenotypes that primarily characterize the senescent state is the stable and long-term loss of proliferative capacity given by the absence of proliferation markers, for which engagement of the p53 and/or $p16^{Ink4a}$–pRb pathways is essential (extensively reviewed in Gorgoulis *et al*, 2019; Hernandez-Segura *et al,* 2018; Rodier & Campisi, 2011;

Salama *et al,* 2014). The tumor-suppressor protein p53 is activated to induce senescence whenever there is a sustained DNA damage response (or DDR), such as in the presence of critical telomere erosion and/or unrepairable DNA breakage (Campisi, 2013). The executer of this signaling pathway is the cyclin-dependent kinase inhibitor 1 protein, a transcriptional target of p53 also known as $p21^{Cip1}$ (Brown *et al*, 1997; Jackson & Pereira-Smith, 2006; Krenning *et al*, 2014; Kulju & Lehman, 1995). While its transient activation arrests cycling cells to ensure efficient DNA repair, prolonged DDR and sustained p53-p21 signaling will prevent cell-cycle progression and force cells to enter the senescent state (Dulic *et al*, 2000; Rodriguez & Meuth, 2006). As a secondary pathway to p53 activation is the $p16^{Ink4a}$–pRb signaling network, which also becomes activated as a result of DDR and oncogene activation (Stein *et al*, 1999). Similarly to $p21^{Cip1}$, $p16^{Ink4a}$ (or cyclin-dependent kinase inhibitor 2A) will prevent entry into S-phase by keeping the retinoblastoma protein (or Rb) in its unphosphorylated (active) form, which ultimately prevents the transcription factor E2F from inducing expression of proliferation-associated genes (Narita *et al*, 2003; Sherr & McCormick, 2002). Although these pathways crosstalk, they can stop cell-cycle progression independently and become engaged differently depending on cell type or species (Campisi & d'Adda di Fagagna, 2007). Also, while p53 signaling-induced withdrawal from the cell cycle can be reversible depending on the duration of the stimulus and the cell cycle stage, the engagement of $p16^{Ink4a}$–pRb axis is generally a way of no return (Beausejour *et al*, 2003; Takahashi *et al*, 2006). Irrespective of the pathway, sustained signaling appears to be key for maintenance of the senescent state as absence of p53 or p16 activity increases susceptibility to tumorigenesis (Dirac & Bernards, 2003; Donehower *et al*, 1992; Jacks *et al*, 1992; Krimpenfort *et al*, 2001; Sage *et al*, 2003; Sharpless *et al*, 2001). Yet, Krenning and collaborators have shown that p53 signaling can drive senescence in G2 phase where, in opposition to G1 phase, a transient activation of p53 is sufficient to drive an irreversible arrest (Krenning *et al,* 2014). Finally, additional changes ensure the maintenance of cell-cycle blockage, namely the heterochromatinization of E2F target genes (Narita *et al,* 2003), the continued production of ROS (Takahashi *et al,* 2006), and changes in ribosome biogenesis (Lessard *et al*, 2018). Also, as discussed next, cells arrested in the cell cycle start to exhibit a characteristic secretory phenotype that allows a cell-autonomous reinforcement of the senescent state.

Secretory phenotype

Senescent cells also undergo dramatic alterations in gene expression and metabolism. Such changes include the development of the senescence-associated secretory phenotype (or SASP). This peculiar secretome encompasses a variety of soluble and insoluble factors, such as cytokines, chemokines, growth modulators, angiogenic

factors and matrix metalloproteinases, that retain the ability to reinforce the senescent state (cell autonomous) and affect surrounding cells and the microenvironment (non-cell autonomous) (Acosta *et al*, 2013; Campisi, 2013; Coppe *et al*, 2010a; Coppe *et al*, 2008; Kuilman & Peeper, 2009). SASP secretion in senescent cells is essentially triggered by genomic damage or epigenetic dysfunction. Thus, as far as a cell experiences DNA damage, telomere erosion or epigenetic abnormalities, senescence induction will be followed by the development of a secretome with varying strength and composition (Acosta *et al*, 2008; Coppe *et al,* 2008; Hernandez-Segura *et al*, 2017; Kuilman *et al*, 2008; Pazolli *et al*, 2012; Rodier *et al*, 2009). Induction of senescence by any other means (*e.g,* ectopic expression of $p16^{Ink4a}$ or $p21^{Cip1}$) originates a cell with senescent features devoid of secretory phenotype (Coppe *et al*, 2011). Consequently, development of the SASP relies on persistent DNA damage signaling, which in turn generally activates factor nuclear kappa B (or NF-κB) via the engagement of the cGAS-STING pathway as very recently shown (Dou *et al*, 2017; Gluck *et al*, 2017; Hari *et al*, 2019; Yang *et al*, 2017) (see also '*cGAS-STING pathway in senescence development*'). Yet, other transcription factors and signaling networks participate in SASP development (Ito *et al*, 2017; Kuilman *et al,* 2008). Those include GATA4 and C/EBPb (Kang *et al*, 2015; Kuilman *et al,* 2008), as well as the mammalian target of rapamycin (mTOR), JAK2/STAT3, NOTCH and p38MAPK signaling pathways (Freund *et al*, 2011; Hayakawa *et al*, 2015; Hoare *et al*, 2016).

The SASP is one of the most striking features of senescent cells and is probably behind almost all consequences of cellular senescence in both health and disease (Childs *et al*, 2015; Coppe *et al,* 2010a; He & Sharpless, 2017). This is certainly related with its complex and highly heterogenous composition, comprising factors that stimulate growth and proliferation (*e.g,* GROs), promote angiogenesis (*e.g,* VEGF), and alter the proliferation and differentiation of stem cells (*e.g,* GROα, IL-6), as well as molecules with the ability to trigger epithelial-to-mesenchymal transition and inflammatory responses with variable durability (*e.g,* IL-6, IL-8, MCPs, MIPs) (Campisi, 2013). The transcriptional programs engaged during the senescent state seem to depend on the cell type (*i.e,* astrocytes, fibroblasts, keratinocytes and melanocytes) and stress (*i.e,* replicative exhaustion, oncogene activation and ionizing radiation), with gene expression in senescent cells being temporarily dynamic (Hernandez-Segura et al, 2017). Furthermore, as SASP factors can exert multiple functions, the context (*i.e,* tissue of origin) is key to define whether their outcome is beneficial or deleterious. As already mentioned, the SASP works as a messenger to neighboring cells: it signals the damaged cell and stimulates the recruitment of an immune response for its clearance, whilst preparing the surrounding tissue for repair. Consequently, it contributes to tissue plasticity during development and wound healing

(Demaria *et al*, 2014; Munoz-Espin *et al*, 2013; Storer *et al*, 2013), and is an appreciated tumor-suppressive mechanism (Campisi, 2001; Serrano *et al*, 1997). Generally, this acute stress-induced senescence is rapidly resolved and clearly beneficial. Conversely, persistent stress-induced senescence generates a chronic pro-inflammatory *milieu* that is believed to fuel aging and aid in cancer progression (Campisi, 2013; Franceschi & Campisi, 2014) (see also '*Senescence in health and disease*').

Apoptosis resistance

Limiting the propagation of damaged cells implies preventing their proliferation, which can be achieved either by entering a senescent state or undergoing death. Commonly, death is ensured by the orderly execution of a series of molecular events, in which the cell systematically destroys itself from within and ultimately becomes engulfed by other cells. In most cases, this is safeguarded by a process termed apoptosis (extensively reviewed in Danial & Korsmeyer, 2004; Ellis *et al*, 1991; Raff, 1998). The intrinsic pathway of apoptosis resumes to a complex integration of pro-survival and pro-death signals, the latter of which must stand out so that the release of cytochrome c and other intermembrane mitochondrial proteins into the cytoplasm triggers the execution of the apoptotic cascade (Jiang & Wang, 2004; Tait & Green, 2013). The BCL-2 family is a major class of apoptotic regulators, comprising pro-apoptotic (*e.g,* Bax/Bak/Bok, Bim, Puma or NOXA) and anti-apoptotic (*e.g,* BCL-2, BCL-xL, BCL-w, or MCL-1) proteins (Czabotar *et al*, 2014; Jiang & Wang, 2004; Willis & Adams, 2005). An additional sensitizer is the tumor-suppressor protein p53, which activates the intrinsic apoptosis network via transcriptional engagement of various pro-apoptotic BCL-2 family members and interaction with several pro- and anti-apoptotic proteins in the cytoplasm and at the mitochondrial membrane (Kruiswijk *et al*, 2015; Vousden, 2005).

Consequently, a senescent cell must develop conditions that allow death signaling evasion to maintain the senescent state when in the presence of high levels of cellular damage (Childs *et al*, 2014). Essentially, senescent cells become resistant to apoptosis by activating several anti-apoptotic factors (*e.g,* pro-survival BCL-2 family members) (Ryu *et al*, 2007; Wang, 1995). As a result, pore-formers Bax and Bak will not release cytochrome c and other mitochondrial components (Uren *et al*, 2017), which then prevents the final steps of the apoptotic cascade namely the apoptosome formation and the caspase cleavage/activation cycles (Kumar, 2007; Yuan & Akey, 2013). Additionally, the epigenetic repression of the apoptotic effector Bax (Sanders *et al*, 2013), and p21 signaling (Rodriguez & Meuth, 2006; Yosef *et al*, 2017), have been implicated in maintenance of the senescent state. Finally, Forkhead box protein O4 (FoxO4) was recently pointed out as a pivot in

senescent cell viability maintenance as its upregulation constrains p53-mediated apoptosis in favor of cell-cycle arrest (Baar *et al*, 2017).

Other hallmarks of cellular senescence

In addition to DNA damage, cell-cycle withdrawal, the characteristic secretome and resistance to cell death, senescent cells display other hallmark features. Amongst them are the profound morphological alterations, including the large and flattened appearance of senescent cells and their increased adhesion to the extracellular matrix (Hernandez-Segura *et al,* 2018; Sharpless & Sherr, 2015). Senescence also induces changes in the content and envelope integrity of the nucleus. A reduction in lamin B1 levels is recurrent in senescent cells (Freund *et al*, 2012; Shimi *et al*, 2011), which has been associated with increased heterochromatinization (senescence-associated heterochromatic foci or SAHFs) (Chandra *et al*, 2012; Shah *et al*, 2013). Other changes include the accumulation of lysosomal content (Cho & Hwang, 2012; Robbins *et al*, 1970) and dysfunctional mitochondria (Correia-Melo & Passos, 2015; Passos *et al*, 2007). The former has been attributed to accumulation of old lysosomes (hence, to reduced clearance activity), and results in increased lysosomal enzyme activity and number of residual bodies (or lipofuscins) in senescent cells (Evangelou *et al*, 2017; Georgakopoulou *et al*, 2013). In addition to lipofuscins, the lysosomal stress results in an increase of lysosome enzyme β-galactosidase. Detection of senescence-associated β-galactosidase (SA-β-gal) activity at a suboptimal pH=6 is probably still one of the most used markers to identify senescent cells (Kurz *et al*, 2000; Lee *et al*, 2006). Similarly to lysosomes, mitochondria become dysfunctional and accumulate with senescence as a result of reduced clearance (or mitophagy) (Tai *et al*, 2017).

In conclusion, the senescence phenotype is highly heterogenous, can exhibit tissue- and cell type-specific differences, and evolves over time. To further complicate the whole picture, certain features are not exclusive to the senescent state and senescent cells do not always exhibit all markers. Also, regardless of being valuable *in vitro*, most markers are not yet reliable for the *in vivo* recognition of senescent cells (Sharpless & Sherr, 2015). Consequently, as it remains still elusive whether universal mechanisms of senescence do truly exist (Sikora *et al*, 2021), features to be analyzed have to be carefully chosen and investigated in combination to ensure the highest accuracy possible when it comes to study and define senescence (discussed in Gorgoulis *et al,* 2019).

I.3.2. cGAS-STING pathway in senescence development

The Cyclic GMP-AMP synthase (cGAS) is the forefront of a conserved pathway aimed at mediating an innate immune response against antimicrobial patterns, namely bacteria, retroviruses and DNA viruses (Margolis *et al*, 2017; Sun *et al*, 2013). It does so by catalyzing the formation of the second-messenger 2′3′-cyclic-GMP-AMP (cGAMP) once bound to double-stranded DNA (dsDNA) (Wu *et al*, 2013). This dinucleotide mediates the dimerization of the adaptor protein Stimulator of Interferon Genes (STING) (Gao *et al*, 2013), which ultimately leads to the transcriptional activation of inflammatory pathways, namely type I interferon (IFN) and NF-κB, to promote clearance of the damaged/infected cells (Galluzzi *et al*, 2018). However, cGAS recognition is not exclusive to antimicrobial patters. It can also sense self-DNA whenever it becomes accumulated in the cytoplasm as a result of perturbations in cellular homeostasis. Defects in cytoplasmic nucleases (DNase2, TREX1 or RNaseH2), errors in chromosome segregation (CIN), DNA damage, issues with nuclear envelope integrity and mitochondrial dysfunction can fuel such cytoplasmic DNA pool (reviewed in Fenech *et al,* 2020; Galluzzi *et al,* 2018; Wu *et al*, 2021; Xiao & Fitzgerald, 2013). These perturbations are amongst the hallmarks of senescence and not surprising a series of recent studies was now able to demonstrate that cGAS-STING pathway activation occurs in senescent cells where it is essential for SASP development (Fig I.8).

Senescent cells exhibit nuclear atypia as a result of changes in nuclear envelope (NE) composition, namely a decrease in lamin B1 (Freund *et al,* 2012; Shimi *et al,* 2011) and various other NE proteins (Lenain *et al*, 2015). Such nuclear envelope integrity issues allow cytoplasmic chromatin fragments (CCFs) to pinch off from intact nuclei of cells (Dou *et al*, 2015; Ivanov *et al*, 2013), which become available to cGAS recognition (Dou *et al,* 2017; Gluck *et al,* 2017; Yang *et al,* 2017). These studies were pioneer in connecting cGAS-STING engagement with senescence, as ablation of cGAS and/or STING functions both *in vitro* and *in vivo* prevented senescence-associated inflammatory genes from being expressed upon damage-inducing insults. Other studies further showed that downregulation of cytoplasmic DNases fosters cytoplasmic DNA accumulation and SASP in senescent cells (Han *et al*, 2020; Takahashi *et al*, 2018). It has been anticipated that cGAS-STING engagement contributes to the non-cell autonomous aspect of cellular senescence mostly by triggering NF-κB-mediated expression of SASP components (Dou *et al,* 2017). Taken together, persistent DNA damage, nuclear atypia and repression of cytoplasmic nucleases in senescent cells result in a jackpot combination that fuels SASP development and is necessary to explain certain consequences of this secretome (Gluck & Ablasser, 2019). This was recently exemplified in some progeroid conditions, for which

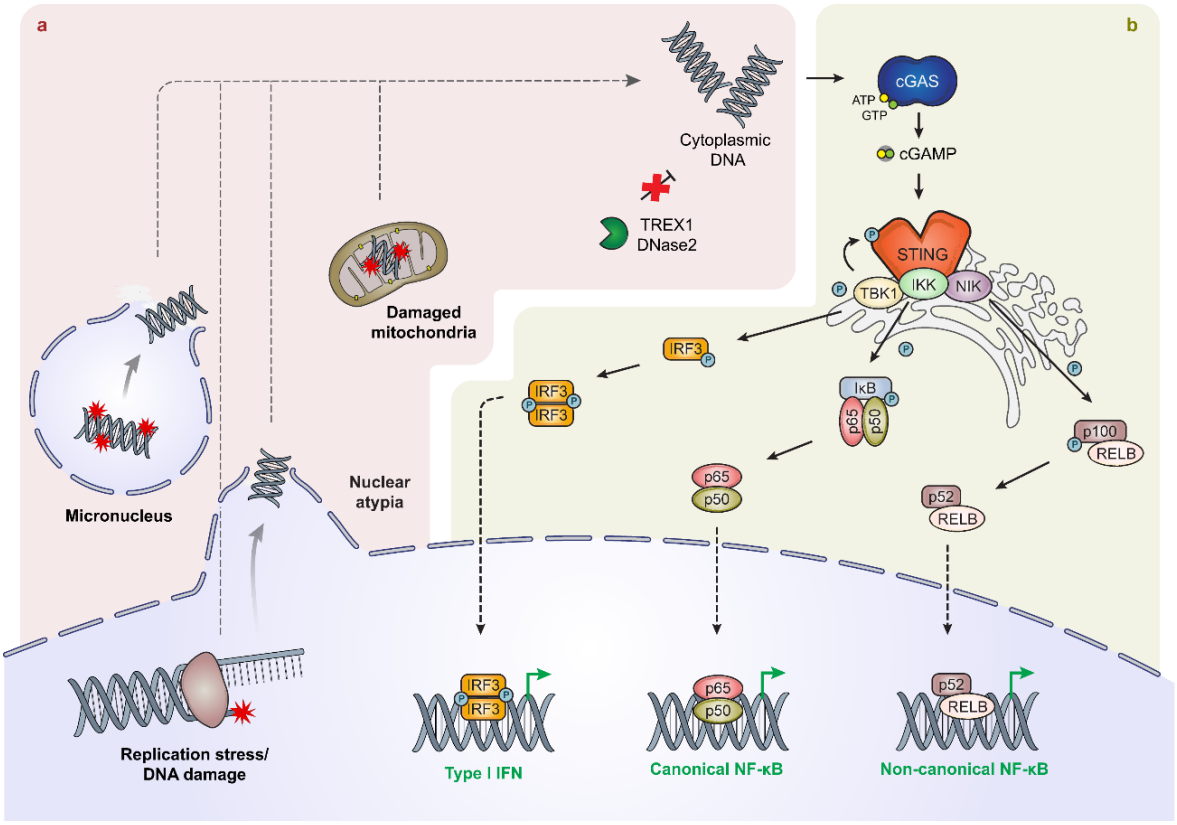

Figure I.8. Putative sources of immunostimulatory cytoplasmic chromatin and activation of the cGAS-STING pathway during cellular aging. (**a**) Faulty chromosome segregation due to mitotic dysfunction leads to the formation of rupture-prone micronuclei, which leak their DNA content into the cytoplasm. Replication stress and DNA damage generates DNA fragments that will bud off from nuclei due to defects in nuclear envelope composition (nuclear atypia). Mitochondrial DNA leakage from damaged mitochondria and defective activity of cytoplasmic DNases, might further contribute to cytoplasmic DNA burden in senescent cells; (**b**) Recognition of cytoplasmic chromatin by the anti-viral cGAS-STING pathway may activate three distinct kinases, namely TBK1, IKK or NIK, that trigger transcription of type I interferon-associated (IFN) genes, canonical NF-κB or non-canonical NF-κB targets, respectively. The SASP, which accompanies the permanent cell cycle arrest of senescent cells, has been attributed to chronic activation of NF-κB signaling, but other signaling pathways might be involved. Adapted from Barroso-Vilares et al. 2019.

accelerated accrual of senescent cells has been acknowledged to contribute to the early development aging phenotypes (discussed in more detail '*Senescence in aging and age-associated pathogenesis*' and '*Senotherapy*'). Kreienkamp and co-workers found that DNA fragments released by progerin-induced replication fork stalling and their nuclease-mediated degradation (*i.e,* replication stress) are recognized by and activate cGAS-STING, which in turn leads to a STAT1-mediated interferon-like response that fuels the cellular aging phenotypes found in the Hutchinson-Gilford Progeria (HGPS) model (Kreienkamp *et al*, 2018). Similarly, accelerated aging phenotypes in two other syndromes – Ataxia telangiectasia and Fanconi anemia – have active contributions of cGAS-STING-mediated interferon response, which becomes upregulated as a result of unrepaired DNA lesions that

are released into and accumulate in the cytoplasm (Aguado *et al*, 2021; Brégnard *et al*, 2016; Hartlova *et al*, 2015). Thus, cGAS-STING seems to be elemental for the establishment of an inflammatory phenotype and by this way will actively contribute to the pathophysiology of conditions where inflammation and senescence accrual play causal roles. In fact, a very recent review article points out that excessive engagement of the cGAS–STING system in the brain can underlie neuroinflammation and neurodegeneration during aging (Paul *et al*, 2021).

I.3.3. Senescence in health and disease

As mentioned in the very beginning of this section, apoptosis and senescence are the two likely outcomes after a cell faces irreversible cellular damage. If we take into consideration the time course of life, senescence is favorable over apoptosis in early stages, as apoptosis would compromise tissue function and decrease the body size of an individual. Indeed, senescence has been shown to play instructive roles during embryonic tissue patterning and growth (Munoz-Espin *et al,* 2013; Storer *et al,* 2013). Also, it actively participates in wound healing and regeneration. For example, it induces myofibroblast differentiation and avoids fibrotic scarring (or fibrosis) during the healing process following injury (Demaria *et al,* 2014; Jun & Lau, 2010; Krizhanovsky *et al*, 2008). Thus, in a healthy context the senescence program ensures that damaged unnecessary or harmful cells become replaced by fit cells, whilst maintaining a proper microenvironment that allows the homeostatic state of a tissue to be kept. This is the ideal scenario and that is probably the reason why senescence has long been regarded to as solely beneficial in the context of tumorigenesis and considered a barrier against cancer development (Collado & Serrano, 2010; Prieur & Peeper, 2008). Precisely, the benefits of an acute and transient presence of senescence contrast with the deleterious consequences of its chronic presence out of proper context and/or later in life. After significantly bridging a certain threshold, factors secreted by senescent cells likely become a threat, which generally occurs in a diseased state. As already mentioned, they cover a wide range of biological activities, which makes it is easy to envision that when chronically secreted, such factors will have unwanted effects (extensively reviewed in Campisi, 2013; Franceschi & Campisi, 2014; He & Sharpless, 2017; Rodier & Campisi, 2011). This paradoxical role of senescence has been referred to as ‘antagonistic pleiotropy of senescence’ (Williams, 1957), and is currently a trending issue in the cancer and aging fields, where a one-size-fits-all description of the causes and consequences of senescence is still elusive.

Senescence and tumorigenesis

In contrast with the importance of senescence over apoptosis in early stages of life, it is still puzzling which fate is favorable when it comes to cancer (He & Sharpless, 2017), a condition generally emerging later in life (Balducci & Ershler, 2005; Campisi, 2003; Serrano, 2016). Cancer, as already mentioned, terms a disease involving dynamic changes in the genome that endow the cells harboring them with a high proliferative potential and the ability to outcompete less fit cells (Hanahan & Weinberg, 2000, 2011). By evading a series of safety breaks (*i.e,* apoptotic and growth suppressive signaling, immune surveillance and replicative exhaustion), such cells endanger health by invading tissues and even metastasize, which ultimately perturbs the balance and proper function of tissues and organs (Hanahan & Weinberg, 2000, 2011). Consequently, the senescence state has been referred to as an exquisite tumor suppressive mechanism, since the irreversible proliferation arrest is generally followed by recruitment of immune-mediated clearance of tumor cells (see Campisi & d'Adda di Fagagna, 2007; Collado & Serrano, 2010). Indeed, it often occurs that cancer cells must acquire mutations in pathways necessary for senescence engagement to evade this cellular state and become aggressive. Further in favor of its anti-tumorigenic role, it has been shown that such mutations increase cancer incidence in mice (Braig *et al*, 2005; Chen *et al*, 2005; Cosme-Blanco *et al*, 2007; Donehower *et al,* 1992) and senescence seems restricted to pre-malignant tumors (Chen *et al,* 2005; Collado *et al*, 2005). Yet, over the past few years it became clear that the role of senescence in cancer is rather a double-edged sword (extensively reviewed in Faget *et al*, 2019; Gonzalez-Meljem *et al*, 2018; Wang *et al*, 2020; Yang *et al*, 2021). Hanahan and Weinberg were the first to entitle inflammation as an ‘enabling characteristic’ of tumorigenesis (Hanahan & Weinberg, 2011), and this paracrine aspect of the senescent state seems to be exactly the reason for the duality of senescence during the cancer process (Coussens & Werb, 2002).

The SASP is composed by several factors some of which have biological activities that are known to fuel phenotypes associated with malignant cancer cells. For example, some SASP components when in particular contexts retain the ability to stimulate angiogenesis (*e.g,* VEGF), while others are known drivers of the proliferative (*e.g,* amphiregulin and GROα) or invasive (*e.g,* IL-6, IL-8 and MMPs) potential of cells (Rodier & Campisi, 2011). In fact, several co-culture experiments and studies with injected or engrafted mice models show that senescent cells can promote tumorigenicity of benign, premalignant, and/or malignant cells (Abdul-Aziz *et al*, 2017; Aifuwa *et al*, 2015; Bartholomew *et al*, 2009; Bavik *et al*, 2006; Cahu *et al*, 2012; Ghosh *et al*, 2020; Krtolica *et al*, 2001; Liu & Hornsby, 2007; Papadopoulou & Kletsas, 2011; Pazolli *et al,* 2012). More recently, two studies observed that senescent cells localize to and increase the SASP

burden at the invasive fronts of human breast (He *et al,* 2018) and papillary thyroid carcinomas (Kim *et al*, 2017), encouraging the invasive phenotype of tumor cells. Also, the secretion of SASP factors by DNA damage-induced senescent hepatic stellate cells was causally linked with hepatocellular carcinoma development in obese mice (Takahashi *et al,* 2018; Yoshimoto *et al*, 2013). Consequently, the chronic presence of senescent cells and their secretome can indeed assist tumor progression in appropriate context. In fact, targeted elimination of senescent cells was able to counteract the survival and malignant phenotype of tumor cells supported by senescence, hence resulting in reduced tumor load and metastization (Abdul-Aziz *et al*, 2019; Baker *et al*, 2016). Importantly, the highly context-dependent effects of the SASP in cancer can also explain how tumors, even though occasionally, emerge from benign or premalignant lesions where senescence normally occurs to arrest incipient tumors (Rodier & Campisi, 2011). Particularly concerning is that most anti-cancer strategies currently being used rely on genotoxic stress induction (Dy & Adjei, 2008; Roninson, 2003), which is known to potentiate local inflammation through the accrual of therapy-induced senescent cells (Demaria *et al*, 2017; Sun *et al*, 2012). A recent pre-clinical validation study addresses this concern and shows that combinatory treatment with palbociclib (chemotherapeutic drug) and navitoclax (senolytic compound) inhibits tumor growth, reduces metastases, and lowers the systemic toxicity of navitoclax (Galiana *et al*, 2020). This underscores the relevance of digging deeper into the causes and consequences of cellular senescence in cancer which simultaneously may also allow to refine anti-cancer therapies (Sieben *et al*, 2018).

Senescence in aging and age-related pathologies

In contrast to cancer, aging correlates with a significant reduction in the proliferative potential of cells and terms the overall decline of physiological functions with time, which is driven by the progressive disruption in the balance of key biological processes (Kirkwood, 2005; Lopez-Otin *et al,* 2013). This time-dependent deterioration of tissue and organ homeostasis that affects most living organisms is caused by cumulative molecular damage, including disruption of proteostasis, loss of genomic stability, telomere attrition and epigenetic dysfunction. As a result, features of aging at both cellular and organismal levels develop, namely mitochondrial dysfunction, deregulated nutrient sensing, stem cell exhaustion, altered intercellular communication and cellular senescence (Lopez-Otin *et al,* 2013). Together, these hallmarks contribute to the rate of aging and fuel the development of major human pathologies emerging later in life including cardiovascular complications, neurodegeneration and diabetes.

Cellular senescence has long been thought to contribute to organismal aging (Campisi, 2013). As stated before, senescence-inducing stimuli are of varying origin and

duration, which likely determines the impact of senescent cells. While acute induction of cellular senescence is assumed as beneficial, chronic persistence of cellular senescence has been proposed to heavily contribute to tissue dysfunction during aging and age-related diseases (Campisi, 2005; van Deursen, 2014). This is likely a result of the chronic inflammatory *milieu* induced by prolonged SASP secretion that perturbs the homeostasis of the environment surrounding senescent cells (*i.e,* extracellular matrix, stem cell niches and healthy cells). Furthermore, a 'senescence-stem lock' model was recently proposed, in which SASP likely inflicts permanent reprogramming of neighboring cells which then hampers tissue rejuvenation by keeping these cells in a state of de-differentiation that impairs the replacement of cells that were lost (de Keizer, 2017). Consequently, SASP likely impacts tissue homeostasis with age by perturbing the integrity of the extracellular microenvironment, whilst compromising the proper clearance and replacement of damaged cells by healthy cells.

Much has been speculated about how senescent cells do accumulate with age and how this then fuels the process. One plausible explanation may be that the number of cells entering senescence increases and that their clearance becomes less effective with age. Indeed, the fidelity and repair abilities of almost all mechanisms weaken in a time-dependent manner (Lopez-Otin *et al,* 2013), which facilitates damage accrual and ultimately increases the likelihood of cells entering into senescence. The intrinsic decline of the immune system, the presence of immune-impairing molecules and a possible ability of senescent cells to evade immune responses (Childs *et al*, 2015; Fulop *et al*, 2017), ultimately allows the senescent cells to accumulate because clearance is compromised. Senescence accrual itself has been proposed to induce changes in the efficacy of the immune system (*i.e,* immunosenescence) with age (Fulop *et al,* 2017). A recent study further showed that changes in immunometabolism can induce senescence in immune cells which, besides compromising clearance as aforementioned, itself is able to fuel age-related degeneration (Desdin-Mico *et al*, 2020). Senescence in aging and age-associated pathogenesis seems thus to be a clear example of snowball effect. Although the use of distinct senescence markers allowed to document that senescent cells accumulate in several age-related conditions (Munoz-Espin & Serrano, 2014), for a long time, it was unclear whether they were the cause or the consequence of the aging process. With the development of models harboring specific transgenes that allow senescence to be monitored *in vivo* (Baker *et al,* 2011; Burd *et al*, 2013; Demaria *et al,* 2014), it became finally clear that senescent cells accrue with age and that they actively participate in the deterioration of tissue and organ function over time. The causal implication of senescence in aging and its associated conditions was first unequivocally shown with the design of the *INK-ATTAC* transgene, that allows identification and drug-inducible elimination of $p16^{Ink4a}$-

positive senescent cells (Baker *et al,* 2011). By studying mice models with this transgene, Baker and co-workers were able to demonstrate that life-long drug-induced removal of $p16^{Ink4a}$-positive senescent cells (or senolysis) delayed or even prevented tissue dysfunction, while extending life and healthspan in $BubR1^{H/H}$ progeroid (Baker *et al,* 2011) and naturally aged mice (Baker *et al,* 2016). Although cellular senescence unlikely explains all changes found during aging, benefits of targeted ablation of senescent cells could be replicated in a series of well-established late-life conditions, including acute myeloid leukemia (Abdul-Aziz *et al,* 2019), atherosclerosis (Childs *et al*, 2016), bone loss (Farr *et al,* 2017), cardiovascular complications (Roos *et al*, 2016), lung problems (Hashimoto *et al*, 2016), liver steatosis (Ogrodnik *et al*, 2017), osteoarthritis (Jeon *et al*, 2017), type 2 diabetes (Aguayo-Mazzucato *et al*, 2019), and neurodegeneration (Bussian *et al*, 2018; Zhang *et al*, 2019). Collectively, these findings not only support a causal implication of senescence in aging and age-related pathogenesis, but also suggest that anti-senescence strategies may have enormous potential in counteracting the aging process and extend healthspan (discussed in more detail in '*Senotherapy*').

I.4. Rejuvenation strategies: how to turn back time

According to the latest world population prospect revision by the United Nations (2019), the elderly population is increasing worldwide. As life expectancy is expected to increase (Vaupel, 2010), age-related diseases will represent a significant challenge to public health and a massive socio-economic burden on health care systems. Thus, the development of strategies to extend and restore healthspan is urging. Extensive research in the aging field paved way by identifying common denominators of aging in different organisms, which helped to establish from the molecular to the organismal level what characterizes the aging process as mentioned before (Lopez-Otin *et al,* 2013). As a result, rejuvenation strategies emerged that target some of the identified aging hallmarks. Essentially, strategies to 'turn back time' can be classified as metabolic manipulations, cellular reprogramming and senolytics (Fig I.9). Even though it remains still a matter of debate whether these approaches indeed reverse or simply delay the aging process, compelling evidence shows that there is a positive impact on several age-associated alterations including inflammation, nutrient-sensing, and epigenome (see Mahmoudi *et al*, 2019b; Melo Pereira *et al*, 2019). This not only suggests that the aging process is malleable (*i.e,* adjusted by genetic and environmental interventions), but most importantly it underscores the potential of the concept to revive aged cells, tissues and organs. The purpose of this section is thus to summarize the promising advances made within each of the three groups and stress some of the pitfalls that may still be a barrier for their viability as anti-aging strategies.

I.4.1. Metabolic manipulations

Dietary restrictions or metabolic manipulations fall into what is probably the oldest of all anti-aging concepts under investigation. Lowering caloric intake without malnutrition represents the most robust non-genetic intervention to date able to expand lifespan and healthspan across several species (recently reviewed in Green *et al*, 2021; Mahmoudi *et al,* 2019b). Thus far, studied metabolic manipulations include long- and short-term caloric restriction (CR), periodic fasting-mimicking diets (FMD), ketogenic dietary regimens and CR mimetics (CRM). CR in rodents is achieved by lowering dietary calories by 10 to 50% and results in substantial improvements that seem to be influenced by genetic and epigenetic factors (Mitchell *et al*, 2016; Weindruch & Sohal, 1997). Essentially, lifespan of mice increases to a maximum as food intake is lowered, and many typical age-associated frailties such as cancer, diabetes, autoimmune conditions or neurodegeneration are either prevented or delayed (Fontana *et al*, 2010; Mitchell *et al,* 2016). This has been largely

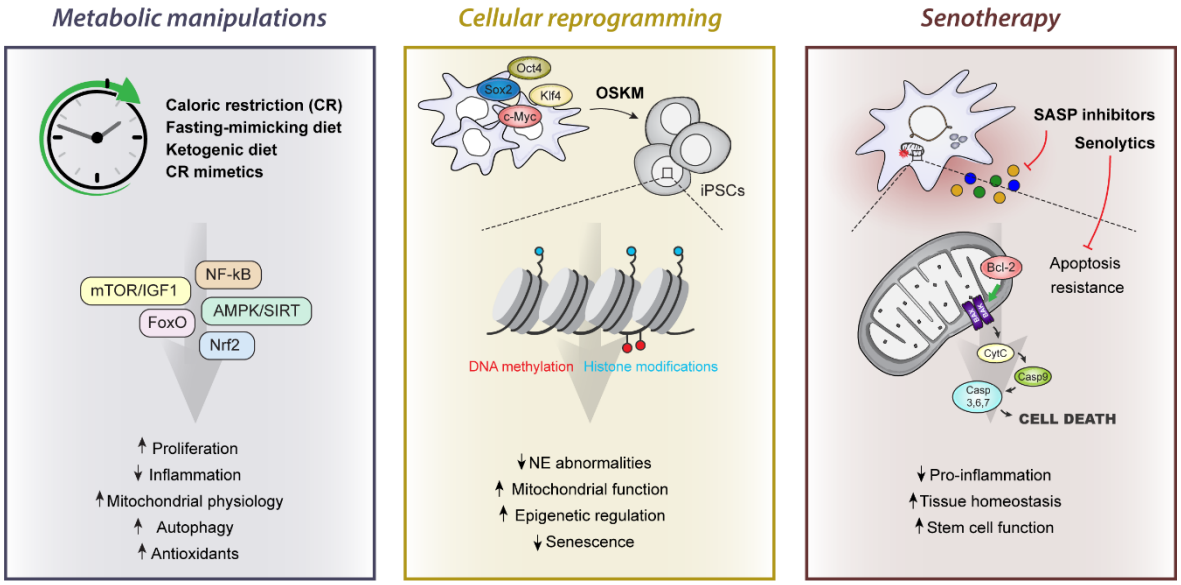

Figure I.9. Emerging anti-aging strategies. Current rejuvenation strategies under investigation, including metabolic manipulations, cellular reprogramming and senolysis, positively impact several age-associated alterations. The figure depicts how and which features are being improved within each of the anti-aging concepts (top to bottom). iPSCs – induced pluripotent stem cells; NE – nuclear envelope; OSKM – Yamanaka factors.

attributed to the effects of CR on metabolic pathways such as mTOR and insulin/IGF1 signaling (Mahmoudi *et al,* 2019b), even though it remains unclear which combination of transcriptional, epigenetic, proteomic, metabolomic and microbiota changes indeed drive the benefits (Green *et al,* 2021). Additionally, it has been proposed that benefits of CR may also be ascribed to a reduction in cellular senescence, either as a result of a decrease in damage-inducing stimuli and an increase in repair efficiency of existing damage (Fontana *et al*, 2018; Kim *et al*, 2020).

Less restrictive dietary regimens and drugs that mimic the metabolic effects of dietary restriction have also been studied which seemingly retain the ability to reverse certain hallmarks of aging without severely reducing or changing at all caloric intake. FMD is such an alternative dietary regimen where rodents are exposed to low caloric intake for 3-4 days twice per month and allowed *ad libitum* feeding in between (Brandhorst *et al*, 2015). Mid- or late-life initiation of FMD in mice resulted in benefits such as reduced cancer incidence, an increase in median lifespan, improved motor and memory performance, and a rejuvenation of the immune system (Brandhorst *et al,* 2015; Roberts *et al*, 2017). It has been proposed that the benefits of FMD depend on a burst in cell proliferation of youthful cells during the refeeding period which, together with an increase in stem cell number, may allow damaged cells to be out-diluted and tissue homeostasis to be re-established (Mahmoudi *et al,* 2019b). Ketogenic dietary regimens, another alternative to CR, substantially reduce carbohydrate intake without altering total caloric intake. By this way,

these diets decrease blood glucose levels and increase ketone body levels and fatty acid oxidation. As in FMD, keto diets improve memory and motor function, with an increase in median lifespan and a lower cancer incidence (Newman *et al*, 2017; Roberts *et al,* 2017). Finally, CRMs or energy restriction mimetics, are compounds the purpose of which is to mimic the anti-aging effects of CR without any dietary restriction (Madeo *et al*, 2014). Rapamycin (mTOR inhibition) and metformin (AMPK activation) are examples of CRMs that reduce risk factors associated with aging (Harrison *et al*, 2009; Martin-Montalvo *et al*, 2013) and are already undergoing clinical trials (see Mahmoudi *et al,* 2019b).

Even though metabolic manipulations show great potential, the concept becomes dimmed by its inherent complexity (Mitchell *et al,* 2016) and by off-target effects of some CRM compounds (Lamming *et al*, 2012). With more and more studies available, it becomes clear that simply lowering calories does not do the trick. A personalized food-as-medicine approach to prevent age-associated degeneration, including optimization of what/when/how much to eat, will be the future of preventive metabolic manipulation because genetic and epigenetic factors, as well as specific changes in nutrients and feeding times seem to significantly influence the outcome of metabolic manipulation induced by dietary regimens (discussed in Green *et al,* 2021).

I.4.2. Cellular reprogramming

Alterations in epigenetic mechanisms have been shown to contribute to the time-dependent deterioration of cells, tissues and organisms with age (Benayoun *et al*, 2015; Issa, 2014). Indeed, changes in DNA methylation patterns over time form the basis of aging clocks (or ‘epigenetic clocks’) (Horvath, 2013). Other age-associated epigenome changes include histone modifications, chromatin remodeling, and non-coding RNAs (reviewed in Sen *et al*, 2016; Zhang *et al*, 2020). Cellular reprogramming refers to the conversion of a specific cell type into another. During the process, terminally differentiated cells are induced to express genes normally active during pluripotency (*e.g,* embryonic stem cells), which afterwards allows their differentiation into any cell type of the body (Buganim *et al*, 2012; Polo *et al*, 2012). By this way, the epigenome becomes remodeled, and the cells rejuvenate into a youthful state. For these reasons, and due the impact of epigenome changes on the aging process, cellular reprogramming is another anti-aging concept that has been devised and tested *in vitro* and *in vivo* (see Ocampo *et al*, 2016a).

The most common form of cellular reprogramming entails transcription-factor induction (*i.e,* Oct4, Sox2, Klf4 and c-Myc; also known as Yamanaka or OSKM factors) (Takahashi & Yamanaka, 2006), but pluripotency can also be induced by somatic nuclear

transfer or cell fusion (Yamanaka & Blau, 2010). Thus, cell fate is malleable as differentiation turns out to be two-way road (Issa, 2014). As a result, several studies with cells from aged and prematurely aged individuals have shown that aging phenotypes can be reversed with cellular reprogramming. For example, induced pluripotent stem cells (iPSC) generated from elderly somatic cells have their epigenome reset, restored telomere length, decreased levels of $p16^{Ink4a}$ and $p21^{Cip1}$, as well as reduced oxidative damage and mitochondrial dysfunction. The same holds true for reprogramming of somatic cells from patients with progeroid syndromes such as HGPS, Werner Syndrome, Nestor-Guillermo Syndrome and Dyskeratosis congenita (discussed in Mahmoudi *et al,* 2019b; Ocampo *et al,* 2016a; Zhang *et al,* 2020). Importantly, this 'youthful state' is maintained not only in pluripotent cells but also in their derivatives. Although 'rejuvenation' includes most aging hallmarks, nuclear and mitochondrial damage do not become reversed (Mahmoudi & Brunet, 2012). Also, cellular reprogramming efficiencies were shown to vary with the genetic background, age and levels of existing damage, as well as the type or even senescence status of cells and tissues (Lo Sardo *et al*, 2017; Mahmoudi & Brunet, 2012; Mahmoudi *et al*, 2019a; Mosteiro *et al*, 2016). Finally, and probably the most concerning observation is that dedifferentiation and loss of cell identity due to persistent OSKM expression predisposes for teratoma development and increases mortality (Abad *et al*, 2013; Ohnishi *et al*, 2014; Takahashi & Yamanaka, 2006).

Remarkably, it was found that short-term induction of OSKM factors (or partial reprogramming) bypasses loss of cellular identity, as cells enter an intermediate state (or partial dedifferentiation) that can be reversed by directed differentiation (Ocampo *et al*, 2016b). Partial reprogramming emerges thereby as an alternative to conventional cellular reprogramming and shows that a more controlled regimen can improve the molecular signature and physiological phenotypes of aging in progeroid and naturally aged mice and/or cells (Lu *et al*, 2020; Ocampo *et al,* 2016b; Sarkar *et al*, 2020; Browder *et al,* 2022). Most importantly, by this way the rejuvenating aspect of reprogramming could be uncoupled from its teratoma-inducing properties (Ocampo *et al,* 2016b). Notwithstanding, the viability of the cellular reprogramming anti-aging concept still faces some issues. OSKM induction times will have to be fine-tuned depending on cell type, tissue or organ, which will then allow to determine whether rejuvenation of specific tissues can positively impact the organism as a whole. Regarding induction of reprogramming factors, different combinations of factors and small molecules have been investigated, but delivery methods remain a hurdle that must also be overcome. Finally, the durability of the rejuvenated state and the actual epigenetic changes that are induced are also not fully understood yet (discussed in Mahmoudi *et al,* 2019b; Ocampo *et al,* 2016a).

I.4.3. Senotherapy

As abovementioned, even though cellular senescence had for long been causally linked to aging based on marker detection, only the development of genetically engineered mice harboring a senescence-specific 'suicide transgene' allowed to unequivocally show that senescent cells can actively participate in the aging process and contribute to the pathophysiology of several age-related conditions. Importantly, studies in these mice models revealed that targeting senescent cells can reverse or delay aspects of the aging process (see '*Senescence in aging and age-related pathologies*'). The healthspan benefits conferred by early-life, mid-life or late-life treatment went from delaying adipose tissue dysfunction, skeletal muscle loss or cataract formation, to attenuating decline in kidney and heart function (Baker *et al,* 2016; Baker *et al,* 2011; Farr *et al,* 2017; Ogrodnik *et al,* 2017), which converged into an overall increase in lifespan. Thus, these proof-of-concept studies encouraged the identification of compounds that can selectively kill senescent cells based on their distinctive molecular signature. Also known as senolytics or senolytic drugs, these compounds antagonize the pro-survival signaling required for the maintenance of the senescent state, including the BCL-2 protein family (*e.g,* ABT263) (Chang *et al*, 2016; Zhu *et al*, 2016), the FOXO4-p53 interaction (*e.g,* FOXO4-DRI) (Baar *et al,* 2017), and other relevant pro-survival cascades (*e.g,* dasatinib and quercetin; 17-DMAG; UBX0101) (Farr *et al,* 2017; Fuhrmann-Stroissnigg *et al*, 2017; Jeon *et al,* 2017; Zhu *et al*, 2015). As in genetically engineered mice models, senolytic drug-induced ablation of senescent cells does extend and restore healthspan, reduce frailty and improve stem cell function, which spurred a number of clinical trials with systemically delivered senolytic compounds (see Paez-Ribes *et al*, 2019; Pignolo *et al*, 2020; Robbins *et al*, 2021).

Although it is one of the furthest anti-aging strategies under investigation, the anti-senescence concept still faces some hurdles that must be overcome for it to become viable (recently discussed in Kirkland & Tchkonia, 2020; Paez-Ribes *et al,* 2019; Robbins *et al,* 2021). As much as senescence is acknowledged to accumulate with aging and contribute to age-dependent tissue deterioration, its definition remains vague. Not only because there is no universal marker available yet that covers all types of senescence and senescent cells, but also because most markers are not exclusive to the senescent state (Sharpless & Sherr, 2015). In addition, as mentioned in the previous section, senescence can play both beneficial and detrimental roles depending on the context (Faget *et al,* 2019; Munoz-Espin & Serrano, 2014). As a result, treatments will have to be extremely fine-tuned, including not only more 'targeted' approaches, but also a tighter control regarding dose regimens, initiation and duration of the treatment, all matters that are still being debated in the field. 'unSASPing' senescence (*i.e,* inhibition of SASP) emerges here as an alternative avenue

to targeted ablation of senescent cells. Compounds that have been tested for this purpose, also known as senomorphics, include metformin, rapamycin, p38MAPK inhibitors and antagonists of specific SASP factors (*e.g,* IL-1, IL-6 and TNF-α) (see Birch & Gil, 2020; Childs *et al*, 2017; Faget *et al,* 2019). However, as with selective killing strategies, abolishing the SASP must be approached with caution. Its composition is very heterogeneous, highly context-dependent, absent in some senescent cells and produced in biological settings not related with senescence (*e.g,* acute inflammation) (Coppe *et al*, 2010b; Cuollo *et al*, 2020). It has been suggested that upcoming senotherapeutic strategies should focus on targeting secondary senescence (*i.e,* cells that became senescent due to cell-to-cell contact or paracrine signaling) (Admasu *et al*, 2021), or subsets of senescent cells induced by age-related dysfunction which are more likely deleterious (Melo Pereira *et al,* 2019).

I.5. Research Aims

An abnormal number of chromosomes – aneuploidy – is often found in tumors and has also been associated with aging. The development of cellular models of aneuploidy allowed to unveil a stress response transversal to single-chromosome gains that severely impacts cellular physiology. Recent studies further reveal that chromosome dosage imbalances can induce cellular senescence and that the non-cell autonomous aspect of the senescent state heavily depends on cytoplasmic accumulation of immunostimulatory DNA. Importantly, cellular senescence has been causally implicated in the aging process. Based on this, our working hypothesis considers that senescence triggered by recurrent DNA damage and/or chromosome mis-segregation (genome instability) fosters a chronic low-grade pro-inflammatory environment that contributes to aging by perturbing tissue and organ homeostasis. In particular, we reason that the mitotic decline of elderly dividing fibroblasts reported by our laboratory coincides with a deregulation of MT dynamics which, by inducing mild CIN in elderly cells, will contribute to the cytoplasmic pool of cGAS-STING-recognized DNA and fuel chronic pro-inflammation through the accumulation of aneuploid senescent cells. Furthermore, we speculate that a similar GIN–senescence correlation occurs in the context of Down Syndrome (or Trisomy 21), where the premature onset of aging phenotypes has been described. With improved medical care, life expectancies are expected to increase, which will place a significant burden on health care systems. Since chronic cellular senescence is acknowledged to participate in the time-dependent tissue and organ degeneration, the identification of GIN-induced senescence in the context of aging and DS would open new possibilities. Modulation of genome instability could offer a novel strategy to delay senescence-associated changes and represent an alternative to the previously mentioned anti-aging concepts. Consequently, as research aims for the here-presented thesis, we set out to:

i) Dissect the impact of human cellular aging on MT dynamics and explore small-molecule modulation of MT dynamics as a means to delay cellular senescence

ii) Investigate the role of trisomy 21-induced genome instability in the development of cellular senescence in Down Syndrome and ascertain the efficacy of pharmacological inhibition of GIN as senomorphic intervention

Chapter – II

Small-molecule inhibition of aging-associated chromosomal instability delays cellular senescence

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II.1. Introduction

Aging is characterized by the progressive disruption of key biological processes and correlates with the extensive accumulation of macromolecular damage over time. As a consequence, tissue and organ homeostasis is perturbed, which contributes to an overall deterioration of physiological functions. Potential drivers of the aging process have been identified and categorized into hallmarks. Molecular hallmarks comprise DNA damage, telomere attrition, epigenetic remodeling, loss of proteostasis, and mitochondrial dysfunction (Lopez-Otin *et al,* 2013). Cellular and organismal features of aging include cellular senescence, deregulated nutrient sensing, and stem cell exhaustion (Lopez-Otin *et al,* 2013). In recent years, several rejuvenation strategies emerged that target these hallmarks (Mahmoudi *et al,* 2019b). Amongst them, metabolic manipulations and senescent cell ablation (or senolysis) have become popular. Senescent cells, which undergo a permanent cell cycle arrest in response to stressors and exhibit stereotyped phenotypic changes, have been shown to contribute to aging (Childs *et al,* 2015; van Deursen, 2014). Their targeted clearance was evidenced to attenuate or even prevent age-associated conditions (Abdul-Aziz *et al,* 2019; Bussian *et al,* 2018; Childs *et al,* 2016; Farr *et al,* 2017; Jeon *et al,* 2017; Roos *et al,* 2016) and to improve lifespan of naturally aged wild-type mice (Baker *et al,* 2016). Senolysis also extended healthspan in progeroid mice that experience chromosomal instability (CIN) as a result of mitotic checkpoint signaling defects (Baker *et al,* 2011). This suggested a possible link between CIN and aging through the accrual of senescent cells. More recently, this correlation was further supported by studies, showing that loss of chromosome segregation fidelity in otherwise karyotypically stable human cells prompts a CIN-driven senescence signature accompanied by the senescence-associated secretory phenotype (or SASP) (He *et al,* 2018; Santaguida *et al,* 2017). Thus, aneuploidy, a state of abnormal chromosome number for long reported to occur with age (Iourov *et al*, 2009; Mosch *et al*, 2007; Mukherjee *et al,* 1996; Mukherjee & Thomas, 1997; Nagaoka *et al,* 2012; Stone & Sandberg, 1995), may significantly contribute to the aging process.

Maintenance of chromosomal stability is ensured through the tightly controlled and timely organization of microtubules (MTs) into a bipolar mitotic spindle and microtubule attachment to the complex proteinaceous structures (kinetochores) at the centromeres of all chromosomes prior to their segregation toward opposite poles. Defects in the spindle assembly checkpoint (SAC) that prevents anaphase onset in the presence of unattached kinetochores, as well as the premature separation of sister chromatids due to cohesion defects, will give rise to aneuploid daughter cells (Compton, 2011). In addition, a major mechanism generating aneuploidy is the persistence of erroneous merotelic kinetochore–

microtubule (k-MT) attachments, in which a single kinetochore bound to MTs from opposite poles is left uncorrected, generating an anaphase lagging chromosome and micronuclei (MN) in telophase (Cimini & Degrassi, 2005). As increases in aneuploidy have been observed with aging, there is the possibility that the mechanisms required to maintain chromosomal stability might deteriorate with age (Macedo *et al,* 2017). Although work on cells and mice with CIN has pointed to a link between chromosomal abnormalities and aging, the mitotic behavior of naturally aged cells was only recently characterized. Analysis of primary human dermal fibroblasts derived from neonatal to octogenarian individuals revealed a progressive loss of proliferative capacity and mitotic dysfunction with age. As a result of the global mitotic gene shutdown caused by the repression of the transcription factor Forkhead box M1 (FoxM1), elderly cells experience chromosome segregation defects that were found to ultimately trigger a full senescence phenotype (Macedo *et al,* 2018). Altogether, this raises the intriguing possibility that loss of mitotic fidelity with aging underlies mild CIN, thus favoring the accrual of aneuploid senescent cells and their paracrine effect on the surrounding microenvironment and neighboring cells. Uncovering the yet unknown mechanism(s) by which aging triggers chromosome segregation defects, and resulting aneuploidy is paramount in light of all recent findings connecting CIN, senescence, and aging.

Here, we show that, in agreement with the previously found mitotic dysfunction, human dermal fibroblasts derived from elderly individuals have lower levels of proteins required for the establishment of proper k-MT attachments, including MT-destabilizing kinesins involved in the correction of merotelic k-MT interactions. As a result of compromised error correction, improper k-MT attachments persist into anaphase giving rise to aneuploid daughter cells. Notably, genetic and pharmacological rescue of MT-destabilizing kinesin-13 activity re-established chromosome segregation accuracy in elderly cells, concomitantly with a reduction in cellular senescence. Consequently, strategic destabilization of k-MT attachments may be a potential strategy to counteract age-associated senescence and thereby act to improve healthspan.

II.2. Materials and Methods

Cell culture

Human dermal fibroblasts (HDFs) retrieved from skin samples of neonatal (No. GM21811, Coriell Institute; No. DFM021711A, Zen Bio) and octogenarian (No. AG07135; AG13993; AG09271; AG10884; all from Coriell Institute) Caucasian males reported as “healthy” were grown in minimal essential medium Eagle–Earle (MEM) supplemented with 15% fetal

bovine serum (FBS), 2mM L-glutamine, and 1x antibiotic-antimycotic (all from Gibco, Thermo Fisher Scientific). Only early passage dividing fibroblasts (up to passage 3–5) with cumulative population doubling level PDL < 24 were used. HT-1080 (ATCC®, CCL-121™) cells were cultured in MEM supplemented with 10% FBS, 2mM L-glutamine, and 1x antibiotic-antimycotic (all from Gibco, Thermo Fisher Scientific).

Drug treatments

Proteasome inhibitor MG-132 (474790, EMD Millipore) was used at 5μM for 2 h to arrest cells at the metaphase stage. Cytochalasin D (C8273, Sigma-Aldrich) was used at 1μM for 24 h to block cytokinesis. Fibroblasts were treated with 2.5μM STLC (2191, TOCRIS) for 5 h to inhibit kinesin-5 activity and induce monopolar spindles, followed by a washout into fresh medium with 500nM of Aurora kinase B inhibitor ZM447439 (S1103, Selleckchem) to potentiate chromosome segregation errors. To enrich the Mitotic Index for mitotic cell shake-off, STLC was used at 5μM during 16 h. To partially inhibit Mps1 kinase activity, 500nM of AZ3146 (3994, TOCRIS) were used during 4 h. 1μM of UMK57 (kindly provided by Dr. Benjamin Kwok) was used to enhance kinesin-13 activity during the time periods indicated for each experiment.

Lentiviral plasmids

To assemble pLVX-Tight-Puro plasmids for lentiviral transduction and expression of GFP-MCAK and mEOS-α-Tubulin, BamHI-NotI-tailed fragments were PCR-amplified from GFP-MCAK (gift from Dr. Linda Wordeman) and mEos2-Tubulin-C-18 (#57432, Addgene), respectively. To generate pLVX-Tight-Puro-GFP-Kif2b, a NotI-MluI-tailed fragment was amplified from GFP-Kif2b (gift from Dr. Linda Wordeman). The PCR products were then ligated into the BamHI and NotI, or NotI and MluI restriction sites of digested pLVX-Tight-Puro vector (Clontech). All primers used for PCR amplifications are listed in Appendix Table II.S1.

Lentiviral production and infection

Lentiviruses were produced according to the Lenti-X Tet-ON Advanced Inducible Expression System (Clontech). HEK293T helper cells were transfected with packaging plasmids pMd2.G and psPAX2 using Lipofectamine 2000 (Life Technologies) to generate responsive lentiviruses carrying pLVX-Tight-Puro, pLVX-TightPuro-H2B-GFP/α-tubulin-mCherry (Macedo *et al,* 2018), pLVX-Tight-Puro-GFP-MCAK, pLVX-Tight-Puro-GFP-Kif2b or pLVX-Tight-Puro-mEOS-α-Tubulin, as well as transactivator lentiviruses carrying the rtTA expressing construct (pLVX-Tet-On Advanced). Human fibroblasts were then co-infected for 6 h with both the responsive and the transactivator lentiviruses (2:1 ratio) in the presence of 8μg/ml polybrene (AL-118, Sigma-Aldrich). Co-transduction was induced with

500ng/ml doxycycline (D9891, Sigma-Aldrich). Transfection efficiencies of all experiments were determined by scoring the number of fluorescent cells, or protein levels by western blot analysis.

Fluorescence-activated cell sorting

Subpopulations of GFP-positive cells were FACS sorted to validate lentiviral transduction of pLVX-Tight-Puro-GFP-MCAK, pLVX-Tight-Puro-GFP-Kif2b and pLVX-Tight-Puro-EOS-α-Tubulin. FACS sorting was performed using a FACSAria™ I Cell Sorter (BD Biosciences), with the laser line of 488 nm. Dead cells and subcellular debris were excluded using gates based on forward scatter area (FSC-A) vs. side scatter area, while cell doublets and clumps were excluded through FSC-A vs. FSC-width plot. The signal was detected using the APC-A channel and gates designed based on the respective auto-fluorescent control.

cGAS siRNA knockdown

Cells were plated and after 1 h transfected with siRNA oligonucleotides targeting cGAS (Dharmacon, M-015607-01-0005, siGENOME 115004) at a final concentration of 25nM. Transfections were performed using Lipofectamine RNAiMAX in Opti-MEM medium (both from Thermo Fisher Scientific) according to the manufacturer's instructions. Transfection medium was replaced with complete medium after 6 h. All experiments were performed 72 h post-transfection, and protein depletion confirmed by western blot and immunostaining analyses.

Fluorescence *in situ* hybridization

FISH was used to score aneusomy indexes (Interphase FISH; Figs II.4D, II.5C and II.EV5E) and chromosome mis-segregation (MS) rates. MS rates were scored by Cyto-D FISH (Figs II.2E and F; II.4E, II.5D and Appendix Fig II.S1), or by FISH on STLC-washed-out fibroblasts (Fig II.1G and H). For all experiments, fibroblasts were grown on Superfrost™ Plus microscope slides (Menzel, Thermo Fisher Scientific) placed in quadriperm dishes (Sarsted). Cells were fixed with ice-cold Carnoy fixative (methanol:glacial acetic acid, 3:1), following an initial 30 min hypotonic shock in 0.03M sodium citrate solution (Sigma-Aldrich). FISH was performed with Vysis centromeric probes CEP7 Spectrum Aqua, CEP12 Spectrum Green, and CEP18 Spectrum Orange (all from Abbott Laboratories) according to manufacturer's instructions. DNA was counterstained with 0.5µg/ml 4',6-Diamidino-2-Phenylindole (DAPI) and microscope slides were then mounted with coverslips in proper anti-fading medium (90% glycerol, 0.5% N-propyl gallate, 20mM Tris pH=8.0).

SA-β-gal assay

Cells were incubated in culture medium containing 100nM Bafilomycin A1 (B1793, Sigma-Aldrich) for 90 min to induce lysosomal alkalization. The fluorogenic substrate for β-galactosidase, fluorescein di-β-D-galactopyranoside (33μM; F2756, Sigma-Aldrich) or DDAO galactoside (10μM; Setareh Biotech LLC), was subsequently added to the medium for 90 min. Cells were fixed in 4% paraformaldehyde for 15 min, rinsed with PBS, and permeabilized with 0.1% Triton-X100 in PBS for 15 min. 0.5μg/ml of DAPI (Sigma-Aldrich) was used to counterstain DNA and coverslips were then mounted on slides.

Calcium-stable k-fiber analysis

Fibroblasts grown on sterilized glass coverslips coated with 50μg/ml fibronectin (F1141, Sigma-Aldrich) were incubated in Calcium buffer (100mM PIPES, 1mM $MgCl_2$, 1mM $CaCl_2$, 0.5% Triton-X100, pH=6.8) for 5 min and fixed with 4% paraformaldehyde/0.25% glutaraldehyde in PBS for 15 min, both at 37ºC. Next, cells were rinsed first in PBS, then in TBS (50mM Tris-HCl, pH=7.4, 150mM NaCl), and permeabilized in TBS + 0.3% Triton-X100 for 7 min. Blocking was performed with 10% FBS + TBS + 0.05% Tween-20 for 1 h and cells were then incubated with mouse anti-α-tubulin (T5168, Sigma-Aldrich) antibody diluted at 1:1500 in 10% FBS + TBS + 0.05% Tween-20. The secondary antibodies AlexaFluor-488 and -568 (Life Technologies) were used at 1:1500 in 5% FBS + TBS + 0.05% Tween-20. DNA was counterstained with 0.5μg/ml DAPI (Sigma-Aldrich) and coverslips mounted on slides.

Immunofluorescence

Fibroblasts were grown on sterilized glass coverslips coated with 50μg/ml fibronectin (F1141, Sigma-Aldrich) and fixed with 4% paraformaldehyde in PBS for 20 min. Following fixation, cells were rinsed in PBS, permeabilized in PBS + 0.3% Triton-X100 for 7 min and then blocked in 10% FBS + PBS for 1 h. Both, primary and secondary antibodies were diluted in PBS + 0.05% Tween-20 containing 5% FBS as follows. Primary antibodies: rabbit anti-53BP1 (4937, Cell Signaling Technology), 1:100; mouse anti-p21 (SC-6246, Santa Cruz Biotechnology), 1:800; mouse anti-Aurora B (Aim-1; 611082, BD Biosciences), 1:500; rabbit anti-cGAS (15102, Cell Signaling Technology), 1:200; mouse anti-Hec1 (ab3613, Abcam), 1:1500; mouse anti-Plk1 (SC-17783, Santa Cruz Biotechnology), 1:2000; rabbit anti-MCAK (Manning *et al*, 2007), 1:5000; mouse anti-α-tubulin (T5168, Sigma-Aldrich), 1:1500; human anti-centromere antibody (ACA; kindly provided by Dr. W. C. Earnshaw), 1:3000; rabbit anti-Aurora B phosphoT232 (600-401-677, ROCKLAND), 1:1000; mouse anti-Retinoblastoma (554136, BD Biosciences), 1:100. Secondary antibodies: AlexaFluor-488, -568 and -647 (Life Technologies), all 1:1500. DNA was counterstained with 0.5μg/ml DAPI (Sigma-Aldrich) and coverslips mounted on slides with proper mounting solution.

Fluorescence dissipation after photoconversion

Human dermal fibroblasts of neonatal and elderly cells transduced with inducible mEOS-tubulin were cultured for 24-48 h in MEM (without phenol red) + 750ng/ml doxycycline on fibronectin-coated glass coverslips. 5μM MG-132 was added prior to rose chamber assembly to prevent mitotic exit. During imaging, cells were maintained at 37°C using a heated stage. Images were acquired with a Plan Apo VC 60x, 1.4 NA, oil immersion objective (Nikon) using a QuorumWaveFX-X1 spinning disk confocal system on a Nikon Eclipse Ti microscope, equipped with an ILE laser source (Andor Technology), a Mosaic digital mirror (Andor Technology), and a Photometrics Evolve 512 Delta camera. Metaphase cells were identified by differential interference contrast (DIC) microscopy. Photoconversion was performed with a 405nm laser (20% power, 500ms pulse) on a rectangular region of interest over one half of the mitotic spindle. Fluorescence z-stacks with a 1μm step size (3 slices) were captured every 15 sec for 4 min. Red fluorescence dissipation after photoconversion was quantified from maximum intensity projections using MetaMorph® software (Molecular Devices). Average pixel intensities were measured within an area surrounding the region of highest fluorescence intensity and background subtraction was performed using an equally sized area from the non-activated half-spindle at each time point. Fluorescence intensities were corrected for photobleaching using values of fluorescence loss obtained from photoconverted 1μM Taxol stabilized spindles. Fluorescence intensities were then normalized to the first time point after photoconversion for each cell. To measure k-MT stability, the average fluorescence intensity at each time point was fit to a two-phase exponential decay curve [$F(t) = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$] using GraphPad software, where A_2 is the percentage of photoconverted fluorescence attributable to the slow decay process with a decay rate of k_2 (Zhai *et al*, 1995). k-MT half-life ($t^{1/2}$) in minutes was calculated as $\ln 2/k_2$. For high stringency, we only considered curves with good fit ($R^2 \geq 0.95$).

Phase-contrast live cell imaging

Fibroblasts grown in ibiTreat polymer-coated μ-slides (Ibidi GmbH, Germany) were imaged using a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Oberkochen, Germany) equipped with a CoolSnap camera (Photometrics, Tucson, USA), XY motorized stage and NanoPiezo Z stage, under controlled temperature, atmosphere, and humidity. Neighbor fields (20-25) were imaged every 2.5 min for 24-48 h, using a 20x/0.3 NA Aplan objective. The “Stitch Grid” (Stephan Preibisch) plugin from ImageJ/Fiji software was used to stitch neighboring fields for image analysis.

Spinning-disk confocal microscopy

Fibroblasts were grown in ibiTreat polymer-coated 35mm µ-dishes (Ibidi GmbH, Germany) and imaged using the Andor Revolution XD spinning-disk confocal system (Andor Technology, Belfast, UK), equipped with an electron-multiplying charge-coupled device iXonEM Camera and a Yokogawa CSU 22 unit based on an Olympus IX81 inverted microscope (Olympus, Southend-on-Sea, UK). The system was driven by Andor IQ software and laser lines at 488 and 561 nm were used for excitation of GFP and mCherry, respectively. Z-stacks (0.8–1.0 µm) covering the entire volume of individual mitotic cells were collected every 1.5 min using a PlanApo 60x/1.4 NA objective. ImageJ/Fiji software was used to edit the movies in which every image represents a maximum-intensity projection of all z-planes.

Fluorescence microscopy

Cells with calcium-stabilized k-fibers or stained for specific kinetochore/centromere-bound proteins (Aurora B/Aim-1, Hec1/Ndc80, MCAK, Plk1, pAuroraB T232 and ACA) were imaged using a Zeiss AxioImager Z1 (Carl Zeiss, Oberkochen, Germany) motorized upright epifluorescence microscope, equipped with an Axiocam MR camera and operated by the Zeiss Axiovision v4.7 software. Z-stacks (0.24 µm) covering the entire volume of individual mitotic cells were collected using a PlanApo 63x/1.40 NA objective. Image deconvolution was performed with the AutoQuant X2 software (Media Cybernetics).

Automated microscopy

For FISH experiments, MN counts, cGAS immunofluorescence and SA biomarkers, images were captured with the IN Cell Analyzer 2000 (GE Healthcare, UK) equipped with a Photometrics CoolSNAP K4 camera and driven by the GE IN Cell Analyzer 2000 v5.2 software, using a Nikon 20x/0.45 NA Plan Fluor objective or a Nikon 40x/0.95 NA Plan Fluor objective, respectively.

Image analysis

Both, live-cell phenotypes (mitotic duration, lagging chromosomes) and fixed-cell experiments (protein intensity, k-fiber intensity, KT distances, FISH, MN counts, mitotic stages, cGAS positivity and SA biomarkers) were blindly quantified using ImageJ/Fiji software. For the analysis of protein intensity levels, the kinetochore area was taken into consideration and Aurora B/Aim-1, Hec1/Ndc80, MCAK, Plk1 and pAuroraB T232 levels were then corrected for the background and normalized to ACA levels (also corrected for the background). For analysis of calcium-stable k-fibers, α-tubulin intensity levels were normalized for the mitotic spindle area of each individual cell and background-corrected. For MN frequencies, interphase cells with DNA aggregates separate from the primary

nucleus were considered, while interphase cells with an apoptotic appearance were excluded. DNA aggregates co-localizing with cGAS and/or Retinoblastoma (Rb) were scored as MN positive for cGAS or Rb, respectively. Intact MN were cGAS-/Rb+ while disrupted MN were cGAS+/Rb-. For the analysis of SA biomarkers (53bp1/p21 and SA-β-galactosidase), fluorescence intensity thresholds were set and used consistently for all samples within each experiment. In case of SA-β-galactosidase activity, only cells displaying >5 fluorescent granules were considered positive.

Western blot

Both asynchronous and mitotic cell populations were analyzed by western blot. Mitotic cell populations were collected by shake-off of cell culture flasks enriched for Mitotic Index following a 16 h treatment with STLC. Cell pellets were resuspended in lysis buffer (150nM NaCl, 10nM Tris-HCl pH 7.4, 1nM EDTA, 1nM EGTA, 0.5% IGEPAL) with protease inhibitors. Protein content was determined using the Lowry Method (DC™ Protein Assay, Bio-Rad) according to the manufacturer's instructions. 20µg of extract were then loaded for SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes for western blot analysis. Non-specific sites were blocked with TBS-T (50mM Tris-HCl, pH=7.4, 150mM NaCl, 0.05% Tween-20) supplemented with 5% non-fat dry milk for 1 h. Both, primary and secondary antibodies were diluted in TBS-T containing 2% non-fat milk as follows. Primary antibodies: mouse anti-Aurora B (Aim-1; 611082, BD Biosciences), 1:250; mouse anti-GFP (46-0092, Invitrogen), 1:2000; rabbit anti-cGAS (15102, Cell Signaling Technology), 1:100); mouse anti-Hec1 (SC-515550, Santa Cruz Biotechnology), 1:500; rabbit anti-MCAK (Manning *et al,* 2007), 1:5000; mouse anti-Plk1 (SC-17783, Santa Cruz Biotechnology), 1:1000; mouse anti-α-tubulin (T5168, Sigma-Aldrich), 1:100,000; and mouse anti-GAPDH (60004, ProteinTech), 1:50,000. Secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-rabbit (SC-2004, Santa Cruz Biotechnology) and goat anti-mouse (SC-2005, Santa Cruz Biotechnology), both at 1:3000. HRP conjugates were detected using Clarity Western ECL Substrate reagent (Bio-Rad Laboratories) according to manufacturer's instructions. A GS-800 calibrated densitometer operated by the Quantity one I-D Analysis Software v4.6 (Bio-Rad Laboratories) was used for quantitative analysis of protein levels.

Cytokine array

Cell medium supernatants from neonatal and octogenarian HDFs for 24 h in the absence of FBS (Rodier, 2013) were harvested and centrifuged for 5 min at 200g to remove dead cells and cell debris. Levels of secreted cytokines were then analyzed using the Proteome Profiler Human Cytokine Array Kit (R&D SYSTEMS) according to the manufacturer's

instructions. Quantitative analysis of dot blots was performed on a GS-800 calibrated densitometer operated by the Quantity one I-D Analysis Software v4.6 (Bio-Rad Laboratories).

Quantitative PCR

Total RNA from both asynchronous and mitotic cell populations was extracted using RNeasy® Mini Kit (Qiagen). 1µg of total RNA was reverse-transcribed using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories). qPCR was performed using iTaq™ Universal SYBR® Green Supermix in a CFX96/384 Touch™ Real-Time PCR Detection System and analyzed using the CFX Maestro Software (all from Bio-Rad Laboratories). Primers used are listed in Appendix Table II.S1.

Targeted transcriptome sequencing and Bioinformatics

Total RNA was extracted as described above. 10ng were reverse-transcribed using the AmpliSeq Whole Transcriptome primers with the included SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific). cDNA was used for target amplification (12 cycles) with Ion AmpliSeq primers and technology. Barcoded adapters were added and ligated to individual reactions according to the instructions of Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific). The pooled libraries were processed on Ion Chef™ System and the resulting 550™ chip was sequenced on a Ion S5™ XL System (all from Thermo Fisher Scientific). Data were processed with the Ion Torrent platform specific pipeline software Torrent Suite v5.8.0 to generate sequence reads, trim adapter sequences, filter and remove poor signal reads, and split the reads according to the barcode. FASTQ and/or BAM files generated with the Torrent Suit plugin FileExporter v5.10.0.0, and were then analyzed using Torrent Suite™ v5.8.0 Software (Thermo Fisher Scientific) running Ion AmpliSeq™ RNA plug-in v5.10.1.2, coverageAnalysis plugin v5.10.0.3 and target region hg19_AmpliSeq_Transcriptome_21K_v1. Differential gene expression analysis and principal component analysis (PCA) was performed using the Transcriptome Analysis Console v4.0.2 (TAC) Software (Thermo Fisher Scientific). Gene expression was defined as significantly different based on p-value < 0.05 and fold change cutoff value < −1.6 or > 1.6 for the comparison between neonatal (HDFN) and elderly (HDF87y) empty vector-transduced (control) cultures (Dataset EV1). Heatmaps were generated using the transcript counts normalized against the sample library size, which were then scaled by gene with normalized scores or z-scores (*i.e*, a value of 0 refers to the mean gene expression of that gene across all libraries; >0 or + indicates expression above and <0 or – indicates expression under this mean gene expression). Hierarchical clustering was performed using the R library heatmap.2. function with R package v3.6.1. Targeted

transcriptome sequencing data represent two independent experimental replicates of each biological sample.

Statistical analysis

All experiments were repeated at least two times unless otherwise stated. Sample sizes and statistical tests used for each experiment are indicated in the respective Figure captions. Data are shown as mean ± SD or mean ± SEM as indicated. GraphPad Prism version 7 was used to analyze all the data. Data were tested for parametric vs. non-parametric distribution using D'Agostino–Pearson omnibus normality test. Two tailed Mann–Whitney or chi-square tests were then applied accordingly to determine the statistical differences between different groups (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, and NS not significant p>0.05).

II.3. Results and Discussion

Defective error correction of improper kinetochore–microtubule attachments in elderly mitotic cells

Minor changes in MT dynamics due to perturbations in the balance of MT stabilizers and destabilizers can profoundly impact the fidelity of chromosome segregation (Bakhoum & Compton, 2012). While a subtle reduction in k- MT stability will delay or prevent the onset of anaphase, small increases in k-MT stability will lead to the persistence of erroneous k-MT attachments (*e.g,* merotelic) into anaphase. Thus, we tested whether changes in k-MT attachment stability underlie the mild CIN observed with age. We started by comparing kinetochore fibers (k-fiber) in dermal fibroblasts (HDFs) derived from young and elderly healthy Caucasian males (see methods section and Macedo *et al,* 2018). Calcium-induced depolymerization of non-kinetochore microtubules (Kollu *et al*, 2009) revealed that elderly cells have increased k-fiber intensity levels at the metaphase stage when compared to neonatal cells (Fig II.1A and B). Intra- and inter-kinetochore distances of aligned chromosomes in elderly metaphase cells were also increased (Maresca & Salmon, 2009) (Fig II.1C and D). However, the stability of k-MT attachments, as measured by the rate of dissipation of fluorescence after photoconversion of Tubulin (Zhai *et al,* 1995), showed no detectable difference between cells derived from young and elderly individuals (Fig II.EV1A–C). Taken together, these data suggest that elderly cells have an increased number of k-MT attachments in metaphase.

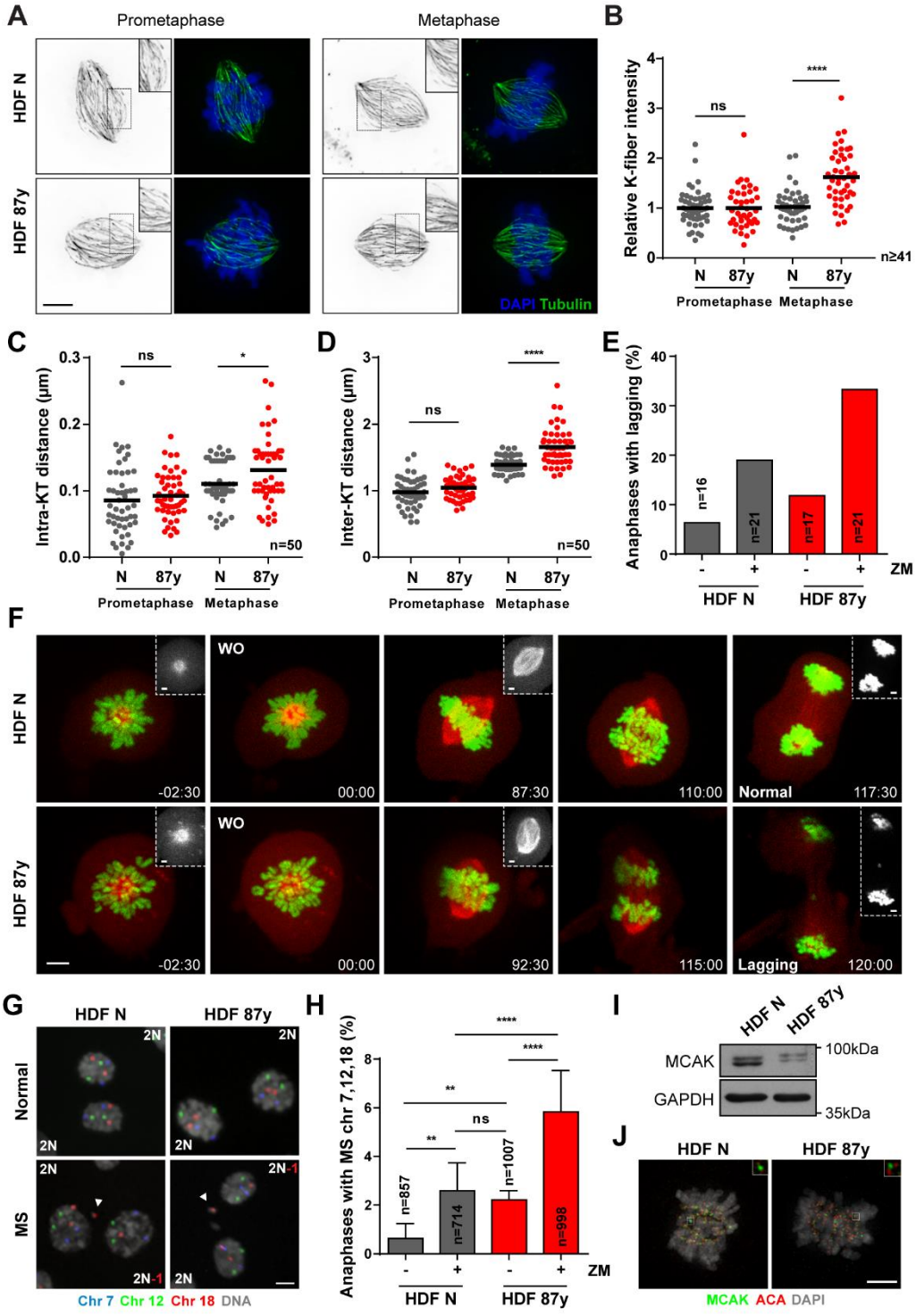


Figure II.1. Impaired k-MT error correction with advancing age. (**A, B**) Representative images (A) and quantification (B) of calcium-stable k-fiber intensity levels by immunofluorescence analysis of n≥41 tubulin-stained mitotic cells of neonatal (N) and elderly (87 years) human dermal fibroblasts (HDF) at prometaphase and metaphase stages. Intensity levels were normalized to neonatal samples. Scale bar, 5 µm; (**C**) Intra-kinetochore distance (between Hec1 and ACA immunostainings of a kinetochore) in n=50 kinetochore pairs scored from 10 elderly versus neonatal cells in prometaphase and metaphase; (**D**) Inter-kinetochore distance (between Hec1 staining of sister kinetochores) in n=50 kinetochore pairs scored from 10 elderly versus neonatal cells in prometaphase and metaphase; (**E, F**) Live-cell imaging of neonatal (N) (***continues in the next page***)

and elderly (87 years) fibroblasts expressing H2B-GFP/α-Tubulin-mCherry treated with kinesin-5 inhibitor (STLC) to induce monopolar spindles, followed by washout (WO) into medium with DMSO (-) or ZM447439 (+; 500 nM). (E) Quantification of anaphases with lagging chromosomes in n=cells scored and (F) representative movie frame series of a young and an elderly dividing cell that underwent correct (Normal) and incorrect (Lagging) chromosome segregation, respectively. Time, min:sec. Scale bars: 5 µm (images) or 2 µm (insets); (**G**) Representative images of anaphases without (top) and with (bottom) mis-segregation (MS), FISH-stained for three chromosome pairs (7, 12, and 18). Arrowheads indicate micronuclei containing centromeric signal for chromosome 18. Scale bar, 10 µm; (**H**) Percentage of anaphases with MS in neonatal (N) versus elderly (87 years) n=cells scored by FISH analysis; (**I**) Western blot analysis of total MCAK protein levels in neonatal (HDF N) and elderly (HDF 87 years) fibroblasts. GAPDH is shown as loading control; (**J**) Immunofluorescence analysis of MCAK levels in neonatal (HDF N) and elderly (HDF 87 years) mitotic cells. Scale bar, 5 µm. *Data information*: All values shown are mean ± SD of at least two independent experiments. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ by two-tailed (B–D) Mann–Whitney test and (H) chi-square test. Source data are available online for this figure.

If MT occupancy at kinetochores is higher in elderly cells, then an increased number of erroneous k-MT attachments could be expected. To test this prediction, we assessed the efficiency of error correction using the reversible inhibition of kinesin-5 with S-trityl-L-cysteine (STLC) to induce transient monopolar spindles and potentiate the formation of erroneous attachments (Lampson *et al,* 2004). Live-cell imaging of cells expressing H2B-GFP/α-Tubulin-mCherry demonstrated that elderly cells are two times more likely to exhibit lagging chromosomes following STLC washout than their young counterparts (11.8% versus 6.3%) (Fig II.1E and F). Fluorescence *in situ* hybridization (FISH) analysis for 3 chromosome pairs showed that chromosome mis-segregation is higher in elderly dividing cells (2.22% versus 0.63%) (Fig II.1G and H), further confirming the live-cell imaging data. We also measured the frequency of errors upon partial inhibition of Aurora B or Mps1 kinase activities using nanomolar concentration of the small-molecules ZM447439 (Ditchfield *et al*, 2003) and AZ3146, respectively. As expected, the frequency of segregation errors significantly increased upon these drug treatments, but a ~2-fold difference was still observed between elderly and neonatal cells (Fig II.1E and H; Appendix Fig II.S1).

Altogether, our data show that aged cells not only generate erroneous k-MT interactions at higher frequency, but also correct them less efficiently. Indeed, gene expression and protein levels of main regulators involved in the establishment of proper k-MT attachments, including the MT-depolymerizing kinesin MCAK, are decreased in elderly cells (Figs II1I and J, and II.EV2A–J).

Overexpression of microtubule destabilizing kinesin-13 proteins restores chromosome segregation fidelity

Efficient k-MT error correction relies on the release of microtubules from kinetochores, and two MT-depolymerizing kinesin-13 proteins, Kif2b and MCAK, have been shown to promote k-MT detachment to effect error correction at distinct phases of mitosis (Bakhoum *et al,* 2009). To directly test whether stimulating this machinery affects mild CIN observed with aging, we overexpressed GFP-tagged versions of MCAK (GFP-MCAK) and Kif2b (GFP-Kif2b) in neonatal and elderly cell cultures (Fig II.2A, Appendix Fig II.S2). We found that overexpression of each kinesin-13 rescues the mitotic delay from nuclear envelope breakdown to anaphase onset that we previously reported for advancing age (Macedo *et al,* 2018) (Fig II.2B), indicating that increased kinesin-13 function facilitates mitotic progression. Overexpression of kinesin-13 proteins also reduces the intensity levels of calcium-stable k-fibers in elderly cells (Fig II.2C and D). To determine whether increased levels of MCAK or Kif2b ultimately impact chromosome segregation fidelity, we combined a cytokinesis-block assay with FISH staining for 3 chromosome pairs to measure the rates of chromosome mis-segregation in both neonatal and elderly cultures (Fig II.2E). Overexpression of MCAK and Kif2b was sufficient to reduce the percentage of elderly binucleated (BN) cells with chromosome mis-segregation, while having no effect on neonatal cells (Fig II.2F). Similarly, the frequency of cells with micronuclei (MN; a common fate of anaphase lagging chromosomes) decreased in elderly cells overexpressing the kinesin-13 proteins when compared to control elderly cells (Fig II.2G). Taken together, these results indicate that restoring kinesin-13 protein levels suffices to improve error correction and mitotic fidelity in elderly cells, confirming that defective error correction of improper k-MT attachments is significantly contributing to the mild CIN observed with aging.

Strategic destabilization of k-MTs delays senescence

Lagging chromosomes and the ensuing MN were recently recognized as a source of pro-inflammatory signaling when loss of compartmentalization exposes MN chromatin to cytosolic cyclic GMP-AMP synthase (cGAS) (Bakhoum *et al*, 2018; Harding *et al*, 2017; Mackenzie *et al*, 2017), a local DNA sensor to initiate innate immune response against foreign DNA (Gao *et al,* 2013; Sun *et al,* 2013), and more recently also reported as an essential player for cellular senescence (Dou *et al,* 2017; Gluck *et al,* 2017; Yang *et al,* 2017). In a previous study, we found chromosome mis-segregation to be a key trigger for the development of full senescence phenotypes in elderly cells (Macedo *et al,* 2018), which consistently with an inflammatory gene expression profile also exhibit increased secretion

of cytokines (Fig II.EV3A). Thus, we tested whether counteracting age-associated mild CIN could delay the development of full senescence in elderly cells.

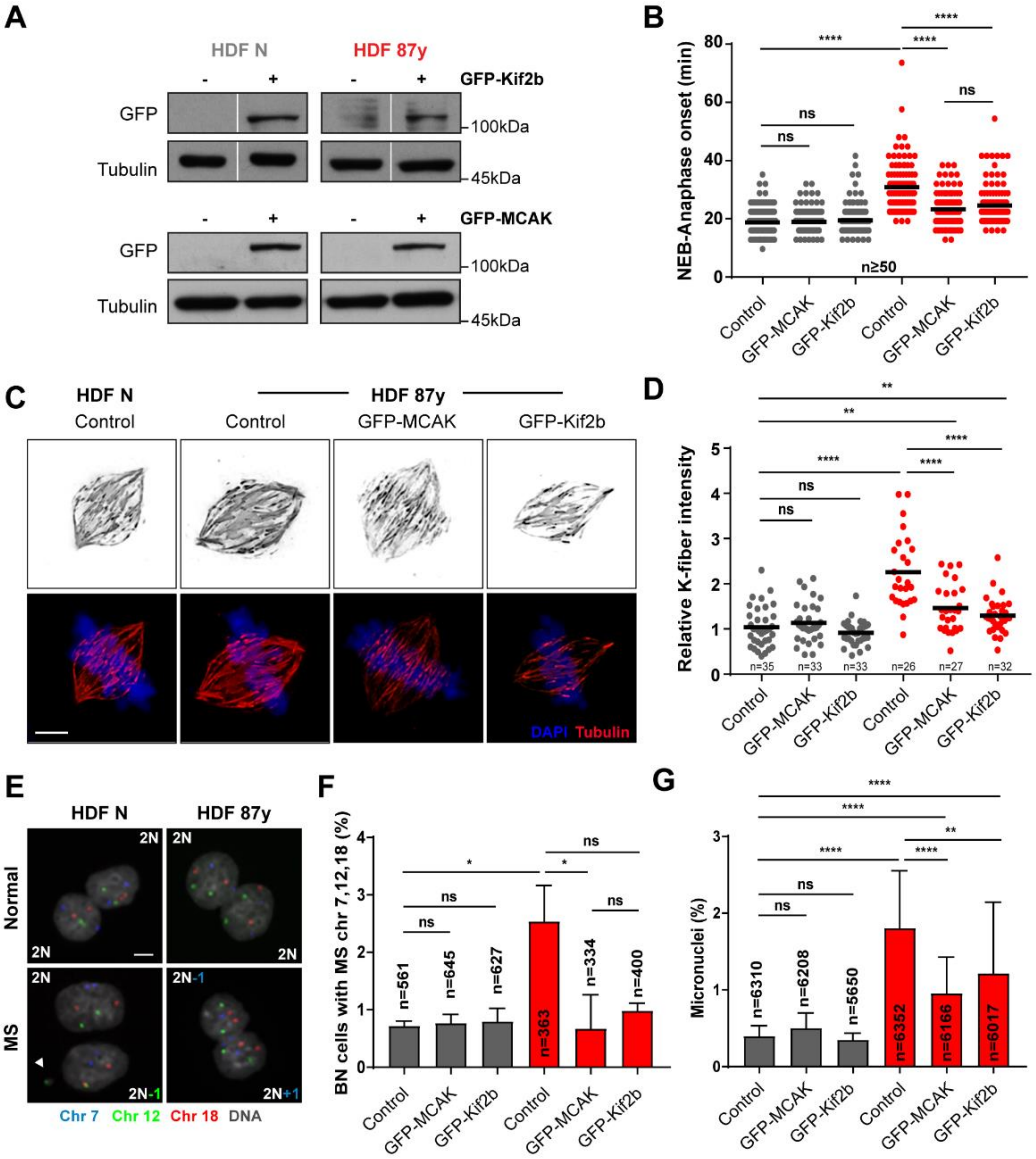


Figure II.2. Overexpression of MT depolymerases, MCAK and Kif2b, restores chromosome segregation fidelity in elderly cells. (**A**) GFP protein levels in FACS-sorted neonatal (N) and elderly (87 years) fibroblasts transduced with empty (control, -), GFP-MCAK (+), and GFP-Kif2b (+) lentiviral plasmids. Tubulin is shown as loading control; (**B**) Mitotic duration scored by time-lapse phase-contrast microscopy of n≥50 cells per condition measured from nuclear envelope breakdown (NEB) to anaphase onset; (**C, D**) Representative images (C) and quantification (D) of calcium-stable k-fiber intensity levels by immunofluorescence analysis of n≥26 tubulin-stained metaphase cells transduced as indicated. Levels were normalized to the control neonatal sample. Scale bar, 5 μm; (**E**) Representative images of cytochalasin D-induced binucleated (BN) cells without (top) and with (bottom) mis-segregation (MS), FISH-stained for three chromosome pairs (7, 12, and 18). Scale bar, 10 μm; (**F**) Percentage of BN cells with MS in n=cells scored; (**G**) Percentage of micronuclei in n=cells analyzed. *Data information*: All values are mean ± SD of at least two independent experiments. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ by two-tailed (B, D) Mann–Whitney and (F, G) chi-square test. Source data are available online for this figure.

Analysis of cGAS gene expression revealed no significant changes between young and elderly fibroblast cultures (Fig II.EV3B), but we found that the percentage of cells with cGAS-positive MN is significantly increased in elderly cultures (Fig II.EV3C and D). cGAS-positive MN were typically Rb-negative, thus suggesting nuclear envelope was disrupted (Fig II.EV3C and D) (Mackenzie *et al,* 2017). The percentage of elderly cells with cGAS-negative/Rb-positive MN was also increased, likely representing a fraction of MN that still did not undergo nuclear envelope disruption. In agreement with the role of this cytosolic DNA sensor in pro-inflammation and senescence development, depletion of cGAS (Fig II.EV3E) was able to decrease the percentage of cells with cGAS-positive MN without changing the total percentage of MN (Fig II.EV3F), and to reduce the senescence-associated phenotypes in elderly cells (Fig II.EV3G and H). These results indicate that cGAS engagement induced by mild CIN may play an important role in the development of cellular senescence with aging. To further test this idea, we analyzed the implications of kinesin-13 overexpression on MN cGAS positivity and senescence. Increased levels of MCAK and Kif2b led to decreased frequency of MN and equivalently fewer staining positive for cGAS (Fig II.EV3I). The percentage of cells exhibiting senescence biomarkers was reduced upon improved error correction efficiency (Fig II.3A–D). Moreover, targeted transcriptomic analysis (see methods section; Fig II.3E and F; Dataset EV1) revealed marked improvements in senescence- associated gene expression profile. From a custom list of senescence and SASP-related genes (Fig II.3G; Dataset EV2), differential expression between young and elderly cells according to the expected was observed for 20 genes, in which 17 were partially recovered following overexpression of kinesin-13 proteins. We additionally extended the analysis to a set of genes that defines a “senescence core signature” common to different cell types and senescence-inducing stimuli (Hernandez-Segura *et al,* 2017). Comparison between octogenarian and neonatal cells revealed 23 genes significantly altered as expected, out of which 17 were modulated upon overexpression of MCAK and Kif2b in elderly cells (Appendix Fig II.S3, Dataset EV3). Moreover, we interrogated the transcriptome dataset for a custom list of 56 genes associated with the cGAS/STING/NF-jB pathway (Dataset EV4), and we found that out of the 25 genes differentially expressed in octogenarian versus neonatal cells accordingly to the expected, 23 were partially rescued by MCAK and/or Kif2b overexpression in old-aged cells (Fig II.3H). Taken together, these data demonstrate that kinesin-13 protein overexpression in aged cells restores chromosome segregation fidelity and reduces the burden of MN-induced pro-inflammatory cGAS signaling. This in turn has a positive impact on the elderly cell population, as significant improvements in the senescence-associated transcriptome signature match with delayed emergence of fully senescent cells permanently arrested in the cell cycle.

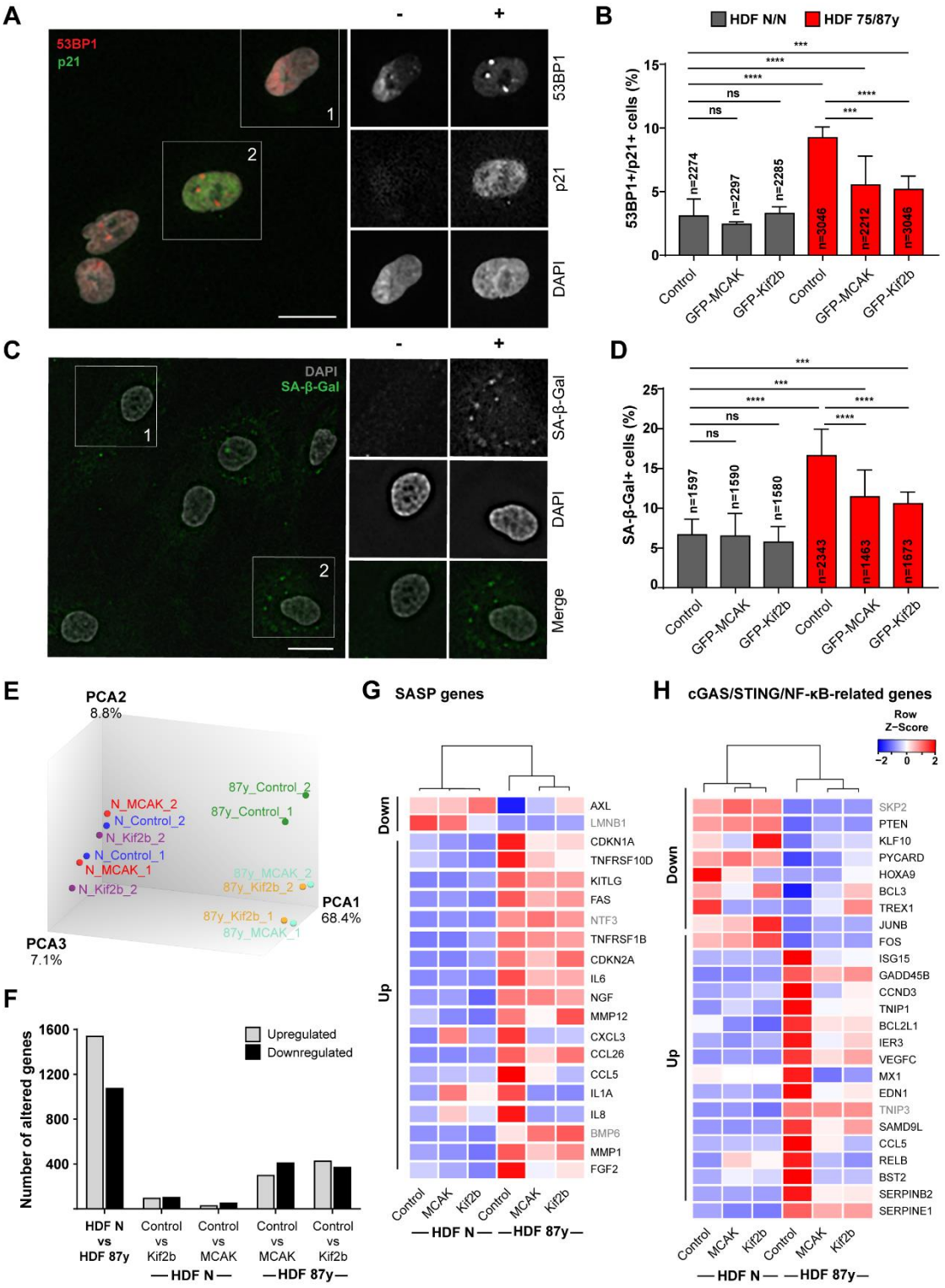


Figure II.3. Overexpression of MCAK and Kif2b delays senescence in fibroblast cultures from elderly donors. (**A**) Representative images of elderly cells scored as negative (-) or positive (+) for Cdkn1a/p21 (cell cycle inhibitor) and 53BP1 (≥ 1 foci; DNA damage) senescence biomarkers. Scale bar 20 μm; (**B**) Percentage of n=cells staining double-positive for Cdkn1a/p21 and 53BP1 in neonatal (N/N) and elderly (75/87 years) human dermal fibroblasts (HDF) transduced with empty, GFP-MCAK or GFP-Kif2b lentiviral plasmids; (**C**) Representative images of elderly cells scored as negative (-) or positive (+) for SA-β-galactosidase (SA-β-gal) activity. Scale bar, 20 μm; (**D**) Percentage of n=cells positive for SA-β-gal in neonatal and elderly HDFs transduced with empty, GFP-MCAK or GFP-Kif2b lentiviral plasmids; (***continues in the next page***)

(**E**) Principle component analysis (PCA) of neonatal and elderly cells transduced with empty-vector (control) or kinesin-13 proteins (MCAK or Kif2b) based on bulk RNA expression data; (**F**) Total number of genes altered (up- or down-regulated, as indicated) for the comparisons shown between control and kinesin-13-overexpressing young (HDF N) and/or aged (HDF 87 years) cells; (**G, H**) Heatmaps of differentially expressed (G) SASP and senescence genes, and (H) cGAS/STING/NF-κB-related genes of neonatal and 87 years HDFs. Down and Up refer to the expected changes as reported for senescent cells (Datasets EV2 and EV4). Gene symbols highlighted in gray indicate genes that were not modulated by overexpression of kinesin-13 proteins. Z-score row color intensities indicate higher (red) to lower (blue) expression. *Data information*: Values are mean ± SD of at least two independent experiments. ns $P > 0.05$, ***$P < 0.001$, ****$P < 0.0001$ by two-tailed chi-square test (B, D).

Small-molecule inhibition of age-associated chromosome mis-segregation delays senescence

A recent study identified a small molecule that specifically potentiates the activity of the kinesin-13 protein MCAK (UMK57), to transiently suppress chromosome mis-segregation in CIN+ cancer cells (Orr *et al*, 2016). Since perturbed error correction contributes to mild CIN in aged cells, we reasoned that this agonist should provide a small-molecule approach, as an alternative to genetic overexpression, to suppress CIN. Thus, we followed cells exposed to increasing concentrations of UMK57 under 24-h long-term time-lapse microscopy and found that 1 µM is sufficient to rescue the increased mitotic duration in elderly cells, while having no noticeable effect on the mitotic progression of neonatal cultures (Fig II.4A). As 1 µM is a 10X higher dose than the previously reported for CIN+ cancer cells, we fine-tuned the titration to lower concentrations using phenotypic readouts for CIN and senescence (Fig II.EV4A–D). We found that 1 µM is indeed required to significantly rescue the age-associated phenotypes. Noteworthy, 1 µM UMK57 did not overtly change the MCAK protein levels in neonatal or elderly cells (Fig II.EV4E). Also, we showed that UMK57 doses > 0.1 µM are unable to suppress, or even increase, CIN in HT-1080 fibrosarcoma cells, which have 60% higher MCAK levels than neonatal fibroblasts (Fig II.EV4F–I). This supports the safe use of 1 µM UMK57 in the context of untransformed aged cells, which is different from the context of cancer cells, which typically overexpress MCAK. All further experiments were conducted with this optimal dose of UMK57. In agreement with the rescued mitotic delay, calcium-stable k-fiber intensity analysis in UMK57-treated elderly cells revealed that enhanced MCAK activity decreases the number of stable k-MT attachments in metaphase (Fig II.4B and C). FISH analysis of 3 chromosome pairs in both interphase cells and cytokinesis-blocked BN cells showed that UMK57 decreases the levels of aneuploidy and the chromosome mis-segregation rate in elderly cell populations (Fig II.4D and E). Also, MN levels were scored and found to be partly decreased in elderly cells upon 24-h exposure to the MCAK agonist (Fig II.4F). These results indicate that age-

associated mild CIN can be rescued using a small-molecule agonist of the kinesin-13 protein MCAK, thus supporting that reduced error correction contributes to mild CIN with age.

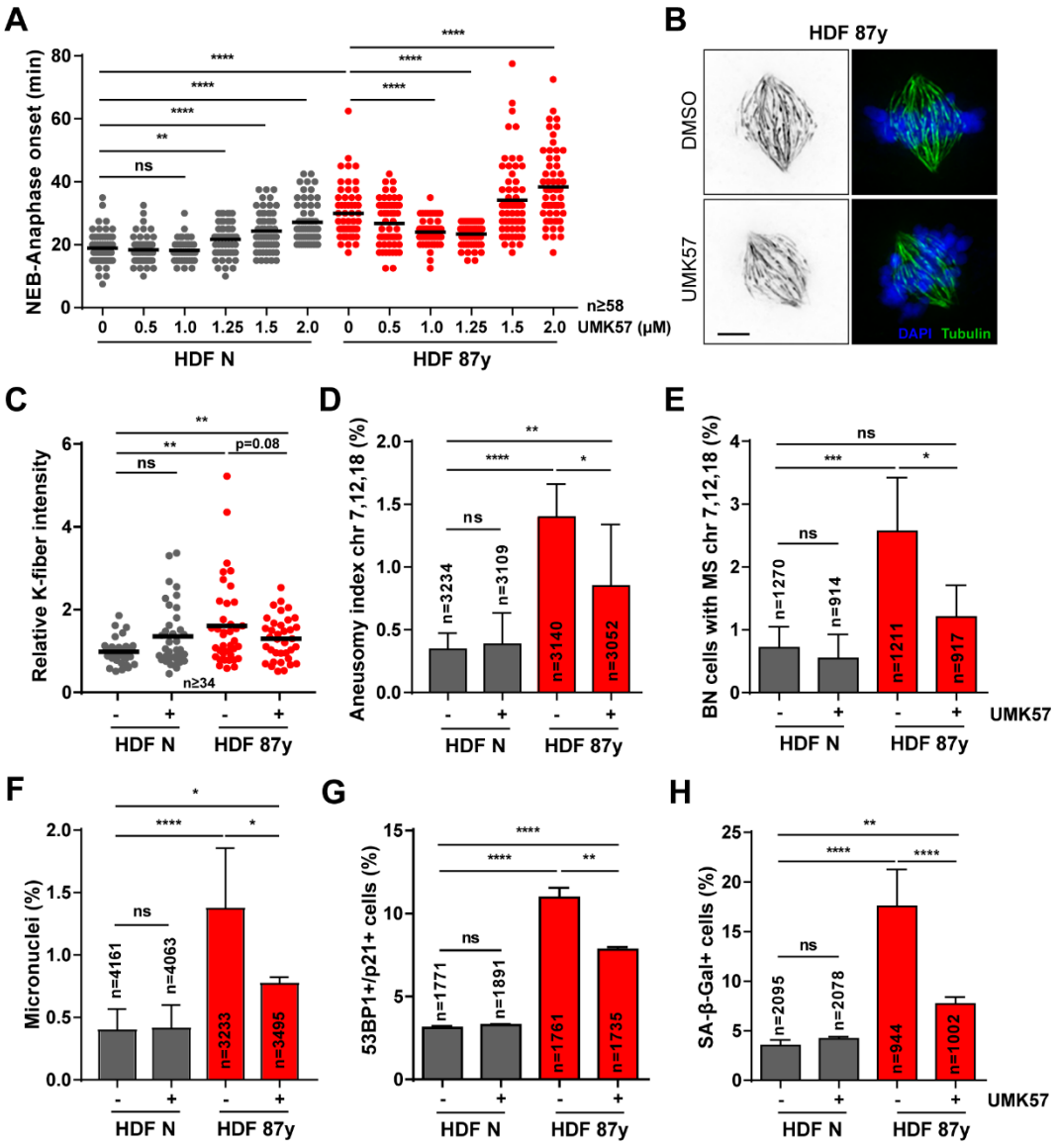


Figure II.4. Small-molecule agonist of MCAK activity rescues age-associated CIN and delays senescence. (**A**) Mitotic duration of neonatal (N) and elderly (87 years) human dermal fibroblasts (HDF) treated for 24 h with different concentrations of UMK57 (MCAK agonist). n≥58 cells were analyzed per condition. For all subsequent experiments, UMK57 was used at 1 μM for 24 h; (**B, C**) Representative images (B) and quantification (C) of calcium-stable k-fiber intensity levels in metaphase, scored by immunofluorescence analysis of n≥34 tubulin-stained mitotic cells of neonatal and elderly samples treated with DMSO (-) and UMK57 (+). Levels were normalized to neonatal DMSO-treated condition. Scale bar, 5 μm; (**D**) Aneusomy index of chromosomes 7, 12, and 18 measured by interphase FISH analysis of n=cells; (**E**) Percentage of cytochalasin D-induced binucleated (BN) n=cells with chromosomes 7, 12, and 18 mis-segregation (MS); (**F**) Percentage of micronuclei in n=cells scored when treated with DMSO or UMK57; (**G**) Percentage of n=cells staining positive for double immunostaining of Cdkn1a/p21 (cell cycle inhibitor) and 53BP1 (***continues in the next page***)

(≥ 1 foci; DNA damage) senescence biomarkers; (**H**) Percentage of n=cells staining positive for SA-β-galactosidase (SA-β-gal) activity. *Data information*: All values are mean ± SD of at least two independent experiments. ns $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ by two-tailed (A, C) Mann-Whitney and (D–H) chi-square tests.

We next tested whether UMK57-induced MCAK activity could delay cellular senescence. We found that 24-h treatment is sufficient to partially rescue the percentage of cells exhibiting the senescence biomarkers 53bp1+p21 and SA-β-galactosidase activity (Fig II.4G and H). Together, these results demonstrate that strategic destabilization of k-MT attachments aids in the correction of improper k-MT attachments, while acting to counteract MN-induced pro-inflammation and cellular senescence with aging.

Efforts to suppress CIN in cancer cell lines using UMK57 unveiled a rapidly arising adaptive resistance mechanism, whereby reversible rewiring of mitotic signaling networks abolishes the CIN-inhibiting effect of the compound (Orr *et al,* 2016). To explore whether such adaptive resistance mechanisms arise in our cellular model of aging, we exposed cells to UMK57 for longer periods. After 96-h treatment, the mitotic delay of elderly cells was rescued (Fig II.5A), indicating that the beneficial effect of UMK57 persists over long-term exposure. In agreement, decreased k-fiber intensity levels in metaphase were still observed after 96 h (Fig II.5B). FISH analyses on interphase and BN cells demonstrated that aneuploidy (Fig II.5C), chromosome mis-segregation (Fig II.5D), and micronucleation (Fig II.EV5A) were also inhibited after 96 h. Furthermore, we found that the decrease in MN, including cGAS+ MN (Fig II.EV5B), correlates with a repression of cellular senescence, demonstrated by the partial rescue in senescence markers (Fig II.5E and F) and mild changes in gene expression of senescence-associated genes, including genes of the "senescence core signature" (Fig II.EV5C). Similar observations were taken for 4 weeks of exposure to UMK57 (Fig II.EV5D), for which decreased levels of aneuploidy and senescence markers were also observed (Fig II.EV5E–G).

Adaptive resistance arising in CIN+ cancer cell lines was shown to be Aurora B-dependent, the activity of which dropped significantly with longer exposures and correlated with increases in k-MT attachment stability specifically in prometaphase (Orr *et al,* 2016). In contrast to CIN+ cancer cells, we found that in elderly cells exposed to UMK57 for 96 h, k-fiber intensity levels in prometaphase were unaltered (Fig II.EV5H), even though analysis of active Aurora B kinase (T232 phosphorylation) at centromeres revealed oscillating levels, a slight decrease in neonatal cells at 96 h and an increase in aged cells at 24 h but not at 96 h (Fig II.EV5I). In metaphase, we found slightly reduced levels of active Aurora B in aged cells at 96 h (Fig II.5G and H), albeit not translated into an adaptive response by functional analysis (Fig II.5B–D). Taken together, these results indicate that suppression of mild CIN

using this small-molecule agonist of MCAK may be a potential strategy to counteract age-associated senescence.

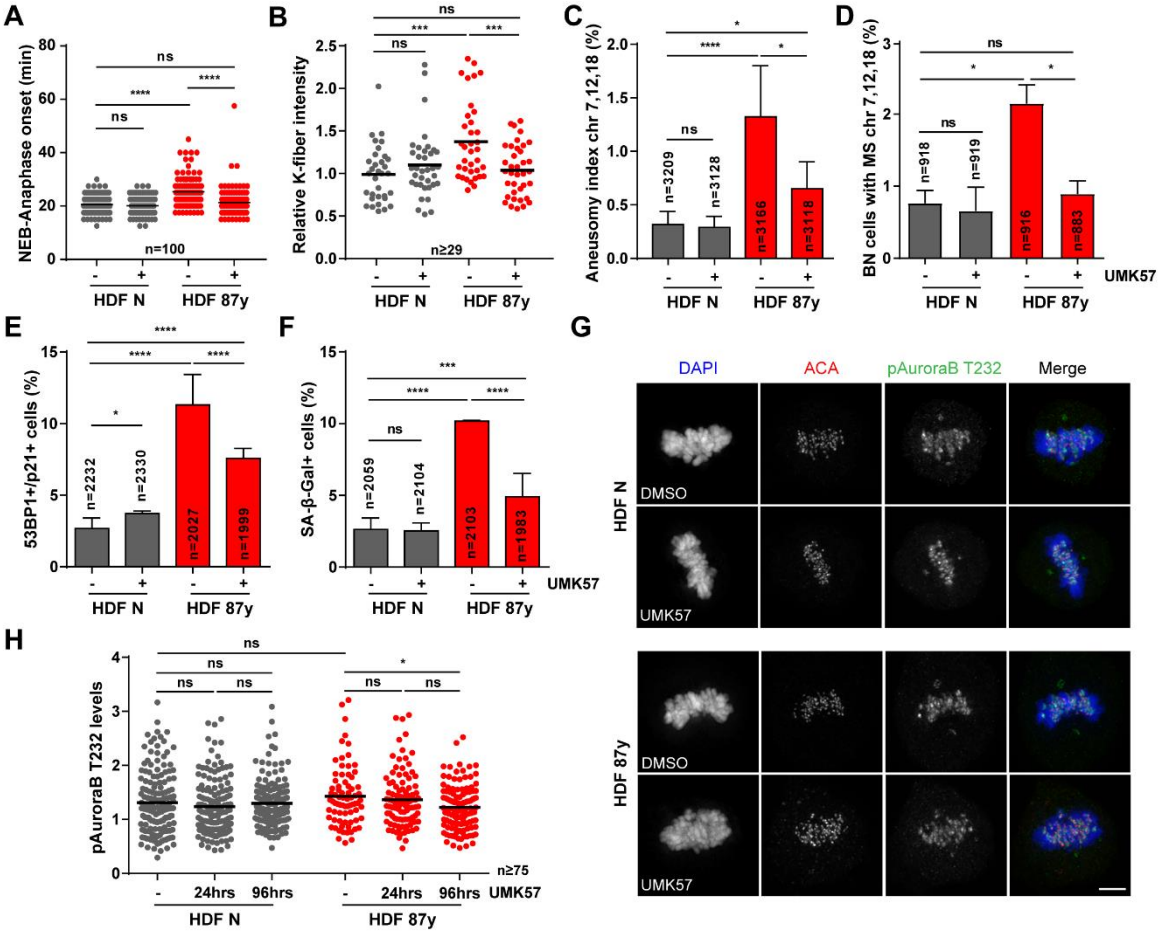


Figure II.5. Long-term treatment with UMK57 does not lead to adaptive resistance in elderly cells. (**A**) Mitotic duration of n=100 neonatal (N) and elderly (87 years) human dermal fibroblasts (HDF) treated with DMSO (-) or UMK57 (+) for 96 h; (**B**) Relative calcium-stable k-fiber intensity levels scored by immunofluorescence analysis of n≥29 tubulin-stained metaphase cells of neonatal and elderly samples treated with DMSO and UMK57 for 96 h. Levels were normalized to neonatal DMSO-treated condition; (**C**) Aneusomy index of chromosomes 7, 12, and 18 measured by interphase FISH analysis of neonatal and elderly n = cells treated for 96 h; (**D**) Cytochalasin D-induced binucleated (BN) n=cells with mis-segregation (MS) of chromosomes 7, 12, and 18 in neonatal and elderly samples treated for 96 h; (**E**) Percentage of n = cells staining positive for double immunostaining of Cdkn1a/p21 (cell cycle inhibitor) and 53BP1 (≥ 1 foci; DNA damage) senescence biomarkers after 96 h of treatment; (**F**) Percentage of n=cells staining positive for SA-β-gal activity when treated for 96 h with DMSO or UMK57; (**G, H**) Representative images (G) and quantification (H) of phospho-Aurora B Thr232 (pAuroraB T232) levels at kinetochores/centromeres in n≥75 neonatal and elderly metaphase cells treated with UMK57 for 24 or 96 h. Intensity levels were normalized to ACA. Scale bar, 5 µm. *Data information*: All values are mean ± SD of at least two independent experiments. ns $P > 0.05$, *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$ by two-tailed (A, B, H) Mann–Whitney and (C–F) chi-square tests.

The mechanistic link between chromosomal instability (CIN) and senescence in the context of aging has remained elusive. Based on our previous findings (Macedo *et al,* 2018), we reasoned that defective k-MT attachments could contribute to the mild levels of CIN observed in mitotically active cell populations from elderly donors. We found that calcium-stable k-fiber intensity and inter-/intra-kinetochore distance are increased in aged fibroblasts, specifically in metaphase. These data suggest that an increased number of k-MT attachments could be responsible for the increased number of lagging chromosomes and micronuclei observed in elderly cell populations (Fig II.6). The importance of MT occupancy for the proper segregation of merotelically attached kinetochores has been previously demonstrated in non-cancerous human cells (Dudka *et al*, 2018). Increased microtubule occupancy at kinetochores may arise as a result of altered microtubule assembly rates (Ertych *et al*, 2014), or altered kinetochore architecture. Cells from aged individuals were reported to exhibit peri/centromeric satellite heterochromatin decondensation, an early indicator of cellular senescence (Swanson *et al*, 2013). The levels of the histone H3 variant centromeric protein A (CENP-A), which epigenetically determines human centromeres, are known to be decreased in senescent cells (Macedo *et al,* 2018). Despite the reduction of CENP-A expression, our previous transcriptomic analysis showed that CENP-C, a crucial component for kinetochore assembly, is upregulated in elderly cells (Macedo *et al,* 2018). Thus, a possibility is that distension of centromeres combined with increased CENP-C levels may provide a structural platform capable of establishing a higher MT occupancy in aged cells. With more MT attachments per kinetochore in elderly cells, the normal rate of k- MT detachment in metaphase is less likely to be sufficient in correcting all erroneous attachments (Drpic *et al*, 2018). In support of this idea, increasing k-MT detachment rate, through the overexpression of kinesin-13 proteins or through treatment with UMK57, rescued k-fiber intensity levels, improved error correction and segregation fidelity in the aged fibroblasts, and ultimately prevented the generation of cGAS-positive micronuclei. Therefore, impaired kinesin-13 activity can be established as a mechanistic link between chromosome mis-segregation and senescence in naturally aged cells, by significantly contributing to cGAS-dependent pro-inflammatory response.

Here, we demonstrated that pharmacological rescue of MCAK activity is a means to delay cellular aging (Fig II.6). Importantly, the optimal concentration of UMK57 that improved chromosome segregation fidelity in elderly cells did not impact the mitotic fitness of younger cells. Also, we excluded the possibility of adaptive resistance to prolonged UMK57 treatment, which has previously impaired its clinical application in CIN+ cancer cells (Orr *et al,* 2016). Even though modulation of CIN solely acts on mitotically active aged cell/tissue populations, there are substantial arguments for it to be taken into consideration as an anti-aging strategy. First, different types of proliferative cells support the function of

stem and differentiated cell pools via paracrine signaling, by secreting bioactive molecules (Bussian *et al,* 2018). Also, emergent rejuvenation strategies such as dietary regimens, cellular reprogramming, and senolysis, primarily target cells with proliferative potential/capacity (adult stem cells, vascular and connective tissue cells) or with loss of proliferative capacity (senescent cells) (Mahmoudi *et al,* 2019b). Second, modulation of CIN encompasses several advantages of senolysis since it prevents the generation of fully senescent cells and their detrimental paracrine signaling. Notably, it bypasses hurdles that still dim the applicability of senolytic therapies, including the need to determine a critical time window for treatment onset and the off-target effects on beneficial senescence (Melo Pereira *et al,* 2019). Finally, reducing the burden of SASP factors via CIN modulation likely has a positive impact on the changes observed in the immune system with age (immunosenescence), which have been ascribed to persistent pro-inflammation in tissues (Fulop *et al,* 2017). The here shown small-molecule modulation of kinesin-13 MCAK acts precisely upstream in the order of events, *i.e,* CIN – micronuclei – cytosolic DNA – cGAS/STING activation – SASP, by reestablishing mitotic competence and diluting out senescent cells (Barroso-Vilares & Logarinho, 2019). Consequently, *in vivo* studies will be paramount to determine the overall impact of chromosome segregation improvement over time at the organismal level.

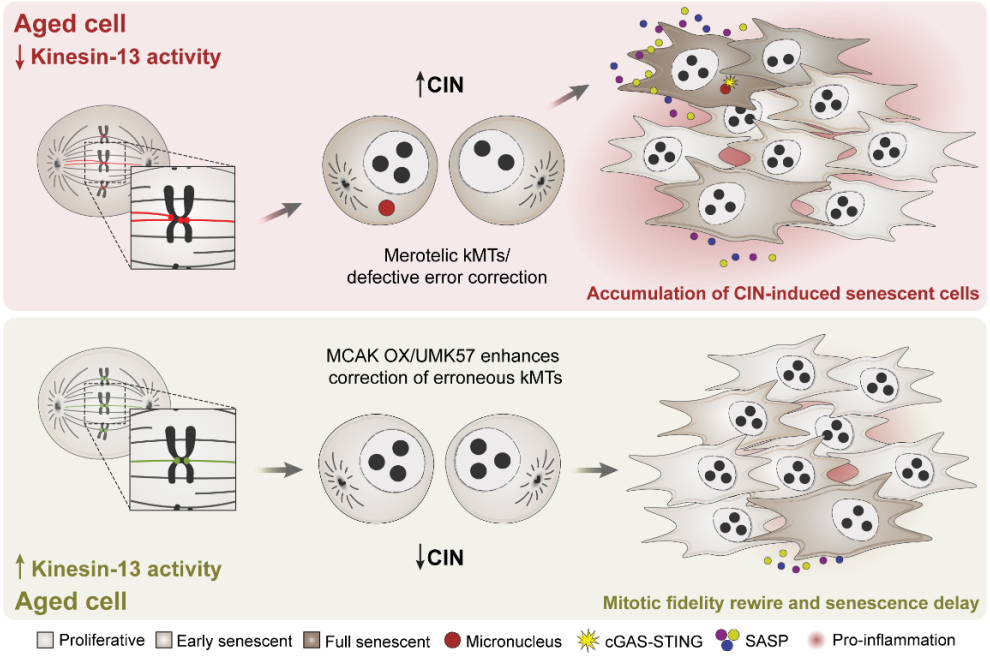


Figure II.6. Disturbed k-MT dynamics is a major driver of CIN with aging and contributes to senescence. The number of k-MT attachments is increased in elderly cells as a result of defective MT-depolymerizing kinesin-13 activity and k-MT error correction. Notably, chromosome segregation fidelity can be restored by genetic and small-molecule enhancement of kinesin-13 activity in old cells and the destabilization of k-MT attachments rescues the cellular aging phenotypes associated with CIN.

II.4. Supplementary Information

This section contains the expanded view (EV) figures and the appendix files related to the main figures included in this chapter. The targeted transcriptome analysis data generated for this study have been deposited at the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-8634. Datasets EV1-EV5, as well as Source Data files can be accessed online (https://doi.org/10.15252/embr.201949248).

II.4.1. Expanded View Figures

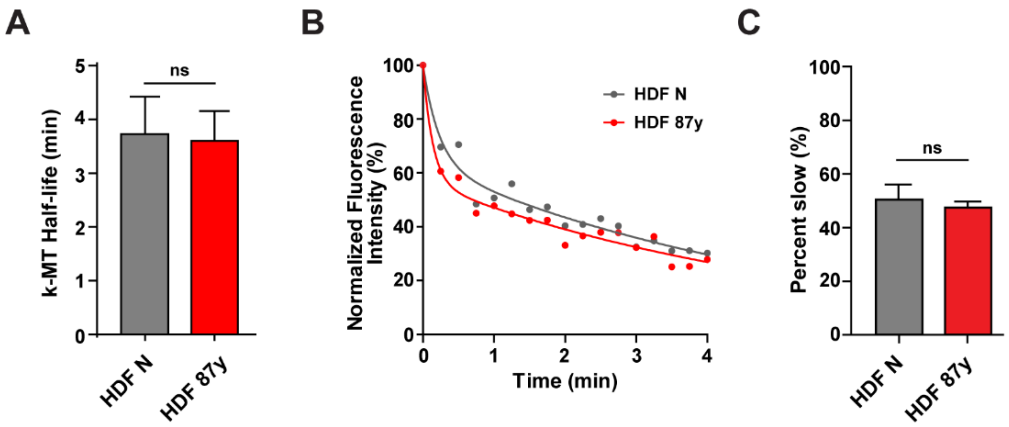


Figure II.EV1. Similar k-MT turnover in young and elderly fibroblasts (related to Fig II.1). (**A**) Average k-MT half-life for n=12 neonatal (HDF N) and elderly (HDF 87 years) metaphase cells expressing mEOS-Tubulin; (**B**) Examples of normalized fluorescence dissipation after photoconversion in neonatal versus 87 years metaphase cells that are representative of the average k-MT half-life shown in (A): (**C**) Percentage of photoconverted fluorescence intensity attributable to the slow decay process for n=12 neonatal versus 87 years cells. *Data Information:* Values shown are mean ± SEM of at least two independent experiments. ns $P > 0.05$ by two-tailed t-test.

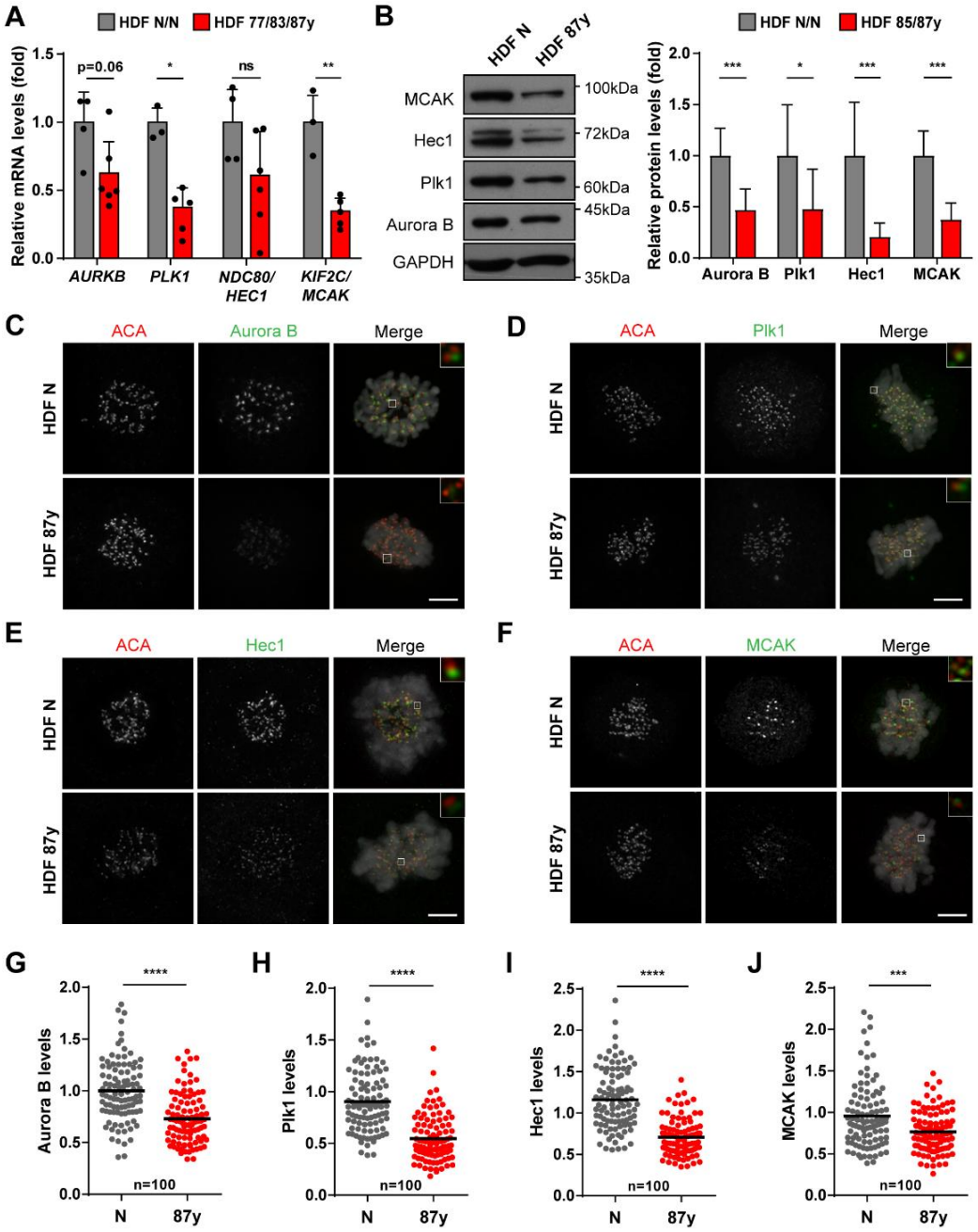


Figure II.EV2. Decreased levels of main regulators of k-MT dynamics in mitotic cells from elderly donors (related to Fig II.1). (**A**) Relative AURKB, PLK1, HEC1, and MCAK transcript levels in total RNA of mitotic fibroblasts from elderly (HDF 77/83/87 years; n>5 replicates) versus neonatal (HDF N/N; n≥3 replicates) donors. TBP and HPRT1 were used as reference genes; (**B**) Western blot analysis (left) and quantification (right) of Aurora B, Plk1, Hec1, and MCAK protein levels in mitotic extracts of elderly (HDF 85/87 years; n≥3 replicates per age) normalized against neonatal (HDF N/N; n=5 replicates per neonatal sample) fibroblasts. GAPDH was used as loading control; (**C–J**) Representative images and immunofluorescence intensity levels of (C, G) Aurora B, (D, H) Plk1, (E, I) Hec1, and (F, J) MCAK, in n=100 kinetochores of mitotic cells (N versus 87 years). Intensity levels were normalized to ACA centromere staining. Scale bars, 5 µm. *Data Information*: All values shown are mean ± SD of at least two independent experiments. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ in comparison to neonatal by two-tailed Mann–Whitney test. Source data are available online for this figure.

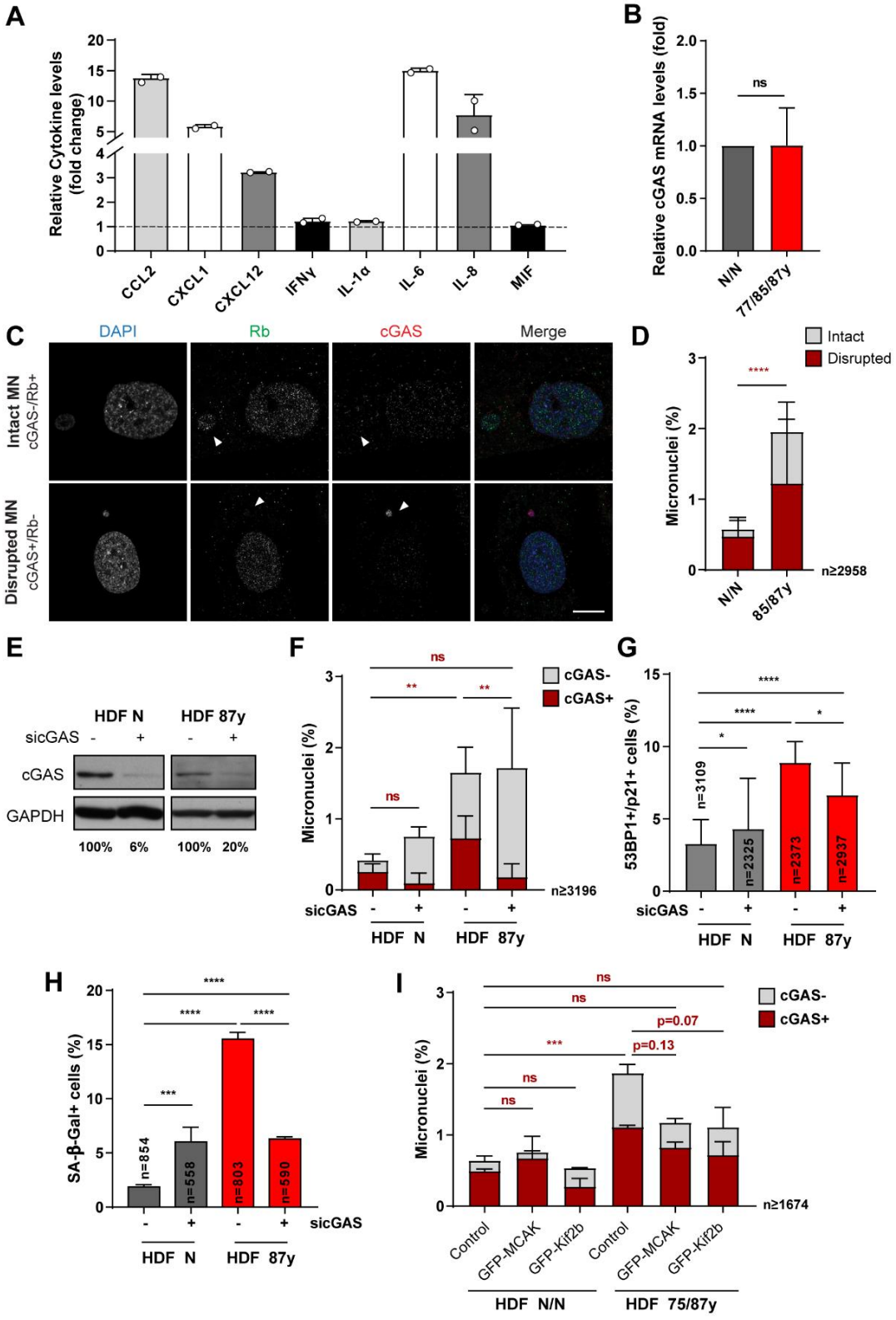


Figure II.EV3. cGAS engagement in aging-associated micronucleation and senescence phenotypes (related to Fig II.3). (**A**) Relative cytokine levels (fold change) in cell medium supernatants of elderly (HDF 87 years) cells normalized to neonatal (HDF N) cells (n=2 replicates per age, shown as individual datapoints); (**B**) Relative CGAS transcript levels in total RNA of fibroblasts from elderly (HDF 87 years; n=5 replicates) versus neonatal (HDF N; n=6 replicates) donors. TBP and HPRT1 were used as (***continues in the next page***)

reference genes; (**C, D**) Representative images (C) and quantification (D) of intact (cGAS-/Rb+) or disrupted (cGAS+/Rb-) micronuclei (MN) in n≥2,958 immunostained cells. Arrowheadsindicate micronuclei. Scale bar, 10 µm; (**E**) Western blot analysis of cGAS protein levels following siRNA depletion (sicGAS). GAPDH is shown as loading control; (**F**) Quantification of cGAS-positive micronuclei in n=cells following sicGAS depletion; (**G**) Percentage of n=cells staining positive for double immunostaining of Cdkn1a/p21 (cell cycle inhibitor) and 53BP1 (≥1 foci; DNA damage) senescence biomarkers following cGAS depletion; (**H**) Percentage of n=cells staining positive for SA-β-galactosidase (SA-β-gal) activity after cGAS depletion; (**I**) Percentage of cGAS-positive micronuclei in n≥1,674 cells of neonatal and elderly cells transduced with empty (control), GFP-MCAK, or GFP-Kif2b lentiviral plasmids. *Data Information*: All values shown are mean ± SD of at least two independent experiments. ns *P > 0.05*, **P < 0.05*, ***P < 0.01*, ****P < 0.001*, *****P < 0.0001* in comparison to neonatal by two-tailed (D, F–I) Mann–Whitney and (B) chi-square tests. Source data are available online for this figure.

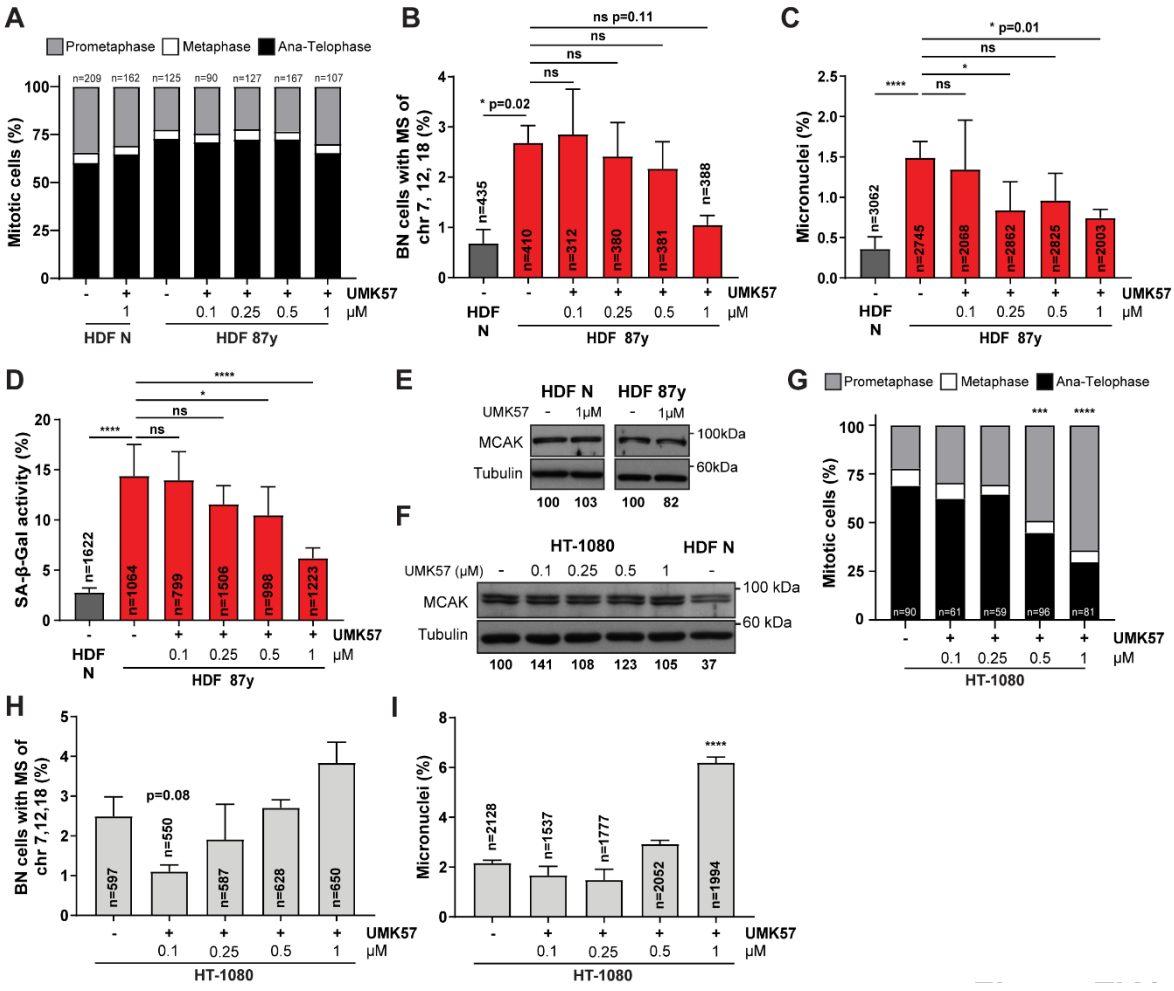
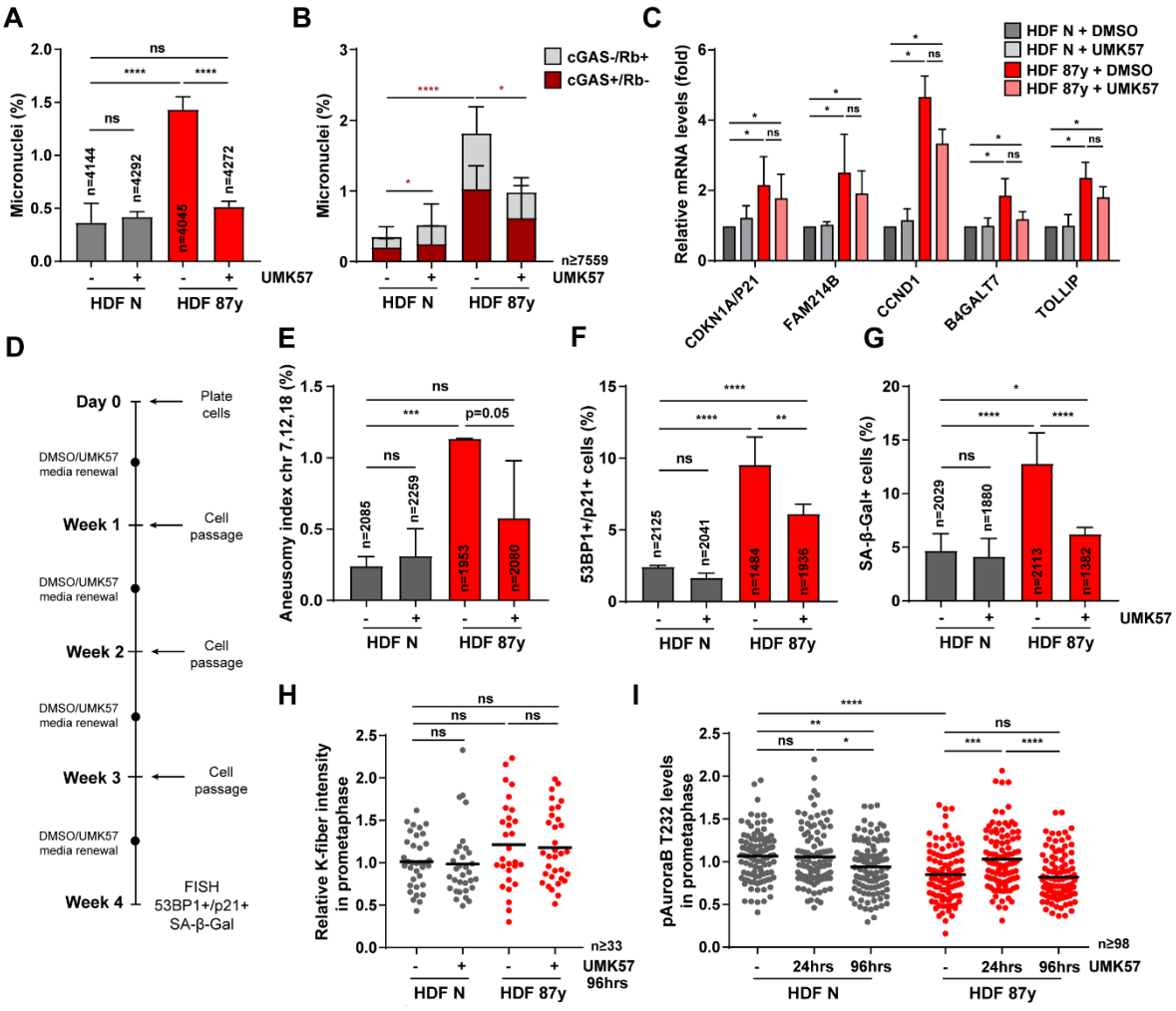


Figure II.EV4. UMK57 optimal concentration for suppressing CIN in dermal fibroblasts versus HT-1080 cancer cells (related to Fig II.4). (**A**) Percentage of n=mitotic cells of neonatal (N) and elderly (87 years) human dermal fibroblast (HDF) cultures in prometaphase, metaphase, or ana-telophase upon 24 h of exposure to DMSO (-) or increasing concentrations of UMK57 (+) as indicated; (**B**) Cytochalasin D-induced binucleated (BN) cells with mis-segregation (MS) of chromosomes 7, 12, and 18 in neonatal (***continues in the next page***)

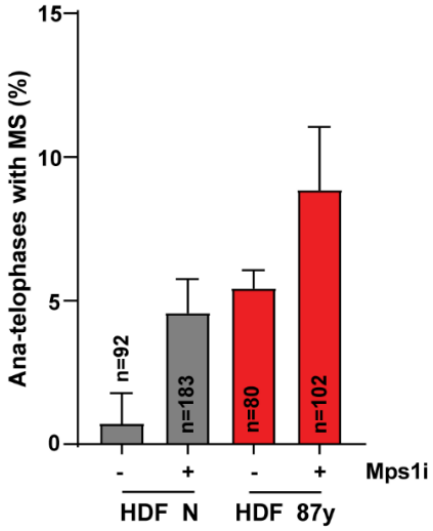
(HDF N) and elderly samples (HDF 87 years) after 24-h treatment with DMSO (-) or increasing concentrations of UMK57 (+) as indicated; (**C**) Percentage of micronuclei in neonatal (N) and elderly (87 years) n = cells treated with DMSO (-) or increasing concentrations of UMK57 (+) as indicated for 24 h; (**D**) Percentage of neonatal versus 87 years n = cells staining positive for SA-β-gal activity upon 24-h treatment with DMSO (-) or increasing concentrations of UMK57 (+) as indicated; (**E**) Western blot analysis of MCAK protein levels in mitotic extracts of neonatal (HDF N) and elderly (HDF 87 years) fibroblasts following treatment with DMSO (-) or 1 μM UMK57 (+). Tubulin is shown as loading control; (**F**) Western blot analysis of MCAK protein levels in mitotic extracts of HT-1080 cells or neonatal fibroblasts treated with DMSO (-) or increasing concentrations of UMK57 (+) for 24 h, as indicated. Tubulin is shown as loading control; (**G**) Percentage of n=HT-1080 mitotic cells in prometaphase, metaphase, or ana-telophase upon 24-h treatment with DMSO (-) or increasing concentrations of UMK57 (+) as indicated; (**H**) Percentage of binucleated (BN) HT-1080 cells with mis-segregation (MS) of chromosomes 7, 12, and 18 upon 24-h treatment with DMSO (-) or increasing concentrations of UMK57 (+) as indicated; (**I**) Percentage of micronuclei in n = HT-1080 cells following 24-h treatment with DMSO (-) or increasing concentrations of UMK57 (+) as indicated. *Data information*: All values shown are mean ± SD of at least two independent experiments. ns $P > 0.05$, *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$ by two-tailed chi-square test. Source data are available online for this figure.



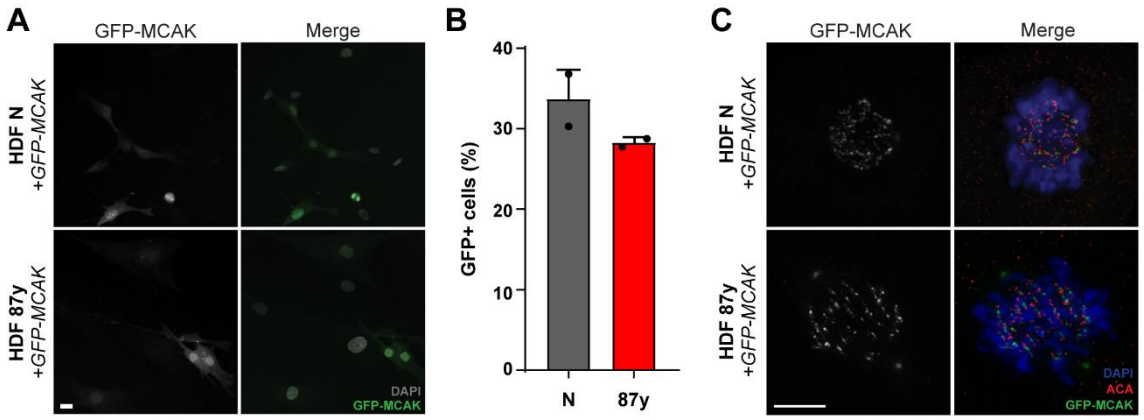
(***Figure II.EV5 legend in the next page***)

Figure II.EV5. Prolonged treatment with UMK57 inhibits CIN and senescence in cells from elderly donors (related to Fig II.5). (**A**) Percentage of micronuclei in n=cells of neonatal versus elderly samples after 96-h treatment; (**B**) Percentage of cGAS+/Rb- and cGAS-/Rb+ micronuclei in n=cells scored by immunofluorescence analysis upon 96-h treatment; (**C**) Relative CDKN1A/P21, FAM214B, CCND1, B4GALT7, and TOLLIP transcript levels in total RNA of neonatal (HDF N; n=5 replicates) and elderly (HDF 87 years; n=3 replicates) fibroblasts treated with DMSO or UMK for 96 h. TBP and HPRT1 were used as reference genes. All levels were normalized to DMSO-treated neonatal sample; (**D**) Experimental layout for prolonged exposure to UMK57 of neonatal (N) and elderly (87 years) fibroblast cultures, with cell passage every week and media renewal halfway each week. At week 4, chromosome segregation and senescence biomarkers were analyzed; (**E**) Aneusomy index of chromosomes 7, 12, and 18 measured by interphase FISH in n=cells; (**F**) Percentage of n=cells staining positive for double immunostaining of Cdkn1a/p21 (cell cycle inhibitor) and 53BP1 (≥ 1 foci; DNA damage) senescence biomarkers; (**G**) Percentage of n=cells staining positive for SA-β-gal activity; (**H**) Calcium-stable k-fiber intensity levels scored by immunofluorescence analysis of n≥33 tubulin-stained mitotic cells in prometaphase of neonatal and elderly samples treated with DMSO (-) and UMK57 (+) for 96 h. Levels were normalized to neonatal DMSO-treated condition; (**I**) Phospho-Aurora B Thr232 (pAuroraB T232) levels at kinetochores/centromeres of neonatal and elderly prometaphase cells treated with DMSO (-) or UMK57 (+) for 24 or 96 h. Intensity levels were normalized to ACA. *Data Information*: All values are mean ± SD of at least two independent experiments. ns *P > 0.05*, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by two-tailed (A, B, E–G) chi-square and (C, H, I) Mann–Whitney tests.

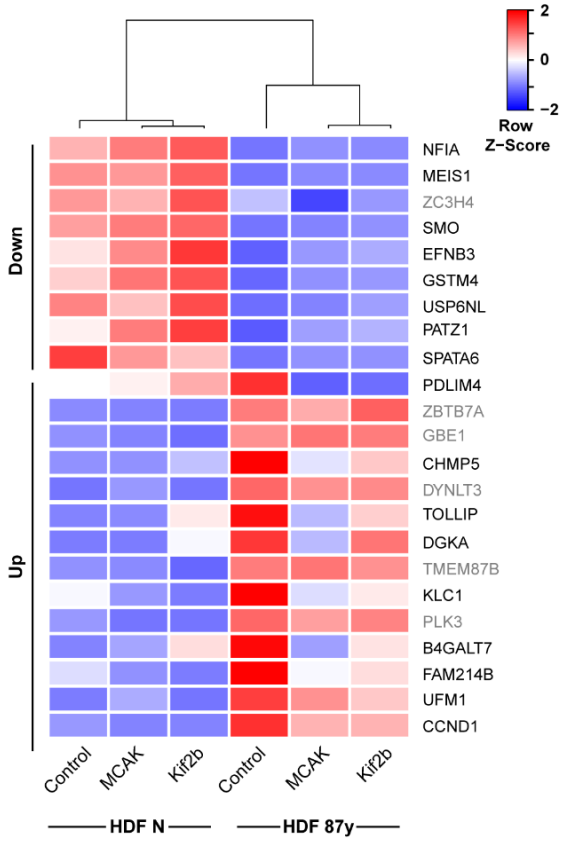
II.4.2. Appendix files



Appendix Figure II.S1 (related to Fig II.1). **Increased frequency of chromosome mis-segregation in elderly vs. neonatal cells following Mps1 partial inhibition.** Percentage of ana-telophases with chromosome mis-segregation (MS) in neonatal vs. elderly cells treated with DMSO (-) or 500 nM AZ3146/Mps1i (+) for 4 h as scored by Fluorescence *in situ* hybridization (FISH) using centromeric probes against chromosomes 7, 12 and 18. *Data information*: values shown are mean ± SD of at least two independent experiments.



Appendix Figure II.S2 (related to Fig II.2). **GFP-MCAK overexpression in young and elderly cells.** (**A, B**) Representative images (A) and percentages (B) of GFP-MCAK positive cells in total cell populations of neonatal (HDF N) or elderly (HDF 87y) after lentiviral transduction. Scale bar, 20 µm; (**C**) Representative images of mitotic cells overexpressing GFP-MCAK in neonatal and elderly cell populations transduced with lentiviruses. Scale bar, 5 µm. *Data Information*: Values shown in (B) are mean ± SD. of two independent experiments, which are indicated as dots.



Appendix Figure II.S3 (related to Fig II.3). **Kinesin-13 overexpression rescues senescence core gene expression signature in elderly fibroblasts.** Heatmap of differentially expressed senescence core signature genes (see Dataset EV3). Down and Up refer to the expected changes reported for senescent cells. Gene symbols highlighted in grey indicate genes whose expression remained unaltered following Kinesin-13 overexpression. Z-score row color intensities indicate higher (red) to lower (blue) expression.

Appendix Table II.S1. List of primers used for lentiviral plasmid assembly and quantitative PCR.

Gene/Fragment name	Gene Symbol	Forward primer (5' to 3')	Reverse primer (5' to 3')	Experiment
BamHI-GFP-MCAK-NotI	-	TATCGCGGATCCATGGTGAGCAAGGGCGAGGAG	TATATAGCGGCCGCTCACTGGGGCCGTTTCTTGCTG	LP
NotI-GFP-Kif2b-MluI	-	TATATAGCGGCCGCATGGTGAGCAAGGGCGAGGAG	TATACGACGCGTTCACTCTACCTTGCTCTTCA	LP
BamHI-EOS-α-tubulin-NotI	-	CGCCGCGGATCCATGAGTGCGATTAAGCCAGAC	TATATAGCGGCCGCTTAGTATTCCTCTCCTTCTTCC	LP
Aurora kinase B	*AURKB*	CGCAGAGAGATCGAAATCCAG	AGATCCTCCTCCGGTCATAAAA	qPCR
Beta-1,4-Galactosyltransferase 7	*B4GALT7*	CACTACCGGCTGTGCAATG	ACCCAGTTGTGATTCCCGAG	qPCR
Cyclin D1	*CCND1*	AGCTGTGCATCTACACCGAC	GAAATCGTGCGGGGTCATTG	qPCR
Cyclin-Dependent Kinase Inhibitor 1A	*CDKN1A/P21*	TGGACCTGGAGACTCTCAGG	CGGATTAGGGCTTCCTCTTGG	qPCR
Cyclic GMP-AMP synthase	*CGAS*	ACGTGCTGTGAAAACAAAGAAG	GTCCCACTGACTGTCTTGAGG	qPCR
Family With Sequence Similarity 214 Member B	*FAM214B*	CACCATCCAAGTGACCTTATTTAACC	AGAAGTCAAAGGTCACAAGGAACAT	qPCR
Hypoxanthine phosphoribosyltransferase 1	*HPRT1*	TGACCAGTCAACAGGqGGACA	CTGCATTGTTTTGCCAGTGTCAA	qPCR
kinesin family member 2C	*KIF2C/MCAK*	CTCAGTTCGGAGGAAATCATGTC	TGCTCTTCGATAGAATCAGTCAC	qPCR
NDC80 kinetochore complex component	*NDC80/HEC1*	CCTCTCCATGCAGGAGTTAAGA	GGTCTCGGGTCCTTGATTTTCT	qPCR
Polo like kinase 1	*PLK1*	AAAGAGATCCCGGAGGTCCTA	GGCTGCGGTGAATGGATATTTC	qPCR
TATA-box binding protein	*TBP*	GAGCCAAGAGTGAAGAACAGTC	GCTCCCCACCATATTCTGAATCT	qPCR
Toll Interacting Protein	*TOLLIP*	GACTTCCTCCGCATCACGC	CCAACTTTGCCTGTACCACC	qPCR

LP - lentiviral plasmid assembly; qPCR - quantitative PCR

Chapter – III

Modulation of genome instability and cellular senescence in cells with trisomy 21

The following chapter includes unpublished data:

Barroso-Vilares M, Silva R, Logarinho E (2021). *Modulation of genome instability and cellular senescence in cells with trisomy 21*. In preparation.

III.1. Introduction

Aneuploidy, an unbalanced number of chromosomes, is a major cause of birth defects and miscarriages in humans (Nagaoka *et al,* 2012). The constitutional presence of a supernumerary chromosome 21 (or trisomy 21), commonly known as Down syndrome (DS), remains the most common chromosomal condition occurring once in every 700 births (Bull, 2020; Mai *et al*, 2019). It is the only autosomal aneuploidy compatible with human survival post-term that allows patients to reach adulthood. However, it results in a collection of clinical symptoms including cognitive and developmental impairments, neurodegeneration, congenital heart defects and a predisposition for leukemias. Also, DS patients exhibit a variety of pathologies commonly associated with premature aging such as Alzheimer's disease, hearing loss and susceptibility to respiratory infections (Antonarakis *et al*, 2004; Antonarakis *et al*, 2020). Intriguingly, certain hallmarks of cellular aging are indeed recapitulated in DS and evidence points towards accelerated biological aging in DS patients (Gensous *et al*, 2020; Horvath *et al*, 2015; Patterson & Cabelof, 2012). Yet, the mechanisms by which trisomy of chromosome 21 fosters these features are poorly understood. It has been speculated that, in addition to the chromosome 21-specific gene imbalances, the sole disruption of cellular physiology due to the aneuploid status of the cell may participate in the development DS phenotypes (Antonarakis, 2017; Hwang *et al,* 2021). Amongst different organisms, aneuploidy elicits abnormalities in cell proliferation, metabolism, proteostasis and genome stability (Chunduri & Storchova, 2019; Zhu *et al,* 2018). It is acknowledged that metabolically altered cells with macromolecular damage that arrest in the cell cycle (or senescent cells) become accumulated with aging and play a role in the development of certain age-related conditions (van Deursen, 2014). This raises the question of whether the cellular distress imposed by the presence of an extra copy of chromosome 21 drives entry into senescence and by this way accelerates aging in DS patients. Advanced maternal age and a rise in life expectancy due to improved medical care has increased the population prevalence of DS (discussed in Antonarakis *et al,* 2020), which urges the need of research dedicated at enlighten the mechanisms that drive age-related phenotypes in DS patients.

Although senescence remains largely elusive in the context of DS (Amiel *et al*, 2013; Nawa *et al*, 2019), several recent studies point-out the senescence-inducing potential of the aneuploid status (Andriani *et al,* 2016; Giam *et al,* 2020; Joy *et al,* 2021; Santaguida *et al,* 2017). By using young to old-aged human dermal fibroblasts, we previously identified a dysfunction of the mitotic machinery with age that perturbs genome stability and contributes to the generation of fully senescent cells (Barroso-Vilares *et al*, 2020; Macedo *et al,* 2018).

Importantly, CIN and other types of genome instability, have been reported as prevalent in different trisomic cellular models (Blank *et al,* 2015; Hintzen *et al,* 2021; Nicholson *et al,* 2015; Passerini *et al,* 2016; Sheltzer *et al,* 2011). This raises the intriguing possibility that disruption of genome stability due to the aneuploid status of the cell may contribute to the generation of fully senescent cells in DS, the accelerated accumulation of which will foster a destructive pro-inflammatory environment in tissues and organs that contributes to premature aging and early onset of age-related pathophysiological features in DS patients.

Here we show that human dermal fibroblasts derived from children to young adults with DS have increased levels of senescence that do scale with age when compared to their healthy diploid counterparts. These increases in senescence markers are accompanied by a higher prevalence of DNA damage and CIN in cells with trisomy 21. Remarkably, small-molecule inhibition of DNA damage and chromosome segregation defects in cells from DS patients translated into a reduction in cellular senescence. Consequently, we found that maintenance of genome stability represents a potential senomorphic approach by delaying senescence in cells with trisomy 21, which opens new avenues for future studies focused on healthspan extension in DS.

III.2. Materials and Methods

Cell culture

Human fibroblasts retrieved from skin samples of young (1-8y) to young adult (13-27y) and elderly (75/87y) caucasian males and females with or without trisomy of chromosome 21 (all from the cell repository of Coriell Institute for Medical Research) were grown in minimal essential medium Eagle–Earle (MEM) with L-glutamine (10-010-CV, Corning) supplemented with 15% fetal bovine serum (FBS) and 1x antibiotic-antimycotic (both from Gibco, Thermo Fisher Scientific). All experiments were conducted with dividing fibroblasts passaged no more than 5 times following purchase. All details about the human dermal fibroblast cultures used in this study are listed in Table III.S1 and the confirmation of diploidy vs. trisomy 21 shown in Fig III.S1.

Drug treatments

Cytochalasin D (C8273, Sigma-Aldrich) was used at 1µM for 24 h to block cytokinesis. Unless otherwise stated, UMK57 (AOB8668, AOBIOUS INC) was used at 1µM to potentiate the activity of Kif2C for 96 h.

cGAS siRNA knockdown

Cells were plated and after 24 h transfected with siRNA oligonucleotides targeting cGAS (Dharmacon, M-015607-01-0005, siGENOME 115004) at a final concentration of 25nM. Transfections were performed using Lipofectamine RNAiMAX in Opti-MEM medium (both from Thermo Fisher Scientific) according to the manufacturer's instructions. Transfection medium was replaced with complete medium after 6 h. All experiments were performed 72 h post-transfection, and protein depletion confirmed by western blot and immunostaining analyses.

Fluorescence *in situ* hybridization (FISH)

FISH was used to confirm the supernumerary presence of chromosome 21 on interphase cells (Fig III.S1) and to measure chromosome mis-segregation rates on cytochalasin D-blocked binucleated cells (Fig III.2). For all experiments, fibroblasts were grown on Superfrost™ Plus microscope slides (Menzel, Thermo Fisher Scientific) placed in quadriperm dishes (Sarsted). For the cytokinesis-block assay, cells were treated with 1µM Cytochalasin D for 24 h prior fixation. Prior to being processed for FISH staining, interphase or cytokinesis-blocked cells were fixed accordingly to the protocol from Macedo *et al.* (2018). Diploidy or trisomy of chromosome 21 were validated using the locus-specific XA 13/21 probe (MetaSystems) and BN cells were hybridized with Vysis centromeric probes CEP7 Spectrum Aqua, CEP12 Spectrum Green, and CEP18 Spectrum Orange (all from Abbott Laboratories) according to manufacturer's instructions. DNA was counterstained with 0.5µg/ml 4',6-Diamidino-2-Phenylindole (DAPI) and microscope slides were then mounted with coverslips in anti-fading medium (90% glycerol, 0.5% N-propyl gallate, 20mM Tris pH=8.0).

Senescence-associated-β-galactosidase (SA-β-gal) assay

For SA-β-gal assay, cell cultures were incubated in medium with 100nM Bafilomycin A1 (B1793, Sigma-Aldrich) to induce lysosomal alkalinization, followed by addition of the fluorogenic substrate of β-galactosidase DDAO galactoside (10µM; Setareh Biotech LLC) and the fixation with 4% paraformaldehyde as in Macedo *et al.* (2018). 0.5µg/ml of DAPI (Sigma-Aldrich) were used to counterstain DNA and coverslips were then mounted on slides.

Calcium-stable k-fiber analysis

Fibroblasts were grown on sterilized glass coverslips coated with 50µg/ml fibronectin (F1141, Sigma-Aldrich). Then cells were incubated in Calcium buffer (100mM PIPES, 1mM $MgCl_2$, 1mM $CaCl_2$, 0.5% Triton-X100, pH=6.8) and fixed with 4% paraformaldehyde/0.25% glutaraldehyde as in Barroso-Vilares *et al.* (2020). Anti-α-tubulin (1:1500; T5168, Sigma-

Aldrich) and the AlexaFluor-568 (1:1500; Life Technologies) were used to label calcium-stabilized k-fibers. DNA was counterstained with 0.5µg/ml DAPI (Sigma-Aldrich) and coverslips mounted on slides.

Immunofluorescence

Fibroblasts grown on sterilized glass coverslips coated with 50µg/ml fibronectin (F1141, Sigma-Aldrich) or 96-well µ-plates (ibidi) were fixed with 4% paraformaldehyde in PBS for 20 min or ice-cold Methanol for 5 min. Next, cells were rinsed in PBS and permeabilized in PBS + 0.3% Triton-X100 for 7 min. Following fixation and permeabilization, cells were washed in PBS + 0.05% Tween-20 (PBS-T) 3 times and blocked in 10% FBS + PBS-T for 1 h. Both, primary and secondary antibodies were diluted in 5% FBS + PBS-T as follows. Primary antibodies: rabbit anti-53BP1 (4937, Cell Signaling Technology), 1:100; mouse anti-p21 (SC-6246, Santa Cruz Biotechnology), 1:500; rabbit anti-cGAS (15102, Cell Signaling Technology), 1:200; human anti-centromere antibody (ACA; kindly provided by Dr. W. C. Earnshaw), 1:3000; mouse anti-Ki-67 (9449, Cell Signaling Technology), 1:1500; mouse anti-lamin B1 (68591, Cell Signaling Technology), 1:100; mouse anti-γH2AX (05-636, Milipore), 1:1000); mouse anti-Retinoblastoma (554136, BD Biosciences), 1:100; mouse anti-α-tubulin (T5168, Sigma-Aldrich), 1:1500. Secondary antibodies: AlexaFluor-488, -568 and -647 (Life Technologies), all 1:1500. Cells were finally washed, DNA counterstained with 0.5µg/ml DAPI (Sigma-Aldrich) and coverslips mounted on slides with anti-fading medium.

Fluorescence microscopy

Cells with calcium-stabilized k-fibers, immunostained for Lamin B1 or hybridized with XA 12/21 FISH probe were imaged using a Zeiss AxioImager Z1 (Carl Zeiss, Oberkochen, Germany) motorized upright epifluorescence microscope, equipped with an Axiocam MR camera and operated by the Zeiss Axiovision v4.7 software. Z-stacks (0.24 µm) covering the entire volume of individual mitotic cells or interphase cells were collected using a PlanApo 63x/1.40 NA objective or an EC-Plan-Neofluar 40x/1.30 objective, respectively. Image deconvolution was performed with the AutoQuant X2 software (Media Cybernetics).

Automated microscopy

For stainings of 53BP1/p21, α-tubulin (cell size), γ-H2AX, ACA (micronuclei), cGAS/Rb, Ki-67, and SA-β-gal, as well as FISH on binucleated cells, images were captured with the IN Cell Analyzer 2000 (GE Healthcare, UK) equipped with a Photometrics CoolSNAP K4 camera and driven by the GE IN Cell Analyzer 2000 v5.2 software. All immunostainings were imaged using the Nikon 40x/0.95 NA Plan Fluor objective, except for 53BP1/p21 and α-tubulin stainings that were imaged with the Nikon 20x/0.45 NA Plan Fluor objective.

Image analysis

All phenotypes were blindly analyzed and, except for cell size measurements, ImageJ/Fiji software was used. Fluorescence intensity thresholds were set and used consistently for all samples within each experiment to analyze 53BP1/p21, γ-H2AX, Ki-67, and SA-β-galactosidase immunostainings. For 53BP1 and γ-H2AX markers, cells displaying ≥1 foci were considered as positive for DNA damage. For SA-β-galactosidase activity, only cells showing >5 fluorescent granules were scored as SA-β-gal-positive. To score MN frequencies, only interphase cells with DNA aggregates separate from the primary nucleus were considered, while interphase cells with an apoptotic appearance were excluded. DNA aggregates co-localizing with ACA, cGAS or Retinoblastoma (Rb) were then classified as being ACA-, cGAS- and/or Rb-positive, respectively. MN were considered as disrupted when positive for cGAS but not Rb, and intact when positive for Rb but not cGAS. For analysis of calcium-stable k-fibers, α-tubulin intensity levels were normalized for the mitotic spindle area of each individual cell and background-corrected. For cell size analysis, first the interactive learning and segmentation toolkit Ilastik v1.3.3 was used to generate probability maps based on a training set of images to discern the object (nuclei/cytoplasm) more accurately from background noise. Then, the morphometric parameter 'cell area' was quantified based on the generated probability maps and α-tubulin/DAPI images using CellProfiler v3.1.8.

Protein detection by western blot

Total protein was extracted from cell pellets resuspended in RIPA buffer (89900, Thermo Fisher Scientific) with protease inhibitor cocktail (87786, Thermo Fisher Scientific) and phosphatase inhibitor cocktail (78420, Thermo Fisher Scientific). Protein content was determined using the Lowry Method (DC™ Protein Assay, Bio-Rad) according to the manufacturer's instructions. 20µg of total protein extract were then resolved on SDS-PAGE gels (10% or 15%) and transferred onto nitrocellulose membranes for western blot analysis. Non-specific sites were blocked with TBS-T (50mM Tris-HCl, pH=7.4, 150mM NaCl, 0.05% Tween-20) supplemented with 5% non-fat dry milk for 1 h. Both, primary and secondary antibodies were diluted in TBS-T containing 2% non-fat milk as follows. Primary antibodies: mouse anti-α-tubulin (T5168, Sigma-Aldrich), 1:100,000; rabbit anti-cGAS (15102, Cell Signaling Technology), 1:500; mouse anti-KIF2C (sc-81305, Santa Cruz Biotechnology), 1:200; rabbit anti-γH2AX (9718, Cell Signaling Technology), 1:1000. Secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-rabbit (111-035-003, Jackson ImmunoResearch) and goat anti-mouse (115-035-003, Jackson ImmunoResearch), both at 1:5000. HRP conjugates were detected using Clarity Western ECL Substrate reagent (Bio-Rad Laboratories) according to manufacturer's instructions. For quantitative analysis of

protein levels, a GS-800 calibrated densitometer operated by the Quantity one I-D Analysis Software v4.6 (Bio-Rad Laboratories) was used.

Statistical analysis

All experiments were repeated at least three times unless otherwise stated. Sample sizes and statistical tests used for each experiment are indicated in the respective Figure captions. Data are shown as mean or mean ± SD as indicated. GraphPad Prism version 8.0.2 was used to analyze all the data. Data were tested for parametric vs. non-parametric distribution using D'Agostino-Pearson omnibus normality test. Two-tailed unpaired t-test, Mann-Whitney or chi-square tests were then applied accordingly to compare pairs of conditions. For multiple comparisons, two-tailed one-way ANOVA or Kruskal-Wallis tests were applied accordingly. Statistical differences between different groups are then indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, and NS not significant $p>0.05$.

III.3. Results

Cellular senescence phenotype in cells with trisomy 21

Cellular senescence terms a non-proliferating state into which cells enter in the presence of sustained stress and unrepairable damage (Hernandez-Segura *et al,* 2017; Rodier & Campisi, 2011). Even though random and/or defined chromosomal imbalances were acknowledged to drive cellular senescence (Andriani *et al,* 2016; Giam *et al,* 2020; Joy *et al,* 2021; Santaguida *et al,* 2017), a systematic analysis of cellular senescence in the context of trisomy 21 (or DS) remains elusive. Thus, we set out to evaluate the senescence phenotype of cells with trisomy 21 and determine whether senescence accrues faster in comparison with normative aging. To this end, we established a human cellular model of trisomy 21 comprising a set of dermal fibroblast cultures with constitutional trisomy 21 (T21) derived from young (1-8y) to young-adult (13-27y) female and male donors, as well as age- and gender-matched diploid (H) counterparts, with 75/87y-old dermal fibroblasts being included as positive control for senescence (Table III.S1). Analysis of interphase nuclei by fluorescence *in situ* hybridization (FISH) with probes specific for chromosomes 13 and 21 showed that T21 fibroblast cultures used in this study were highly homogenous (> 89.9 ± 1.7 %) for the presence of an additional chromosome 21 (Fig III.S1). To evaluate the cellular senescence phenotype of cells with trisomy 21, we analyzed a set of well-established and commonly used markers (Gorgoulis *et al,* 2019; Hernandez-Segura *et al,* 2017). The number of cells exhibiting senescence biomarkers was significantly increased in T21 fibroblasts when compared with their diploid counterparts (Fig III.1).

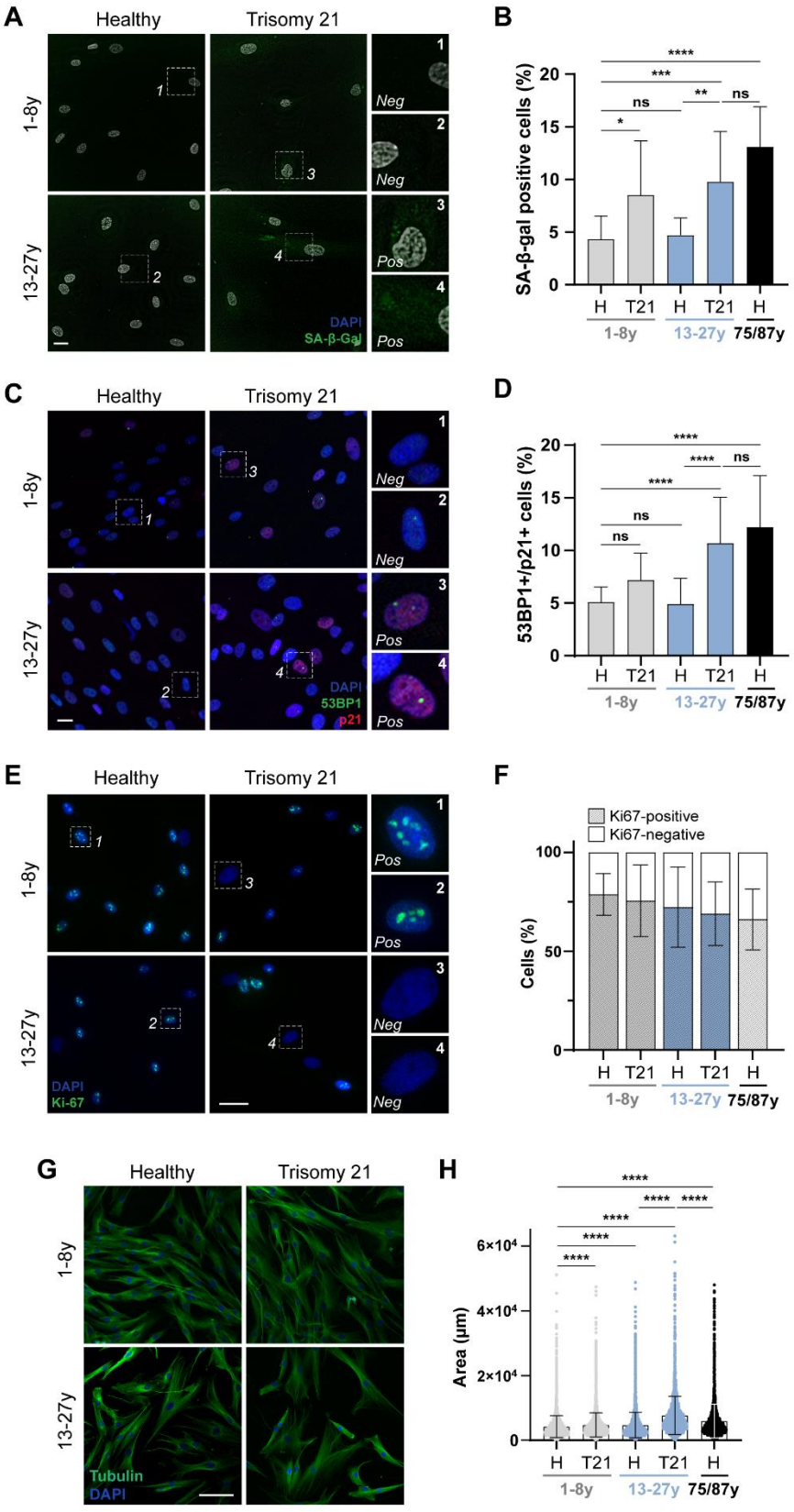


Figure III.1. Cellular senescence phenotype in cells with trisomy 21. (**A, B**) Representative images (A) and quantification (B) of SA-β-galactosidase (SA-β-gal) activity (green) in fibroblast cultures from healthy (H) and trisomy 21 (T21) donors. DNA is shown in grey. Insets show cells scored as SA-β-gal-negative (Neg) or -positive (Pos). Scale bar 20 µm; (**C**) Representative images of fibroblasts from healthy (***continues in the next page***)

and trisomy 21 donors immunostained for Cdkn1a/p21 (red; cell cycle inhibitor) and 53BP1 (green; DNA damage) senescence biomarkers. DNA is shown in blue. Insets show cells scored as negative (*Neg*) and positive (*Pos*) for the indicated markers. Scale bar 20 µm; (**D**) Frequencies of cells staining double-positive for Cdkn1a/p21 and 53BP1 (≥1 foci); (**E, F**) Representative images (E) and quantification (F) of cells immunostained for Ki-67 (green) in H vs. T21 fibroblast cultures. DNA is shown in blue. Insets show cells scored as negative (*Neg*) and positive (*Pos*) for Ki-67. Scale bar 40 µm; (**G, H**) Representative images (G) and cell size measurements (H) scored in fibroblasts from H vs. T21 cultures immunolabeled for tubulin (green). DNA is shown in blue. Scale bar 100 µm. *Data Information*: (B) n>3700, (D) n>3800, (F) n>3600 and (H) n>4800 cells were analyzed for each condition and values shown are mean ± SD of at least three independent experiments. ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by two-tailed (B) non-parametric Kruskal-Wallis; or (D) parametric one-way ANOVA test.

In fact, senescence-associated-β-galactosidase (SA-β-gal) activity is higher in T21 and, in agreement with accelerated aging, the levels of SA-β-gal scale in 13-27y vs. 1-8y cultures to levels similar to those detected in 75/87y-old fibroblasts (Fig III.1A and B). Similar results were observed for the combined analysis of the cell cycle inhibitor p21 and the DNA damage marker 53BP1 (Fig III.1C and D). Also, although the impact of an additional chromosome on proliferation is modest with fibroblasts retaining a fairly good proliferative capacity, T21 fibroblast cultures exhibit an age-dependent decline in Ki-67 positivity (Fig III.1E and F). This matches the previously mentioned markers and is also congruent with increases in cell size observed in T21 vs. H fibroblasts (Fig III.1G and H). Taken together, our results show that the constitutional presence of an additional chromosome 21 correlates with an increase in senescence phenotypes that aggravate with age, suggesting that cellular senescence accumulates faster in DS when compared to healthy normative aging, and thus matches with the early onset of aging phenotypes in these patients.

Loss of genome stability in cells with trisomy 21

The changes in cellular physiology induced by the aneuploid status overlap in part with molecular and cellular features that characterize the aging process (Lopez-Otin *et al,* 2013). Intriguingly, it has been proposed that aging hallmarks influence chromosome segregation fidelity (Macedo *et al,* 2017), and it was shown that chromosome mis-segregation can drive elderly cells into full senescence (Barroso-Vilares *et al,* 2020; Macedo *et al,* 2018). Thus, loss of chromosomal stability may possibly contribute to cellular senescence development in cells with trisomy 21. In fact, single-chromosome imbalances have been demonstrated to affect not only segregation but also duplication of the genetic material, thereby increasing the tendency of genome alterations over cell divisions (or genomic instability) (Blank *et al,* 2015; Hintzen *et al,* 2021; Nicholson *et al,* 2015; Passerini *et al,* 2016; Sheltzer *et al,* 2011). To confirm such genome instability (GIN) phenotype in

cells derived from DS patients, we started by investigating the effect of constitutional aneuploidy of chromosome 21 on chromosome segregation. FISH staining with probes specific for chromosomes 7, 12, and 18 on cytokinesis-blocked binucleated (BN) cells revealed that chromosome mis-segregation (MS) rates are increased in cells with trisomy 21, with the difference being higher in the 13-27y (2.62 ± 1.22 % vs. 0.26 ± 0.55 %) than in the 1-8y (1.53 ± 0.67 % vs. 0.28 ± 0.34 %) age group (Fig III.2A and B). Furthermore, we found that micronuclei (MN) levels increase in T21 vs. H, following a similar trend as MS rates (Fig III.2C and D). MN represent an exquisite biomarker of chromosomal damage and instability, and they can originate during anaphase from lagging acentric chromosome fragments or intact chromosomes that become excluded from the daughter nuclei during mitosis (Fenech *et al*, 2011; Fenech *et al,* 2020). Notably, immunofluorescence analysis with anti-centromere antibody (ACA) shows that only half of the detected MN originate from whole-chromosome mis-segregation events (ACA+) (Fig III.S2A and B). This means that the other half contains acentric fragments (ACA-), which suggests that cells are undergoing DNA damage and further supports that DS patient-derived fibroblast cultures experience genome instability. Accordingly, we observed that the DNA double-strand break marker γ-H2AX accumulates in T21 with the difference being most marked in 13-27y fibroblast cultures (Figs III.2E and F, and III.S2C). Altogether, our findings indicate a GIN phenotype in cells with trisomy 21 and that there is a time-dependent yet accelerated degeneration in the proficiency of DNA repair and chromosome segregation mechanisms in DS.

Cellular senescence phenotype in cells with trisomy 21 is cGAS-dependent

The senescence-associated secretory phenotype (SASP) is probably the most prominent feature of senescent cells, comprising amongst other factors cytokines, chemokines, and metalloproteinases. These factors retain the ability to reinforce the senescent state (cell-autonomous), but can also influence the surrounding environment and neighbor cells (non-cell autonomous) (Acosta *et al,* 2013; Kuilman & Peeper, 2009). Importantly, SASP development relies on the innate immune signaling pathway cGAS-STING which recognizes and becomes activated in response to cytoplasmic chromatin fragments (CCFs) (Dou *et al,* 2017; Gluck *et al,* 2017; Han *et al,* 2020; Yang *et al,* 2017). CCFs accumulate due to changes in nuclear envelope (NE) composition and integrity (Dou *et al,* 2015; Ivanov *et al,* 2013), but recent findings demonstrate that the cytoplasmic DNA pool can also be fueled by replication stress, DNA damage and chromosome segregation errors (Fenech *et al,* 2020; Galluzzi *et al,* 2018). Interestingly, in agreement with Hwang *et al.* (2019), we found that the percentage of cells exhibiting nuclear atypia is significantly increased in DS-derived fibroblast cultures (Fig III.S3A). Based on this and the above-

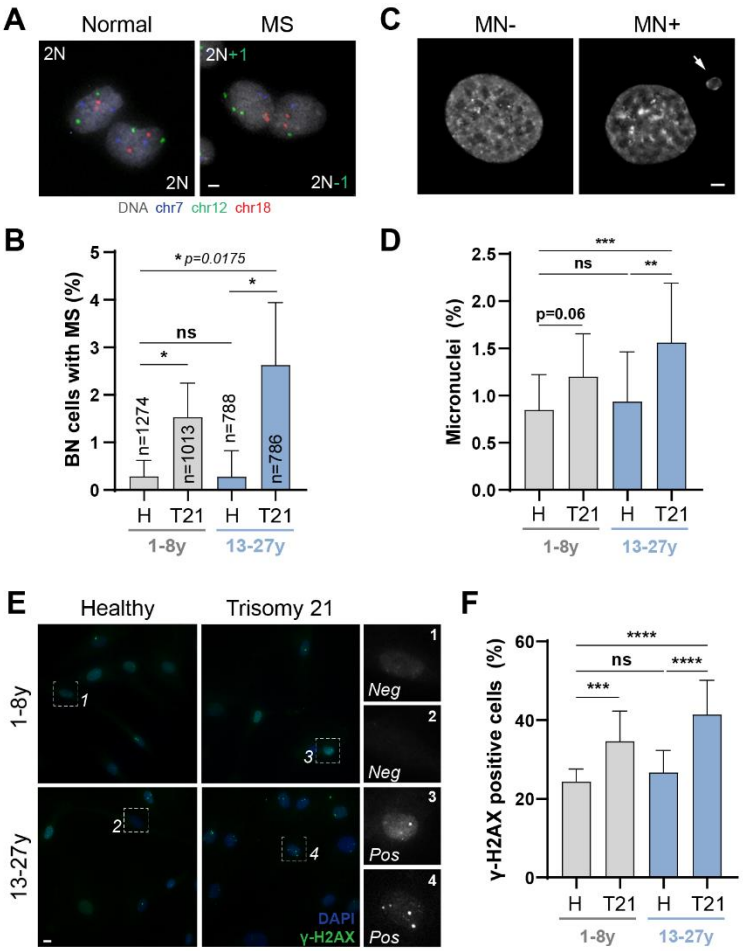


Figure III.2. Loss of genome stability in cells with trisomy 21. (**A**) FISH staining in cytochalasin-D-blocked binucleated (BN) cells with centromeric probes specific for chromosomes 7 (blue), 12 (green), and 18 (red). DNA is shown in grey. Scale bar 10 µm; (**B**) Percentage of BN cells with chromosome mis-segregation (MS) in healthy (H) and trisomy 21 (T21) donors. Sample size (n) for each condition is indicated in the graph; (**C**) Representative images of micronuclei-negative (MN-) vs. micronuclei-positive (MN+) cells. DNA is shown in grey. Arrows point to MN. Scale bar 5 µm; (**D**) Frequencies of MN+ cells scored in 4',6'-diamino-2-fenil-indol (DAPI)-stained fibroblast cultures from H vs. T21 donors; (**E, F**) Representative images (E) and frequencies (F) of fibroblasts from healthy and trisomy 21 donors staining positive for immunostaining of Phospho-Histone H2A.X (Ser139) (γ-H2AX; red) DNA damage biomarker (≥1 foci). DNA is shown in blue. Insets show cells scored as negative (*Neg*) and positive (*Pos*) for γ-H2AX staining. Scale bar 10 µm. *Data Information*: (D) n>9400 and (F) n>6600 cells were analyzed for each condition. Values shown are mean ± SD of at least three independent experiments. ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by two-tailed (B) chi-square test, (D) non-parametric Kruskal-Wallis; or (F) one-way ANOVA test.

described genome instability phenotype, we reasoned that cellular senescence in cells with trisomy 21 may depend on cytoplasmic DNA recognition by cGAS. Even though there is no significant change in total levels of the cytosolic DNA sensor cGAS (Fig III.S3B), we observed an increase in the number of ruptured MN exposed to cGAS (cGAS+/Rb-) in T21 vs. H (Fig III.3A and B). In fact, as a result of improper NE assembly and recruitment of DNA replication/repair machineries (Crasta *et al,* 2012; Liu *et al,* 2018; Maass *et al,* 2018),

MN are particularly liable to rupture and massive DNA damage (Hatch *et al,* 2013; Zhang *et al,* 2015), exposing MN chromatin to cGAS (Harding *et al,* 2017; Mackenzie *et al,* 2017). To further investigate the potential role of cGAS in trisomy 21-mediated cellular senescence, we depleted cGAS using RNAi (Fig III.3C and D). Remarkably, previously analyzed senescence-associated phenotypes become significantly decreased in all T21 fibroblast cultures following cGAS depletion, while as expected there is no effect on their diploid counterparts (Fig III.3E and F). Consequently, our findings so far suggest that genome instability may be contributing to cellular senescence development in cells with trisomy 21 via cGAS-STING pathway engagement.

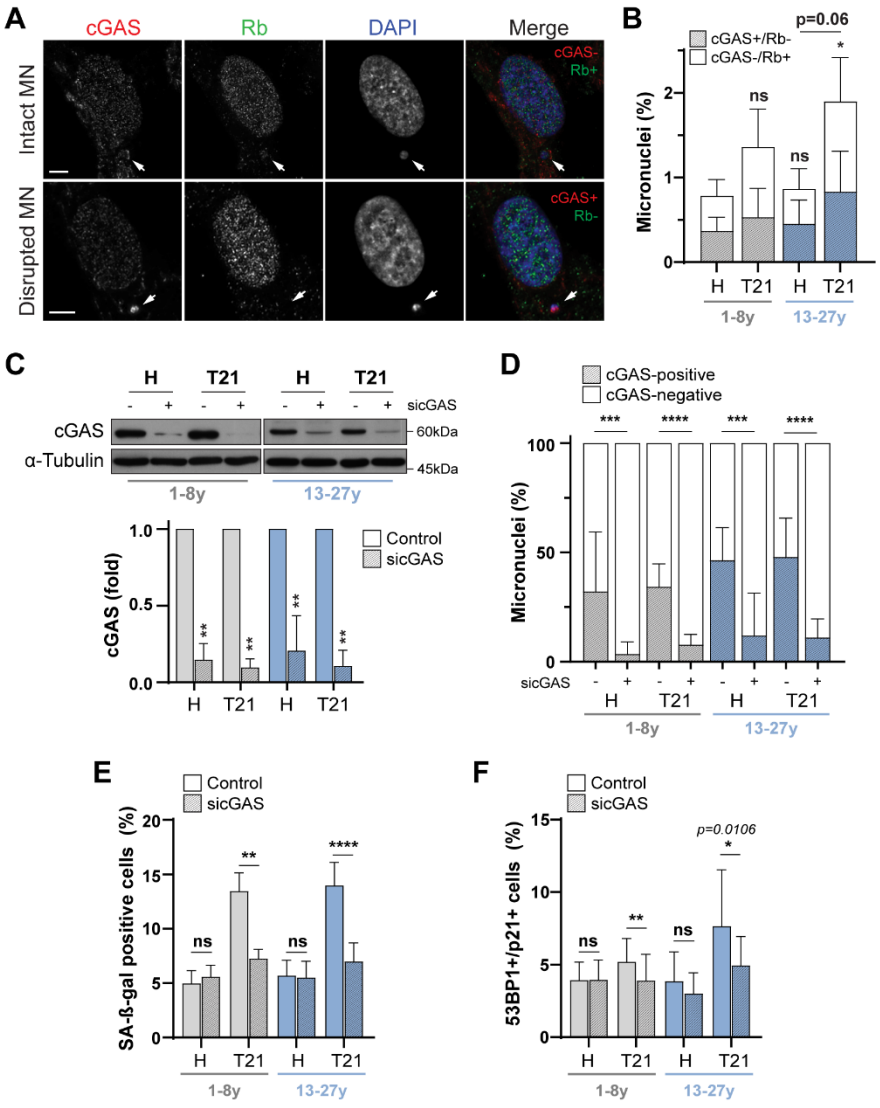


Figure III.3. cGAS engagement in cellular senescence phenotype of cells with trisomy 21. (**A**) Representative images of cells with an intact (cGAS-/Rb+) or a disrupted (cGAS+/Rb-) micronucleus (MN) upon immunostaining of cGAS (red) and Retinoblastoma (Rb; green) in fibroblast cultures from healthy (H) and trisomy 21 (T21) donors. Arrows indicate micronuclei. Scale bar 10µm; (**B**) Frequencies of disrupted (cGAS+/Rb-) micronuclei; (**C**) cGAS protein levels following RNAi-mediated depletion (sicGAS). GAPDH is shown as loading control; (**D**) Quantification of cGAS-positive micronuclei in H (***continues in the next page***)

vs. T21 fibroblast cultures immunostained for cGAS and Rb following depletion of cGAS; (**E**) Percentage of cells with detectable SA-β-galactosidase (SA-β-gal) activity after RNAi-mediated depletion of cGAS; (**F**) Frequencies of cells staining double-positive for cell cycle inhibitor Cdkn1a/p21 and DNA damage marker 53BP1 (≥1 foci) following sicGAS depletion. *Data Information*: (B) n>5500 cells, (D) n>5300, (E) n>2400 cells, and (F) n>6100 cells. All values shown are mean ± SD of at least three independent experiments. ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by two tailed (B,D) chi-square; or (E,F) non-paired Mann-Whitney test.

Genomic instability in cells with trisomy 21 fosters the cellular senescence phenotype

Kif2C, also known as Mitotic Centromere Associated Kinesin (or MCAK), is a microtubule (MT) depolymerizing kinesin acknowledged to intervene in MT dynamics by destabilizing overstable improper kinetochore-microtubule (k-MT) interactions to prevent chromosome mis-segregation (Kline-Smith *et al*, 2004; Manning *et al,* 2007). In fact, a small-molecule agonist of Kif2C – UMK57 – has been shown to suppress chromosomal instability (Barroso-Vilares *et al,* 2020; Orr *et al,* 2016). However, Kif2C was recently also established as a new factor involved in DNA double strand break (DSB) repair, as it facilitates DSB mobility and participates in the formation, fusion, and resolution of DNA damage foci (Macedo *et al,* 2021; Zhu *et al*, 2020). Since we found a genome instability phenotype in DS patient-derived fibroblasts that may be contributing to cellular senescence development, we reasoned that targeting Kif2C with UMK57 could provide a means to further validate our hypothesis. Indeed, we find that total levels of Kif2C are steadily decreasing in cells with trisomy 21 in an age-dependent manner (Fig III.S4A). Consistent with the MT destabilizing function of Kif2C, calcium-induced depolymerization of non-kinetochore microtubules (Kollu *et al,* 2009) revealed changes in k-fiber intensity that indicate k-MT hyperstability in cells with trisomy 21 both during prometaphase and metaphase (Fig III.S4B). Thus, the observed changes in Kif2C may be contributing to trisomy 21-induced GIN. Treatment of all fibroblast cultures for 96 h with the by us previously established optimal dose of UMK57 did not overtly change total levels of Kif2C (Fig III.4A) (Barroso-Vilares *et al,* 2020). However, as expected, genome stability in cells with trisomy 21 was significantly improved (Fig III.4B and C), with a slight reduction in the burden of MN chromatin accessible to cGAS (Fig III.4D). Remarkably, although the impact on cell size and proliferative capacity was trivial (Fig III.S5B and C), counteracting genome instability translated into a significant decrease in SA-β-gal activity and double-positivity of 53BP1 and p21 (Fig III.4E and F). Together, these findings support a role for DS-induced GIN in cellular senescence development and suggest that maintenance of genome stability may

offer a potential senomorphic approach to be considered for healthspan extension in the context of DS.

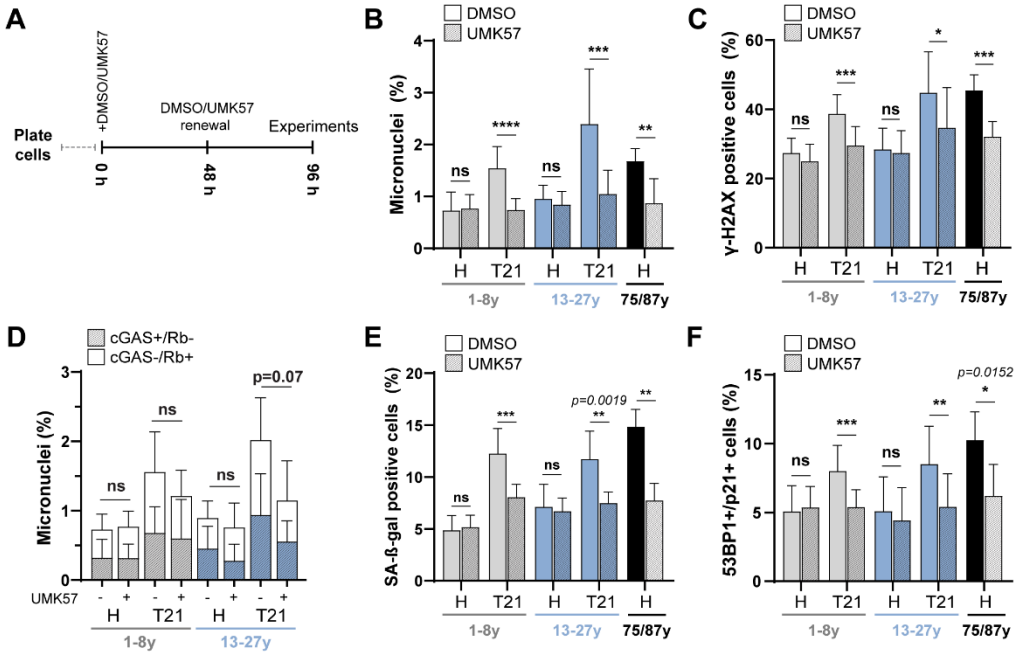


Figure III.4. Small-molecule modulation of genome instability counteracts the accumulation of cellular senescence in cells with trisomy 21. (**A**) Fibroblast cultures from healthy (H) and trisomy 21 (T21) donors were plated, and after 24 h DMSO/UMK57 was added to the culture medium. Halfway through the experiment (48 h) media containing DMSO/UMK57 was renewed. All analyses were performed after a total of 96 h of exposure to DMSO/UMK57; (**B**) Percentages of micronucleated cells scored in DAPI-stained fibroblast cultures from healthy and trisomy 21 donors following exposure to UMK57; (**C**) Frequencies of cells staining positive for γ-H2AX (≥1 foci) after UMK57 treatment; (**D**) Quantification of cGAS-positive micronuclei in UMK57-treated healthy and trisomy 21 fibroblast cultures immunostained for cGAS and Rb; (**E**) Percentages of cells scored as positive for SA-β-galactosidase (SA-β-gal) activity following UMK57 treatment; (**F**) Percentages of cells staining double-positive for cell cycle inhibitor Cdkn1a/p21 and DNA damage marker 53BP1 (≥1 foci) following treatment with UMK57. *Data Information*: (B) n>7100, (C) n>5600, (D) n>4900, (E) n>3300, and (F) n>5800 cells were analyzed for each condition. Values shown are mean ± SD of at least three independent experiments. ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<$ by two-tailed (B,D,F) non-parametric Mann-Whitney test; (C) parametric t-test; or (D) chi-square test.

III.4. Discussion

Although the supernumerary presence of chromosome 21 is acknowledged as the underlying cause of Down syndrome, how altered gene expression and downstream molecular events lead to the onset of the typical symptoms in DS remains poorly defined. Our findings support a model in which the trisomy 21-induced genome instability phenotype contributes to a faster accrual of cellular senescence in DS when compared to the normative

aging process. This likely encourages the early onset of clinical symptoms of DS generally present only later in life or in premature aging conditions, as it fuels a destructive pro-inflammatory environment that compromises tissue homeostasis and function (Fig III.5).

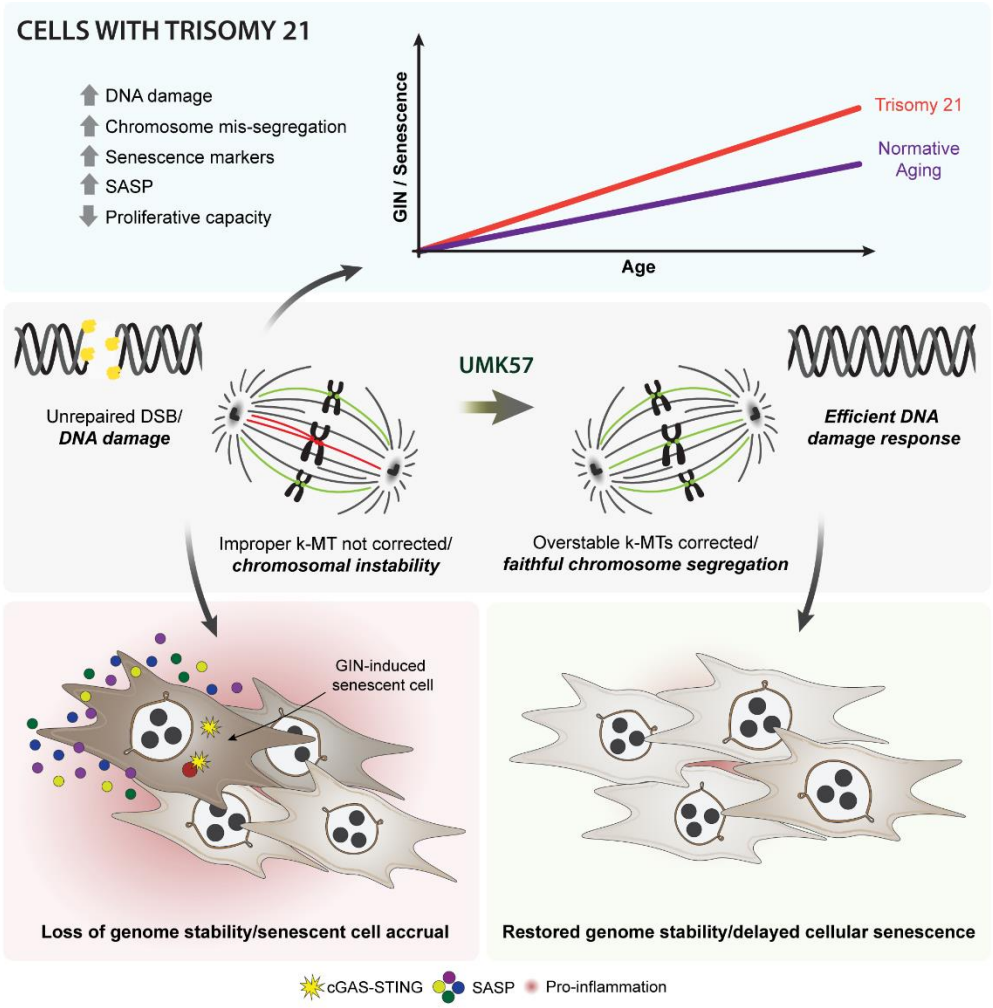


Figure III.5. Trisomy 21-driven genome instability triggers transition into cellular senescence. The constitutional presence of a supernumerary chromosome 21 compromises the DNA damage repair and chromosome segregation proficiency of the cell. The resulting genome instability (GIN) phenotype of cells with trisomy 21 then accelerates the accumulation of cellular senescence when compared to the normative aging process. The small-molecule inhibition of trisomy 21-associated GIN via modulation of DNA damage repair and chromosome segregation acts to prevent the accrual of cellular senescence. Thus, the accumulation of GIN-driven senescent cells likely fuels a destructive pro-inflammatory environment that compromises tissue homeostasis and contributes to the accelerated aging phenotypes in DS.

It is widely accepted that single-chromosome imbalances compromise duplication and segregation of the genome during cell division (Passerini & Storchova, 2016), yet the mechanistic link remains vague. In addition to GIN, aneuploidy also induces abnormalities in metabolism and proteostasis (Chunduri & Storchova, 2019; Zhu *et al,* 2018). We reason that the GIN phenotype in cells with trisomy 21 is being fueled by this aneuploidy-induced changes in cellular physiology. For example, DNA repair factors can be sequestered by

existing protein aggregates, compromising by this way their function (Gao *et al*, 2015; Pfeiffer *et al*, 2017). Loss of proteostasis has also been suggested to impact chromosome segregation, as chaperones and protein degradation pathways are involved in spindle formation and kinetochore assembly being by this way required for proper function (Macedo *et al,* 2017). Ensuing genome instability becomes a spiral as DNA replication and chromosome segregation fidelity become compromised generating further DNA damage and chromosomal abnormalities (Bakhoum *et al,* 2014; Burrell *et al,* 2013; Soto *et al,* 2018). The result is a nearly 'infinite' source of immunostimulatory cytoplasmic DNA, that can engage the cGAS-STING pathway (Galluzzi *et al,* 2018). In agreement with its role as a cell-intrinsic stress sensor required for senescence (Gluck & Ablasser, 2019), we observed that RNAi-mediated depletion of cGAS ameliorates the senescence phenotype in DS patient-derived fibroblast cultures. To our knowledge, the here-presented study is the first to systematically characterize cellular senescence in cells with trisomy 21 taking into consideration patient age. We find that senescence accrues faster in DS when compared to normative aging, with young-adult DS patients having a senescence status comparable to that of elderly individuals. Consistent with our observations of a GIN-induced and cGAS-dependent cellular senescence phenotype in DS, previous studies reported an upregulation of type I interferon (IFN)-related players in cells with trisomy 21 suggestive of chronic pro-inflammation (De Toma *et al*, 2021; Krivega *et al*, 2021; Sullivan *et al*, 2017; Sullivan *et al*, 2016).

Persistent cellular senescence may be contributing to the clinical symptoms of DS reminiscent of aging-associated conditions and premature aging. In fact, SASP-induced low-grade chronic inflammation (or 'inflammaging') disturbs tissue homeostasis and has thereby been implicated in the aging process (Franceschi & Campisi, 2014; Franceschi *et al*, 2018). Even though pro-inflammation in the context of aneuploidy can signal cells with complex karyotypes for clearance (Santaguida *et al,* 2017), it was found that the interferon signature of cells with trisomy 21 is accompanied by a deregulation in the kynurenine pathway which can cause immunosuppression (Powers *et al*, 2019). Also, quantitative and qualitative changes in the immune system of DS subjects have been well documented, with a potential acceleration of immunosenescence (Gensous *et al,* 2020). Thus, as in aging, a faulty immune-mediated clearance of stress- and damage-induced cellular senescence can encourage accrual of senescent cells, contributing by this way to the age-related phenotypes and accelerated aging in DS. For example, neurodegeneration is highly prevalent and associated with neuroinflammation in DS, where certain pro-inflammatory cytokine signatures do correlate with an increased risk for dementia (Ballard *et al*, 2016; Iulita *et al*, 2016). Senescence may be fueling this neuroinflammation (Carreno *et al*, 2021; Guerrero *et al*, 2021), which fits recent observations showing that selective killing of

senescent cells (or senolysis) can delay or even prevent neurodegenerative conditions (Bussian *et al,* 2018; Zhang *et al,* 2019). Counteracting senescence accrual should thus be further explored in the context of DS. Notably, we disclose here a small-molecule inhibition of GIN that acts on DNA damage repair and chromosome segregation, able to delay the accrual of cellular senescence in cells with trisomy 21. Therefore, our study offers an interesting alternative to the widely studied senolysis concept (recently reviewed in Robbins *et al,* 2021), whilst paving way for future studies aimed at healthspan extension in the context of DS. *In vivo* studies will be paramount not only to determine whether cellular senescence accrues over time at the organismal level, but also to investigate which benefits arise from genome stability improvement.

III.5. Supplementary Information

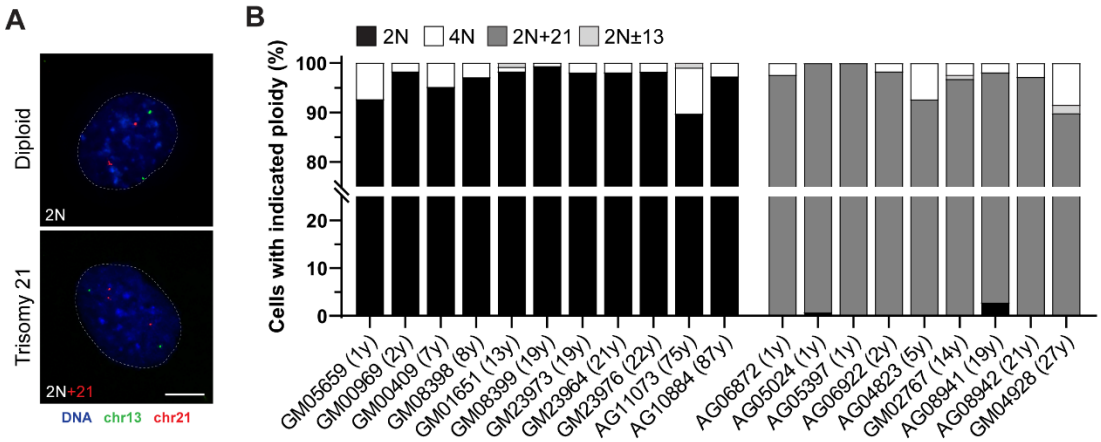


Figure III.S1. Trisomy 21 in fibroblast cultures used in this study. (**A**) Representative images of chromosome-specific FISH staining for chromosomes 13 (green) and 21 (red) in diploid (top) and trisomy 21 (bottom) interphase cells. DNA is shown in blue. Scale bar 5 μm; (**B**) Characterization of all fibroblast cultures from healthy (right) and trisomy 21 (left) male and female individuals by interphase FISH at passage 1–2 following purchase with probes against chromosomes 13 and 21. Cells were classified as diploid (two signals for chr13 and two signals for chr21), having gained a copy of chromosome 21 (three nuclear signals for chr21, two signals for chr13), displaying gain/loss of chromosome 13 (one/three signals for chr13 and two/three signals for chr21), or displaying tetraploidy (four signals for chr13 and four/six signals for chr21). *Data Information*: n>100 cells were analyzed for each condition and values shown are mean of at least two independent experiments.

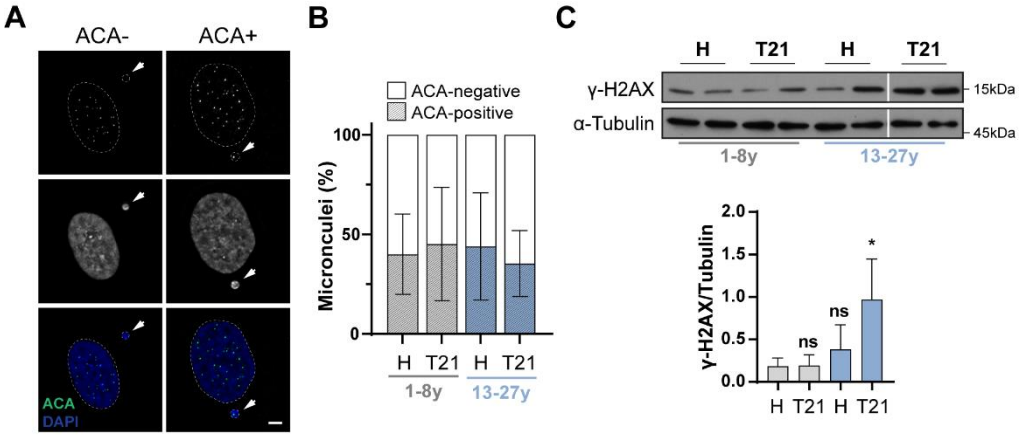


Figure III.S2 (related to Fig III.2). Genome instability phenotype of cells with trisomy 21. (**A**) Representative images of micronuclei-positive cells scored as negative (ACA-) or positive (ACA+) for the anti-centromere antibody (ACA; green) immunostaining. DNA is shown in blue. Arrows point to micronuclei. Scale bars 5 µm; (**B**) Percentages of ACA-positivity in micronuclei scored from healthy (H) vs. trisomy 21 (T21) fibroblast cultures; (**C**) γ-H2AX protein levels in total extracts from healthy and trisomy 21 fibroblasts. Tubulin is shown as loading control. *Data Information*: (B) n>5500 and (C) n>6600 were analyzed for each condition. Values shown are mean ± SD of at least three independent experiments. ns p>0.05 and * p<0.05 by two-tailed (C) non-parametric Mann-Whitney test.

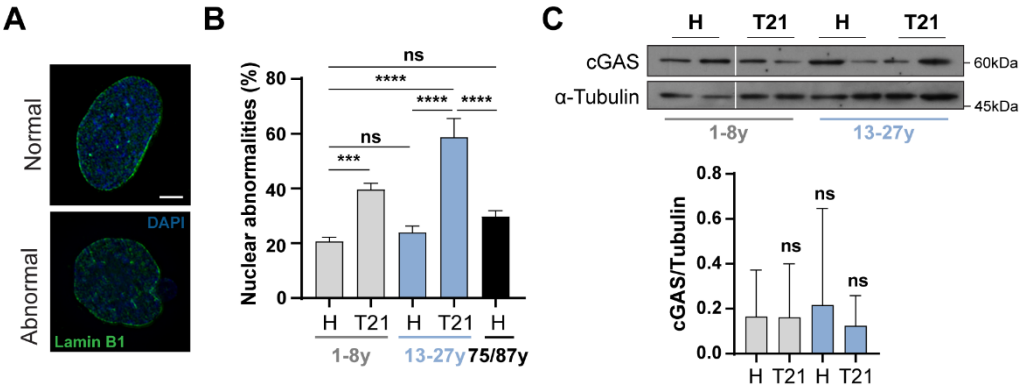


Fig III.S3 (related to Fig III.3). cGAS in diploid vs. trisomy 21 fibroblast cultures. (**A**) Examples of a normal vs. abnormal cell shape in cultures immunostained for Lamin B1 (green). DNA is shown in blue. Scale bar 5 µm; (**B**) Frequencies of cells with nuclear abnormalities scored in Lamin B1-stained fibroblast cultures from H vs. T21 donors; (**C**) cGAS protein levels in total extracts of fibroblasts from healthy (H) and trisomy 21 (T21) donors. GAPDH is shown as loading control. *Data Information*: (B) n>250 cells were analyzed for each condition. Values shown are mean ± SD of at least three independent experiments. ns p>0.05, *** p<0.001, **** p<0.0001 by two tailed (B) one-way ANOVA or (C) non-paired Mann-Whitney tests.

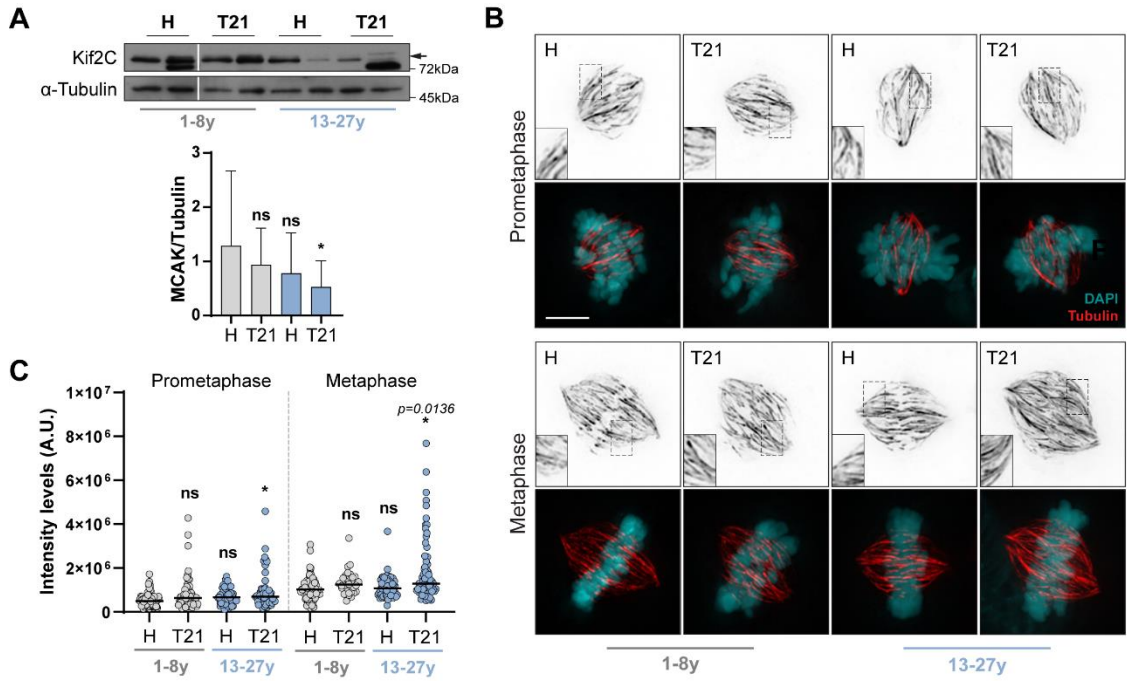


Figure III.S4 (related to Fig III.4). Kif2C downregulation and MT hyperstability in cells with trisomy 21. (**A**) Kif2C protein levels (arrow) in total extracts from healthy (H) and trisomy 21 (T21) fibroblasts. Tubulin is shown as loading control; (**B, C**) Representative images (B) and quantification (C) of calcium-stable k-fiber intensity levels in prometaphase and metaphase, scored by immunofluorescence analysis of tubulin-stained (red) mitotic cells of healthy and trisomy 21 samples. DNA is shown in cyan. Scale bar 5µm. *Data Information*: n>30 cells were analyzed for each condition in (C). Values shown are mean ± SD of at least three independent experiments. ns $p>0.05$, * $p<0.05$ by two-tailed (A) non-parametric Mann-Whitney; or (C) non-parametric Kruskal-Wallis test.

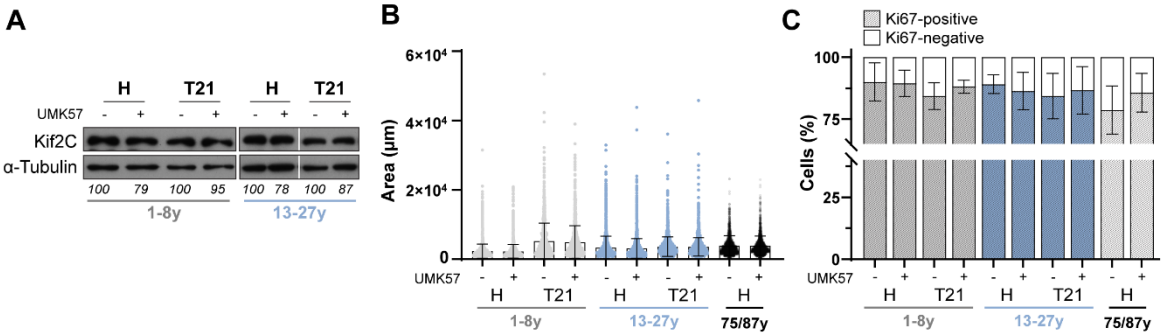


Figure III.S5 (related to Fig III.4). Small-molecule enhancement of MCAK in cells with trisomy 21. (**A**) Kif2C protein levels in total extracts of fibroblasts from healthy (H) and trisomy 21 (T21) donors treated for 96 h with UMK57. Tubulin is shown as loading control; (**B**) Percentages of cells staining positive for Ki67 in H vs. T21 fibroblast cultures following 96 h of exposure to UMK57; (**C**) Cell size analysis of tubulin-stained fibroblasts from healthy vs trisomy 21 cultures after UMK57 treatment for 96 h. *Data Information*: (B) n>2185 and (C) n>655 cells. Values shown are mean ± SD of at least three independent experiments, except for (A) which shows data from one independent experiment. All ns $p>0.05$ by two tailed non-paired Mann-Whitney test.

Table III.S1 (related to Fig III.S1). Fibroblast cultures from healthy and trisomy 21 male and female individuals used in this study.

Reference	Repository	Gender	Age	Condition	PDL at freeze	Passage frozen
GM05659	Coriell	Male	1 year	Healthy	4.57	10
AG06872	Coriell	Female	1 year	Trisomy 21	6.43	5
AG05397	Coriell	Male	1 year	Trisomy 21	5.67	4
AG05024	Coriell	Female	1 year	Trisomy 21	8	6
GM00969	Coriell	Female	2 years	Healthy	6.32	14
AG06922	Coriell	Male	2 years	Trisomy 21	7	4
GM00409	Coriell	Male	7 years	Healthy	5.3	3
GM08398	Coriell	Male	8 years	Healthy	5.77	4
AG04823	Coriell	Male	5 years	Trisomy 21	5.63	8
GM01651	Coriell	Female	13 years	Healthy	4.66	14
GM02767	Coriell	Female	14 years	Trisomy 21	n/a	11
GM23973	Coriell	Male	19 years	Healthy	3.34	6
GM08399	Coriell	Female	19 years	Healthy	6.01	4
GM23964	Coriell	Male	21 years	Healthy	4.33	6
GM23976	Coriell	Male	22 years	Healthy	4.98	4
AG08941	Coriell	Female	19 years	Trisomy 21	n/a	14
AG08942	Coriell	Male	21 years	Trisomy 21	5.68	8
GM04928	Coriell	Male	27 years	Trisomy 21	n/a	13
AG11073	Coriell	Male	75 years	Healthy	8	n/a
AG10884	Coriell	Male	87 years	Healthy	5	2

Chapter – IV

General Discussion and Concluding Remarks

Parts of the following publication were reproduced in this section:

Barroso-Vilares, M & Logarinho, E (2019). *Chromosomal instability and pro-inflammatory response in aging*, Mechanisms of Ageing and Development, 182: 111118. https://doi.org/10.1016/j.mad.2019.111118

IV.1. General Discussion

During the last two decades, increasing efforts in aging research allowed to significantly enlighten the basic mechanisms underlying the aging process (Lopez-Otin *et al,* 2013). This obviously spurred the interest to develop strategies to revive cells and/or postpone cellular aging with the ultimate goal to ameliorate age-related conditions (Mahmoudi *et al,* 2019b). Changes in metabolism, epigenetic dysregulation and accumulation of cellular senescence are amongst the hallmarks targeted by the current approaches under investigation. The here-described work offers an interesting alternative to be explored, the focus of which is another hallmark of the aging process – genome instability.

Although former work from our laboratory and others allowed to document that an age-dependent decline in chromosome segregation fidelity may contribute to aging phenotypes via induction of cellular senescence (Baker *et al,* 2013; Baker *et al,* 2004; Baker *et al,* 2006; Baker *et al,* 2011; Macedo *et al,* 2018) (Fig IV.1A), mechanistic insights into how aging compromises chromosomal stability were still elusive. Here we demonstrate that MT dynamics becomes dysregulated with aging and by this way induces mild CIN in elderly cells. As a consequence of their defective nature (Krupina *et al*, 2021), generated MN contribute to the cytoplasmic pool of immunostimulatory DNA sensed by the cGAS-STING pathway, fueling an inflammatory phenotype in aneuploid senescent cells. In opposition to the well-established senescence-inducing potential of DNA damage (Hernandez-Segura *et al,* 2018; Munoz-Espin & Serrano, 2014; Shay & Wright, 2019), chromosomal imbalances are only now starting to be recognized as drivers of senescence and SASP, thanks also to observations made in human cell lines (He *et al,* 2018; Santaguida *et al,* 2017), and wing primordium of *Drosophila* (Joy *et al,* 2021), both treated to experience varying levels of aneuploidy. Based on these data and our findings showing that pharmacological inhibition of aging-associated CIN prevents cellular senescence accrual, it is plausible that CIN-driven senescence might contribute to the chronic low-grade pro-inflammatory state observed during and known to fuel aging (also known as ‘inflammaging’) (Franceschi & Campisi, 2014; Franceschi *et al,* 2018). Thus, modulation of CIN could represent a novel strategy to delay senescence-associated changes with age in alternative to senolytic and senostatic approaches (Fig IV.1B). In fact, it was found that sustained overexpression of BubR1 in *BubR1* hypomorphic mice can correct premature aging phenotypes and extend lifespan by preserving genome integrity (Baker *et al,* 2013). Similarly, expression of a constitutively active form of the transcription factor FoxM1, which amongst others drives the expression of BubR1 (Fischer *et al*, 2016), re-established mitotic proficiency of elderly human and

mouse fibroblasts thereby rescuing aneuploidy-induced senescence (Macedo *et al,* 2018). Notably, recent efforts of our lab allowed to extend these findings from elderly fibroblasts into the *in vivo* setting and show that the negative consequences of FoxM1 repression with aging at the organismal level can be counteracted by overexpressing the transcription factor, translating into a delay in tissue deterioration and an extended lifespan (Ribeiro *et al,* 2021).

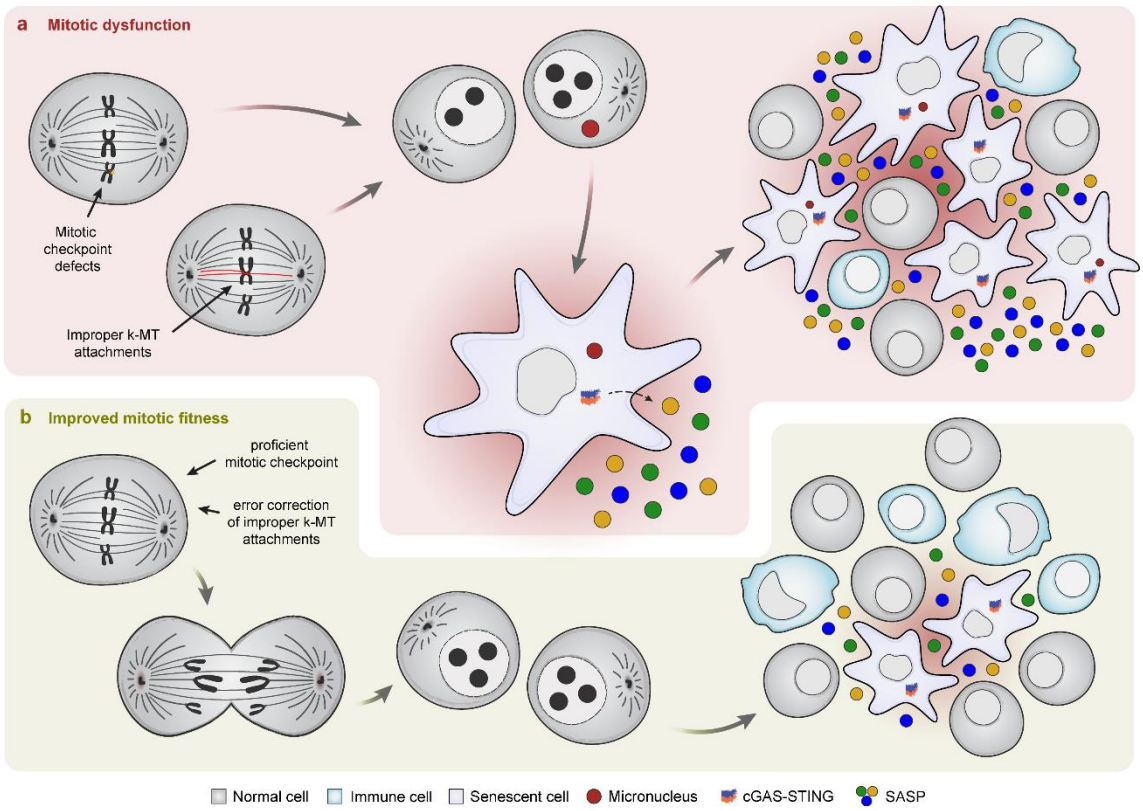


Figure IV.1. Modulation of age-associated CIN as an anti-aging strategy. (**a**) Mitotic dysfunction observed in aged cells causes chromosome mis-segregation and micronuclei formation, which is crucial for the transition into a permanent cell cycle arrest (full senescence), presumably due to cGAS-STING activation by cytosolic DNA released from micronuclei, leading to active secretion of pro-inflammatory molecules (SASP). In combination with the decreased efficiency of the immune system, which itself senesces (immunosenescence), accrual of aneuploid senescent cells leads to a chronic inflammatory state that perturbs tissue function contributing to the development of aging-associated phenotypes; (**b**) By improving mitotic fitness of proliferative elderly cells (inhibition of CIN through BubR1 and FoxM1 overexpression, or UMK57 treatment, for example), generation of aneuploid senescent cells is prevented, which is expected to alleviate the pro-inflammatory status of aged tissues and thus act to delay the onset of age-related conditions. Adapted from *Barroso-Vilares et al. 2019*.

Our work also focused on Down Syndrome, a condition caused by the supernumerary presence of chromosome 21 (Bull, 2020). Different cellular models allowed to establish that single-chromosome gains increase the tendency of genomic alterations (or genomic instability) (Blank *et al,* 2015; Hintzen *et al,* 2021; Nicholson *et al,* 2015; Passerini

et al, 2016; Sheltzer *et al,* 2011). This in turn further compromises DNA replication and chromosome segregation fidelity, generating more DNA damage and chromosomal abnormalities (Bakhoum *et al,* 2014; Burrell *et al,* 2013; Soto *et al,* 2018). Accordingly, we show that the constitutional trisomy of chromosome 21 correlates with increased CIN and DNA damage, but additionally we found a higher prevalence of cellular senescence in DS-patient derived fibroblasts. As patient age was taken into consideration in our analysis, we provide unprecedent evidence that cells with trisomy 21 accumulate GIN and senescence faster, which might explain how DS patients exhibit premature onset of symptoms reminiscent of aging and progeroid conditions (Antonarakis *et al,* 2004; Antonarakis *et al,* 2020). Indeed, similarly to the aging model, we predict that the changes in cellular physiology induced by the extra chromosome 21 lead to a dysfunction in mechanisms required for maintenance of genome stability, which then results in cGAS-STING proinflammatory signaling driving cellular senescence. The persistent generation and inefficient clearance of senescent cells will compromise tissue homeostasis and contribute to the premature aging symptoms observed in DS. Considering our rationale, it would thereby be reasonable to extend the 'genome stability maintenance anti-aging concept' into Down Syndrome. DNA damage can be corrected in differentiated cells and proliferating cells will be allowed to divide properly. This is expected not only to lower the burden of pro-inflammatory signals, but also to replenish the tissues with 'fit' cells, positively impacting the surrounding environment whilst out-diluting existing secreted molecules and senescent cells which arose by other means. In fact, factors secreted by proliferative cells assist stem and differentiated cell pools, the function of which is known to degenerate over time (Chien & Karsenty, 2005; Ermolaeva *et al*, 2018; Oh *et al*, 2014; Schultz & Sinclair, 2016).

It is curious that the mainstream rejuvenation strategies under investigation (*i.e,* metabolic manipulation, cellular reprogramming and senotherapeutics) seem to directly or indirectly act on proliferative capacity, with the primary cell types targeted being stem cells (lose renewal ability with aging), vascular and connective tissue cells (lose proliferative capacity with aging), and senescent cells (permanently arrest in the cell cycle) (Mahmoudi *et al,* 2019b). Yet, as mentioned in Chapter I, each of the three approaches has flaws. Here we disclose a small-molecule strategy that offers a valuable alternative to be tested for healthspan extension and which overcomes some of the concerns raised for the rejuvenation strategies currently under investigation. The small molecule used in our work enhances the activity of Kif2C, a MT depolymerizing kinesin well-known for its role in MT dynamics during mitosis (Kline-Smith *et al,* 2004; Manning *et al,* 2007), and more recently also implicated in DSB repair during interphase (Zhu *et al,* 2020). As we found that Kif2C in elderly and DS samples becomes compromised, the small molecule would be preventing the generation of senescent cells with detrimental impact in aging and DS pathologies. This

would allow to circumvent drawbacks of senotherapy, namely the off-target effects on beneficial senescence (*i.e,* tumor-suppressing senescence and senescence required during development and regeneration) and pathways that are not exclusive to senescence. Moreover, preventing the generation of GIN-induced senescence can be advantageous over senolysis, as the latter is unable to overturn the damage and changes already inflicted by the secretory phenotype on microenvironment and neighboring cells. Finally, because senescence and SASP are acknowledged as very heterogeneous and highly context-dependent (Coppe *et al,* 2010b; Cuollo *et al,* 2020; Gorgoulis *et al,* 2019; Hernandez-Segura *et al,* 2018), it is likely that the aging and DS senescence signatures diverge. But being GIN a hallmark of accelerated aging syndromes (Burla *et al*, 2018; Burtner & Kennedy, 2010; Foo *et al*, 2019), as well as being upstream in the order of events (*i.e,* CIN/DSBs – cytoplasmic DNA – cGAS/STING engagement – SASP) (Galluzzi *et al,* 2018; Gluck & Ablasser, 2019), modulation of genome stability is expected to work regardless of senescence/SASP heterogeneity. With an emerging functional link between persistent DNA lesions and age-related epigenetic changes (Hayano *et al*, 2019; Siametis *et al*, 2021), there is the intriguing possibility that in addition to preventing senescence, re-establishing DNA damage repair efficiency may also counteract epigenome changes associated with aging phenotypes. However, in opposition to cellular reprogramming which revives cells by erasing aging-related epigenetic alterations (Ocampo *et al,* 2016a), it is unlikely that modulation of genome stability becomes tumorigenic. A major concern of the cellular reprogramming concept is in fact that dedifferentiation and loss of cell identity predispose for teratoma development (Abad *et al,* 2013; Ohnishi *et al,* 2014). Ultimately, by focusing on a specific molecular player involved in DNA repair and chromosome segregation, our small-molecule approach narrows down the possibilities and complexity of what is being improved and/or modulated, which also represents an advantage over existing strategies. For example, it is still unclear which combination of transcriptional, epigenetic, proteomic and metabolic improvements ultimately promotes health and longevity in response to dietary restriction. Also, the outcome of metabolic manipulations is influenced by genetic and epigenetic factors, and depends heavily on the diet itself (*i.e,* nutrients and feeding times) (Green *et al,* 2021). Obviously, as in all other rejuvenation approaches, treatment and dose regimens will have to be fine-tuned to ensure efficacy and safety, whilst determining potential benefits on healthspan. This is particularly important since chromosome segregation fidelity relies on the tight balance of MT dynamics and thereby over-enhancement of Kif2C would rather be detrimental (Bakhoum & Compton, 2012). Remarkably, our small-molecule treatment also positively impacts the fitness of fibroblast cultures from patients with HGPS (Macedo *et al,* 2021), a progeroid syndrome in which cellular aging was recently shown to be mediated by a replication stress-driven Interferon-

like Response (Kreienkamp *et al,* 2018). Based on all of this, our laboratory decided to devise and move into a pre-clinical validation of the compound UMK57 *in vivo* using the HGPS-mimicking *Lmna*$^{G609G/G609G}$ (or LAKI) mouse model (Osorio *et al*, 2011).

IV.2. Outlook and future directions

Despite being an exquisite barrier against damage propagation and being beneficial during development or regeneration following injury, a wealth of studies focused on senescent cell and/or SASP ablation allowed to state that persistent senescence contributes to the pathophysiology of aging and premature aging conditions. In fact, even a relatively small number of senescent cells transplanted into young mice suffices at causing persistent physical dysfunction and spreading cellular senescence to host tissues (Xu *et al*, 2018). Consequently, with the observation that cellular senescence can be prevented by restoring genome stability in three distinct contexts, namely normative aging (Chapter II and Macedo *et al,* 2018), DS (Chapter III) and HGPS (Macedo *et al,* 2021), efforts from our laboratory using cellular models raise the possibility that inhibition of GIN rates may have potential as senotherapy. Intriguingly, although GIN is typical in progeroid syndromes and has been recently proposed as a unifying cause of aging (Schumacher *et al*, 2021), a strategy as the one disclosed in this thesis effective at correcting DNA damage and chromosome segregation defects was lacking. It will be relevant to ascertain whether CIN and other forms of genome instability do accumulate preferentially or faster in particular tissues of naturally aging mice and mice models of Trisomy 21. Also, a greater understanding of senescence *in vivo* is required. A number of mice models have been developed to study p16^{Ink4a}-driven senescence *in vivo* (Baker *et al,* 2011; Burd *et al,* 2013; Demaria *et al,* 2014), and recent studies also disclosed 'probes' that signal cellular senescence *in vivo* when in the presence of lysosomal β-Gal activity (Lozano-Torres *et al*, 2020; Lozano-Torres *et al*, 2021; Munoz-Espin *et al*, 2018). However, both p16^{Ink4a} and SA-β-Gal are not exclusive to senescent cells (Robbins *et al,* 2021). Identification of reliable *in vivo* markers of senescence is thus urging and would be helpful to more accurately determine whether GIN-induced senescent cells do occur and accrue in mice. Finally, should *in vivo* studies underscore their prevalence in naturally aging mice and murine models of Trisomy 21, it will be interesting to investigate whether our small-molecule approach promotes healthspan. To this end, besides evaluating how tissue aging (*e.g,* senescence status and stem cell function) and overall organismal performance is influenced by the modulation, it will be important to determine the impact on known cellular hallmarks of the aging process (Lopez-Otin *et al,* 2013). For example, benefits on epigenome

regulation and metabolic function should be assessed by measuring whether inhibition of GIN restores common epigenetic changes (*e.g,* DNA methylation patterns or clocks, histone modifications and chromatin accessibility) and dysregulated metabolic pathways (*e.g,* mTOR and insulin/IGF1). In fact, a recent review discusses how aging-associated DNA damage can underlie molecular consequences such as epigenome alterations, proteotoxicity and compromised mitochondrial function (Schumacher *et al,* 2021). Thus, the collective addressment of this questions will be fundamental to support the senomorphic potential of the here-disclosed GIN-inhibiting small molecule.

In summary, the data presented within this thesis suggest that loss of genome stability, acknowledged to be a consequence and hallmark, may after all also be a cause of aging or premature aging conditions. Furthermore, our findings anticipate an unprecedented value for senotherapy in the context of DS which, as far as we are aware, has not been investigated. Therefore, it will be worthwhile to devote future studies at exploring the contribution of GIN-induced senescence to the pathophysiology of aging and DS.

Chapter – V

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