Organization of centromere during senescence

Camellia Chakraborty

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Préparée à l'Institut Curie
UMR 144- Biologie Cellulaire et Cancer
Laboratoire Mécanismes Moléculaires de la Dynamique des Chromosomes

Organization of centromere during senescence

Organisation du centromère pendant la sénescence

Soutenue par
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6 Novembre 2024

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To every person and aspect
Who have inspired me
To step beyond the mighty Brahmaputra
Because ahead lays the vast, deep Ocean

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It is said that PhD is about knowing more and more about less and less. The journey has been not just about exploring the physical dimensions of senescent cell and building scientific maturity but also identifying the inner personality that lay dormant in each of us.

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Where the mind is without fear
and the head is held high;
Where knowledge is free;

Where the world has not been broken up
into fragments by narrow domestic walls;

Where words come out from the depth of truth;
Where tireless striving stretches its arms toward perfection;
Where the clear stream of reason has not lost its way
into the dreary desert sand of dead habit;

Where the mind is led forward by Thee
into ever-widening thought and action —
Into that heaven of freedom,
my Father, let my country awake.

– Rabindranath Tagore

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Abstract

Cellular stresses promote senescence, an irreversible exit from the cell cycle, that in turn promotes an inflammatory response. An early event in senescence is the formation of Senescence Associated Distension of Satellite DNA (SADS) where satellite DNA, a type of repetitive DNA, unfolds. How SADS originate and what is their role in promoting senescence is unknown. To answer these questions, I study a particular type of repetitive satellite DNA, α-satellite, that is present at chromosomal regions called centromeres. Centromeres are loci essential to mediate accurate sister chromatid segregation during both meiosis and mitosis. Within centromeres, α-satellite DNA folds into unique secondary structures and plays a role in centromere functionality, highlighting the importance of maintaining its architecture.

To induce SADS formation I subjected cells to several cellular stresses to induce senescence like DNA damage, aneuploidy or p53 stabilization. By investigating the factors promoting SADS formation and its cellular consequences, I found that SADS occurs through p53 activation in a DNA damage independent manner. However, upon DNA damage the presence of p53 becomes dispensable in promoting SADS formation. Within the DNA damage response cascade, I have found that inhibition of the DNA damage response factor ATM minorly prevents SADS formation, but only following DNA damage. Additionally, I discovered that the centromeric proteins CENP-A, CENP-B and CENP-C are important to maintain centromeric DNA compaction in non-cycling cells, with CENP-B having a stronger effect. Importantly, ectopic CENP-B once bound to centromeric DNA partially delays SADS formation and minorly impacts the downstream senescence phenotype. This highlights a direct connection between SADS and other senescence phenotypes.

Centromeres have a unique epigenetic landscape. I have found that during senescence onset centromeres undergo histone modifications and that such epigenetic modifications might influence centromere compaction. I am currently looking into the chromatin accessibility at SADS by measuring histones abundancy and changes of DNA methylation, known to drive important physiological functions like transcription. Here, I found that centromeric transcription is altered during senescence, but this is differently regulated depending on different senescence conditions. This suggest that while SADS is an early universal hallmark of senescence, its outcome might be a context-driven phenomenon.

To this end, I am testing if SADS can influence other senescence hallmarks like Senescence Associated Secretory Phenotype (SASP). SASP occur during senescence and depend on cGAS, a DNA sensor protein that can activate the innate immune response. cGAS has been shown to self-oligomerise to cause DNA compaction and it is preferentially enriched at centromeres during interphase. Its role at centromere, especially during senescence onset, is not known. Here, I found that cGAS is depleted from centromeres as cells proceed to a senescent state, suggesting that its absence

might influence centromere decompaction. I also found that in the interphase of cycling cells, cGAS is enriched at CENP-B bound centromeres.
In summary, this work sheds light on the events occurring at centromere during early senescence. This will enhance our understanding of senescence progression that is relevant in physio- and patho-logical contexts such as cancer.

Key words: Senescence, Centromere, DNA compaction, Epigenetics, Innate immune response, cGAS

Résumé

Les stress cellulaires favorisent la sénescence, une sortie irréversible du cycle cellulaire, qui favorise une réponse inflammatoire. Un événement précoce de la sénescence est la formation d'une distension de l'ADN satellite associée à la sénescence (SADS), où l'ADN satellite, un type d'ADN répétitif, se déplie. Comment se forment les SADS et quel est leur rôle dans la promotion de la sénescence reste un mystère. Pour répondre à ces questions, nous étudions un type particulier d'ADN satellite répétitif, α-satellite, présent dans des régions chromosomiques appelées centromères. Les centromères sont des loci essentiels à la ségrégation précise des chromatides sœurs au cours de la méiose et de la mitose. Au centromère, l'ADN α-satellite se replie en structures secondaires uniques et joue un rôle majeur dans la fonctionnalité du centromère et le maintien de son architecture.

Pour induire la formation de SADS, j'ai soumis les cellules à plusieurs stress cellulaires visant à induire la sénescence, tels que les dommages à l'ADN, l'aneuploïdie ou la stabilisation de la p53. En étudiant les facteurs favorisant la formation des SADS et leurs conséquences cellulaires, j'ai découvert que les SADS se produisent par l'activation de p53 de manière indépendante des dommages à l'ADN. Cependant, lorsque l'ADN est endommagé, la présence de p53 devient inutile pour favoriser la formation des SADS. Dans la cascade des dommages à l'ADN, j'ai découvert que l'inhibition du facteur de réponse aux dommages à l'ADN, ATM, empêche modérément la formation des SADS, mais seulement après un dommage à l'ADN. En outre, j'ai découvert que les protéines centromériques CENP-A, CENP-B et CENP-C sont importantes pour maintenir la compaction de l'ADN centromérique dans les cellules qui ne se divisent pas, notamment pour CENP-B. Il est important de noter qu'une fois liée à l'ADN centromérique, la CENP-B ectopique retarde partiellement la formation des SADS et a un impact mineur sur le phénotype de sénescence en aval. Cela met en évidence un lien direct entre les SADS et d'autres phénotypes de sénescence.

Les centromères ont un paysage épigénétique unique. J'ai découvert qu'au cours de la sénescence, les centromères subissent des modifications des histones qui pourraient influencer la compaction des centromères. J'étudie actuellement l'accessibilité de la chromatine aux SADS en mesurant l'abondance des histones et les changements de méthylation de l'ADN, connus pour piloter d'importantes fonctions physiologiques telles que la transcription. Ici, j'ai trouvé que la transcription centromérique est altérée pendant la sénescence, mais qu'elle est régulée différemment selon les conditions de sénescence. Cela suggère que si la formation de SADS constitue un marqueur universel précoce de la sénescence, leurs conséquences pourraient varier en fonction du contexte.

À cette fin, je cherche à savoir si le SADS peut influencer d'autres caractéristiques de la sénescence telles les phénotypes sécrétoires associés à la sénescence (SASP). Ceci se produisent au cours de la sénescence et dépendent de cGAS, senseur d'ADN

cytosolique, qui peut activer la réponse immunitaire innée. Il a été démontré que cGAS s'auto-oligomérise pour provoquer la compaction de l'ADN et qu'elle est préférentiellement enrichie au niveau des centromères pendant l'interphase. Son rôle au niveau des centromères, en particulier lors de l'apparition de la sénescence, est inconnu. Ici, j'ai découvert que cGAS est dépourvu aux centromères lorsque les cellules sont à l'état sénescent, ce qui pourrait influencer la décompaction des centromères. J'ai également découvert que lors de l'interphase, cGAS est enrichi aux centromères liés au CENP-B.
En résumé, ce travail met en lumière les événements qui se produisent au niveau du centromère au cours de la sénescence précoce. Cela améliorera notre compréhension de la progression de la sénescence retrouvée dans le contexte physio- et pathologique tel que le cancer.

Mots clés : Sénescence, centromère, compactage de l'ADN, épigénétique, réponse immunitaire innée, cGAS

Abbreviations

5mC	5-methylcytosine
6mA	6-methlyadenine
ACA	Anti-centromere Autoantibody
AID	Auxin-Inducible Degron
APC	Anaphase Promoting Complex/Cyclosome
ASF1A	Anti-silencing Factor 1A
ATM	Ataxia telangiectasia mutated
ATR	RAD3-related
BAF	Barrier to Autointegration Factor 1
BRCA	BReast Cancer
BrdU	Bromodeoxyuridine
BubR1	Budding uninhibited by benzimidazole related 1
CATD	CENP-A Targeting Domain
CCAN	Constitutive Centromere-Associated Network
Cdc	Cell Division Cycle
CDK	Cyclin Dependent Kinase
cenDNA	centromeric DNA
CENP	CENtromere Protein
cGAMP	cyclic Guanosine monophosphate-Adenosine MonoPhosphate
cGAS	cyclic GMP-AMP synthase
ChIP	Chromatin ImmunoPrecipitation
CHK1/2	Checkpoint kinase 1/2
CREST	Calcinosis, Raynaud's phenomenon, Esophogeal dysmotility, Sclerodactyly, Telangiectasia
CTCF	CCCTC binding factor
DAPI	4',6-DiAmidino-2-PhenylIndole
DDR	DNA damage Response
DDR	DNA Damage Response
DNA	Deoxyribose Nucleic Acid
DNA SCARS	DNA segments with chromatin alterations reinforcing senescence
Dox	Doxycycline
DoxR	Doxorubicin
ds	double strand
DSB	Double strand break
EYFP	Enhanced Yellow Fluorescent Protein
FACS	Fluorescence Activated Cell Sorting
FACT	FAcilitates Chromatin Transcription

FAIRE	Formaldehyde-Assisted Isolation of Regulatory elements
FBS	Fetal Bovine Serum
FISH	Fluorescence *In Situ* Hybridization
FOXO	Forkhead Box O
GOI	Gene of Interest
HAC	Human Artificial Chromosome
HGPS	Hutchinson-Gilford progeria syndrome
HIRA	Histone Repressor A
HJURP	Holliday Junction Recognizing Protein
HMGB1	High-mobility group box 1
HOR	Higher Order Repeat
HP1	Heterochromatin Protein 1
HR	Homologous recombination
IAA	Indole-3-acetic acid
ICF	Immunodeficiency, Centromeric instability, and Facial anomalies
IF	Immunofluorescence
IFN	Interferon
ISGs	Interferon Stimulated Genes
KO	Knock-Out
LINES	Long Interspersed Nuclear Elements
M18BP1	Mis18 Binding Protein 1
MAC	Mammalian Artificial Chromosome
MAPK	Mitogen Activated Protein Kinase
MDC1	Mediator of DNA damage Checkpoint protein 1
MDM2	Murine Double Minute-2
Mis18	MISsegregation 18
MMEJ	Microhomology Mediated End Joining
MPS1	MonoPolar Spindle 1
MRE11	Meiotic Recombination 11
MRN	MRE11-Rad50-Nbs1
mTORC	Mammalian target of rapamycin complex
MTs	Microtubules
Myc	Myelocytomatosis oncogene
NCP	Nucleosome Core Particle
Ndc80	Nuclear division cycle 80
NES	Nuclear Export Signal
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non Homologous End Joining
NT	Non-Treated
OE	Overexpression

OIS	Oncogene induced senescence
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PH	Pelckstrin Homology
PI3	phosphatidyl- inositol 3-kinase
PML	Promyelocytic Leukemia
PTM	PostTranslational Modification
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma virus
RB	Retinoblastoma
RNA	Ribo Nucleic Acid
RNF8	RING Type Ubiquitin ligase 3
RS	Replicative senescence
SADS	Senescence associated distension of satellite DNA
SAHF	Senescence associated heterochromatin foci
SASP	Senescence Associated Secretory Phenotype
SMC	Structural Maintenance of Chromosome
SMURF2	Smad ubiquitin regulatory factor 2
ss	single strand
STING	Stimulator of Interferon Genes
SUMO	Small Ubiquitin MOdifier
TAD	Topologically associating domains

Introduction

Cellular foundation of life

Human consciousness has always contemplated about the very basic definition of life. Life is a process governed by nature to sustain simple to complex dynamic systems called living beings. Though advances in science have enabled us to study the physical, chemical and biological laws that govern these processes, a lot remains to be understood *(Gómez-Márquez, 2021)*. From domains like biodiversity on earth, evolution, ecosystem, physiology and response to stimuli, to molecular biology, genetics, biochemistry, etc., an entire plethora of life sciences have been subjects of investigation.

The organization of living systems is based on a hierarchy of scale right from atoms to a complete individual organism. When looking at all life forms in terms of matter, the basic structural, functional and self-sustained living unit that they are composed of is called a cell. A cell carries out different functions like, absorbing nutrients from food, generating energy or providing structure, etc. A single cell itself can form a complete organism like a bacteria and yeast or cells can undergo specialization and co-ordinate among themselves to form tissues, organs, organ systems and finally multicellular organisms like humans, which are made of around 28-35 trillion cells (*Hatton et al., 2023)*. A size scale ranging from the atom, which is the unit of matter, to a cell that is the unit of life, is shown in **figure 1** *(Charles Molnar and Jane Gair, 2015*).

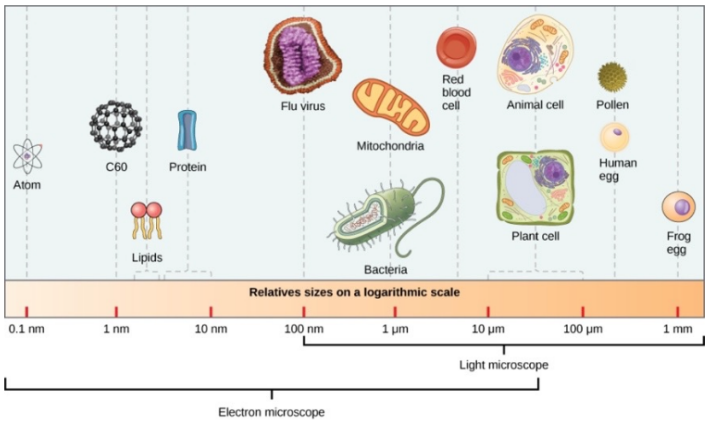


Figure 1: Spectrum of the relative sizes of various microscopic entities (Charles Molnar and Jane Gair, 2015).

A typical cell has functional structures called organelles that carry out specialized functions as mentioned above. It has a Nucleus which is the master regulator of all the processes happening inside a cell. The nucleus contains the genetic material called genome which holds the genetic and epigenetic information needed to functionalize a cell. In the life span of a cell, it proceeds through different phases of growth also known as cell cycle wherein a cell grows, duplicates its proteins and genetic material and then gives rise to daughter cells as a way of continuity of life from one cell to the next.

1. The Inner world of a cell:

1.1 Genome and its structure

A genome is the complete set of genetic material in a cell (including the nucleus and the mitochondria). It is made of DNA (2' Deoxy ribose nucleic acid) which functions to store and transmit genetic information through its unique structurization. DNA is composed of a ring of pentose sugar moiety attached to a nitrogen base (Adenine, Thymine, Cytosine or Guanine) and phosphate group (forming a nucleotide). Many such nucleotides come together through establishment of a phosphodiester bond to form a single DNA strand of sugar-phosphate backbone. A DNA molecule is a link of two such strands at the core through weak H-bonding between complementary nitrogen bases (A=T, C≡G), to form a double stranded structure that interwind and form a double helix (as shown in **figure 2**). The structure of DNA was resolved for the first time in 1953 by Watson and Crick with the contribution of Rosalind Franklin through x-ray crystallography, after almost 150 years after DNA was first observed by Swiss biochemist Frederich Miescher.

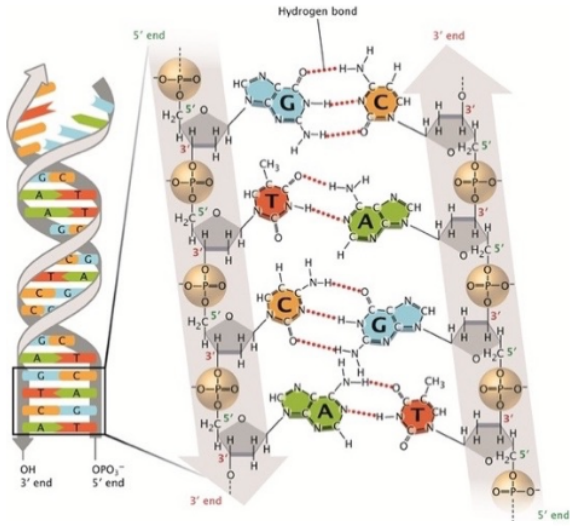


Figure 2: The DNA double helix model.

This unique structure of DNA can be critical for various biological functions: its function as being the genetic information, the packaging and storage of the genetic information in limited space and the transmission of this information to other cells.

1.1.1 Genetic information

The sequence of nitrogen bases throughout the DNA template serves as the code for production of functional proteins or RNA. Not all DNA is meant to code for proteins; the parts of DNA that code to form a protein are called genes. Proteins are comprised of sequences of amino acids that undergo proper folding. An amino acid is formed from a combination of three nucleotides, also known as codon. Any rare change, addition or deletion in a nucleotide can lead to clinical implications. For example, a point mutation of adenine to thymine in the b-globin gene can lead to sickle cell anemia, often observed in individuals of African descent, and can limit the carrier's lifespan to 40 years (*Jack Y. Ghannam; Jason Wang; Arif Jan. 2023*).

1.1.2 Packaging and storage of genetic information

Information about how the genome is organized in a nucleus can give a critical overview about how it functions. With recent major advances in relevant technologies and methods that include computational and modelling analyses, sequencing-based techniques and imaging-techniques like super-resolution microscopy, significant exploration of the 3D organization of the genome has been made.

The telomere-to-telomere consortium presented the complete sequence of the human genome consisting of 3.055 billion base pairs, now used as the reference human genome (in CHM13 cell line without chromosome Y) *(Nurk et al., 2022)*. This corresponds to approximately 2 meters long DNA in a cell, contained in 46 chromosomes. Packaging of this enormous length of a DNA molecule in a cellular space of 10-20 μm diameter together with other organelles requires effective strategizing. As outlined in **figure 3 A**, DNA is hierarchically organized into 3D structures starting from nucleosome units to nucleosome clutches, to chromatin loops and chromosomal domains called topologically associated domains (TADs) and then chromosome territories. The chromosomes remain like a loose thread in their own territories, thereby avoiding entanglement with other chromosomes but just before cell division, individual chromosomes package into compact 'X' shaped structures for their segregation into daughter cells (Jerkovic ´ & Cavalli, 2021).

The DNA is sequentially coiled, starting from wrapping double stranded DNA around proteins called histones to form nucleosomes (**figure 3 A**) *(Weaver et al., 2017)*, (R. Chen et al., 2014). The wrapping of DNA around histones is achieved through the electrostatic interaction between the negatively charged DNA phosphate backbone and positively charged amino acids of histone proteins. Around 147 base pairs of DNA coil 1.8 times around the core nucleosome, i.e., eight histone proteins (two each H2A, H2B and tetramers of H3 and H4). Between two core nucleosomes, at the linking DNA of 20-90 bp, the linker histone H1 further compacts the chromatin by binding to the start and end of DNA wound around the nucleosome, forming the famous model of "beads on string" (**figure 3 B**) (Duzdevich et al., 2014).

Due to technical limitations, before it was thought that such "beads on string" would fold-up to form a 30 nm broad fiber but now, thanks to super-resolution microscopy, we know that a group of few nucleosomes are organized further into unordered groups called "nucleosome clutches" (**figure 3 C**). These "nucleosome clutches" assemble to form 150-300 nm wide irregular clusters of nucleosomes called "Chromatin Nanodomain" (CND) (Jerkovic´ & Cavalli, 2021).

Next in the hierarchy of organization is the formation of dynamic heterogenous structures called TADs consisting of several CNDs forming 500 nm wide super clusters of nucleosomes. During interphase, the chromatin can undergo loop extrusion mediated by cohesin until it encounters CCCTC binding factor (CTCF) bound to specific sequence motif at the borders, and this extruded chromatin fiber organizes into clutches and CNDs ultimately giving rise to the formation of TADs.

A gene within a chromosome can be controlled by a group of regulatory sequences that can be situated up to thousands of base pairs away along the length of the chromosome. TADs act to keep the gene and its regulatory elements within the local molecular environment to enable their proper expression when necessary. Any defect in TADs boundaries can cause mis-separation of individual genes and cause mis regulation of gene expression, for example, in certain cases improper rearrangement can cause activation of genes implied in cancer.

Many such TADs then come together to form compartments harboring distinct types of chromatin: compartment A containing active genes and compartment B containing inactive genes. Current studies with chromatin capture techniques like Hi-C are trying together with existing ChIP-seq signals to predict the factors driving such compartmentalization. For example, a study by Harris et al., 2023 achieved up to 500 bp resolution and found that for active genes, their promoters and distal enhancers are present in compartment A but not even the flanking regions are present in A. In fact, for paused genes, the transcription start site (TSS) and transcription termination site (TTS) are often present in different compartments.

Finally, the individual chromosomes are packed in their own chromosomal territories that allow the chromosomes to stay segregated from other chromosomes. Such kind or organization allows segregation of chromosomes into daughter cells during cell division.

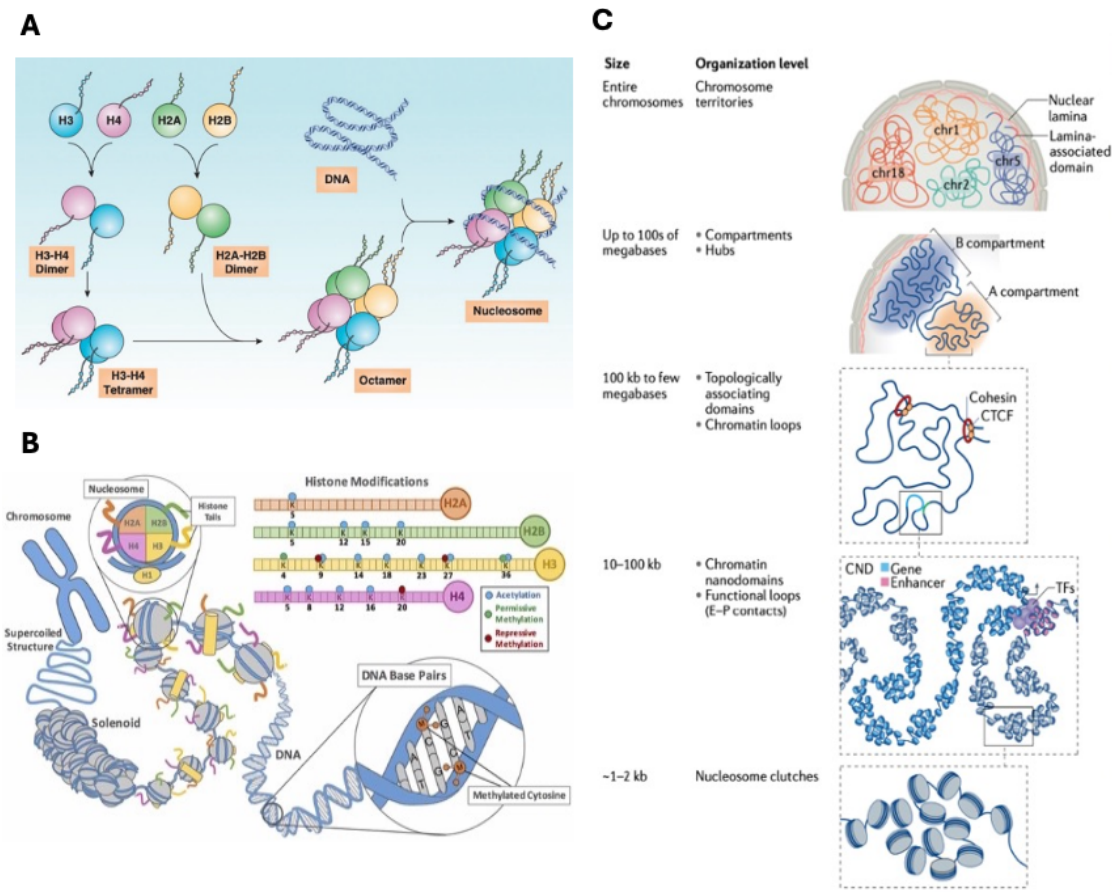


Figure 3: Packaging and storage of genetic information inside the cell:
A) Assembly of dimers of histone H2A, H2B, H3, H4 to form histone octamer which together with DNA assembles the nucleosome unit (R. Chen et al., 2014).
B) Dynamic (epi)genome: DNA methylation, histone post translation modification and chromatin structural organization in eukaryotic nucleus. Histone H1 protein stabilizes the wrapping of DNA around the nucleosome (Weaver et al., 2017).
C) Hierarchical 3D organization of the genome showing DNA folds at multiple scale (indicated in left) (Jerkovic´ & Cavalli, 2021).

1.1.3 Decoding and transmission of genetic information:

The information coded in DNA is transformed into protein via formation of an intermediate molecule termed RNA, through a process called transcription. Passage of DNA from the parent to the daughter cells requires accurate and precise duplication of the genome, a process termed replication. Both transcription and replication require access to the DNA, which is obtained by the reversible unwinding of DNA strands through breaking of its weak H-bonds. The ability of DNA to self-assemble helps restore the structural stability making it ideal to function as a long-term storage of genetic information.

In addition to DNA and its classical double helical structure, DNA secondary structures, chemical modifications and DNA-Histone interactions further modulate genome structure. These impact on the extent to which DNA strands are accessible and are wrapped around nucleosomes, serving beyond the genetic code as an "Epigenetic" way of regulating gene expression or even processes like DNA replication. Modifications in the nucleosome core, both in DNA or histones, can alter the associative strength between these two elements. Weak DNA-histone associations are permissive for transcription of DNA, and are mostly present in open chromatin regions, whereas strong DNA-histone interactions repress transcription. The different epigenetic regulatory mechanisms regulating DNA accessibility for DNA replication and gene expression are:

i. <u>DNA modifications</u>:
One of the most prevalent chemical modifications in DNA is the addition of a methyl group, that typically occurs at the 5th carbon position of the cytosine ring (5mC) and at the 6th carbon position of the adenine ring (6mA). 5mc is the most prevalent type of DNA methylation and occurs in the C-G dinucleotide sites called CpG sites. It is carried out by a family of DNA methyltransferase (DNMTs) from S-adenosyl methionine (SAM) as a donor, to form 5- methyl cytosine (Jiang & Guo, 2020) that is either involved in *de novo* deposition of new methyl groups in the cytosine or maintenance of methylation patterns as shown in **figure 4** (Moore et al., 2013).

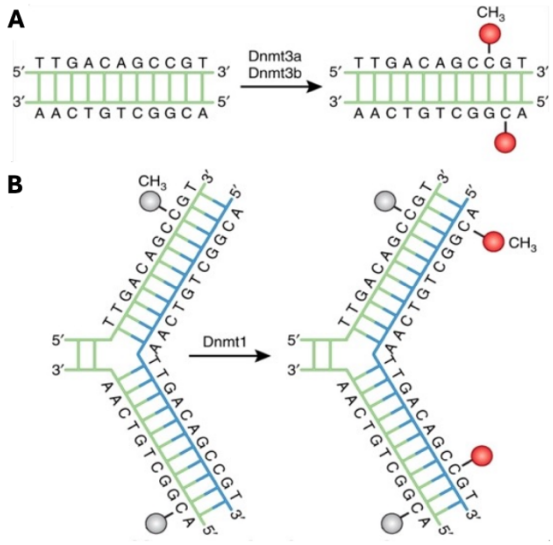


Figure 4: DNA methylation pathways: A family of DNA methyltransferase (DNMTs) catalyzes the transfer of –CH_3 from SAM to 5th carbon of cytosine (Moore et al., 2013).
A) DNMT3A and DNMT3B are de novo methyltransferase and transfer –CH_3 group to naked DNA.
B) DNMT1 is the maintenance methyltransferase and maintain methylation during replication. The parental strand retains original –CH_3 (in grey). DNMT1 associates at the replication foci and precisely adds –CH_3 to the daughter strand.

Although methylated cytosine was discovered around 1948 and its natural existence in DNA was already assumed, it was only around the 1980s that DNA methylation was recognized to be involved in gene expression and cell differentiation. DNA methylation acts as an inheritable regulatory mechanism for gene expression without changing the DNA sequence. It has been linked with many cellular processes like X-chromosome inactivation or silencing of retroviral elements, and it influences different genes based on the underlying genetic sequence. One key example is genomic imprinting, where one of the two alleles of a gene is silenced through methylation rather than genetic mutation. This mechanism plays a crucial role in cellular differentiation, and the phenomenon of genomic imprinting is both stable and heritable.

Some parts of the genome are particularly rich in cytosine and guanine nucleotides and form the so called “CpG islands”. They may be around 1000 bases long and ~70% promoters are associated with CpG islands, especially promoters of housekeeping genes. The CpG islands have been evolutionary conserved between mice and humans implying the functional importance of these regions. CpG DNA regulates chromatin structure and also the binding of transcription factors (TFs). CpGs have less nucleosomes than other regions of DNA and these few nucleosomes in CpGs have modifications in them such that they promote gene expression. Therefore, DNA methylation in these sites, which mostly harbor TSS regulate the silencing of these genes. (Moore et al., 2013).

There are also many studies about the role of DNA methylation in tissue differentiation and development, for example, DNA methylation state has been seen to vary across the endometrium tissue during endometrial phase (Retis-Resendiz et al., 2021). Therefore, the effect of DNA methylation on gene expression depends on the genomic context, is tissue specific and, when dysregulated, can even be implicated in various diseases.

Indeed, DNA methylation has been shown to induce differential expression of genes involving synaptic processes and it also impacts the binding of Methyl CpG binding protein 2 (MeCP2) affecting gene expression related to autism. Changes in DNA methylation have been associated with many conditions like the ICF (Immunodeficiency Centromeric instability Facial anomalies) syndrome due to loss of methylation from the (peri)centromeric regions through chromosome instability (Jin & Liu, 2018).

More than 75% of the CpG sites are methylated in mammalian genome and changes in DNA methylation have also been related to age as it is seen that with aging, methylation gradually declines (Wilkinson et al., 2021). Indeed, in mice embryos, high 5mC was observed which gradually decreased with age, inversely relating to the mice lifespan. Further, links between 5mC and senescence can be seen when normal fibroblast from mice, hamster and human had reduced 5mC, when the cells were cultured over a period whereas immortal mouse cell line had stable DNA methylation (Wilson & Jones, 1983) (Jung & Pfeifer, 2015). Studies are undertaken to identify links between DNA methylation and aging. This led to identification of age associated biomarkers subject to methylation changes, in model organisms of mice and rats, leading to the development of Epigenetic clocks. Additionally, methylation rates of DNA have recently been seen to correlate negatively with the maximum lifespan of a species (**figure 5**) (Crofts et al., 2023).

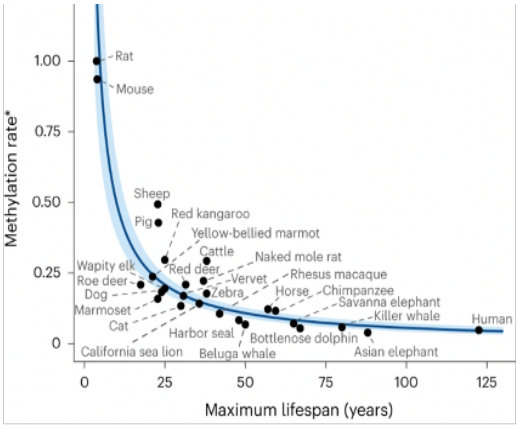


Figure 5: Regression line showing inverse correlation between methylation rate versus maximum life span (Crofts et al., 2023).

ii. <u>Histone post translational modifications (PTMs) and histone variants</u>:

The negative backbone of DNA makes it electrostatically unfavorable to DNA compaction, therefore wounding of DNA around positive histone proteins neutralizes the negative charge of DNA up to some extent. This allows higher order of chromatin structure. DNA-templated processes like DNA replication, transcription, recombination or repair require the DNA to be accessed by the required machinery. The accessibility of DNA depends on how compactly the DNA is wound around the histones. The open-chromatin conformation whereby nucleosomes unwind and is accessible for transcription is called euchromatin whereas when the DNA is wound tightly around the nucleosome, it's called heterochromatin. This compaction can be altered by affecting the DNA-histone affinity which can be achieved by PTMs of histone

proteins, alteration in the nucleosome positioning or by insertion of histone variants into chromatin.

Covalent PTMs of histone proteins is another epigenetic factor known to regulate chromatin structure and arrangement. Modified histones can exert their effect in two ways: first in Cis, where the modified histone can directly impact the physical properties and the local chromatin structure thereby impacting DNA access to various machineries of genome processing; and the second in Trans, where the modified histone in turn recruits other effector proteins that have downstream effects of remodeling the chromatin or transcriptional activity (S. Zhao et al., 2021). More than 100 different modifications have been identified in histones, thanks to the breakthrough of mass spectrometry (MS) proteomics. The most common types of histone modifications are acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP ribosylation, and they are to some extent reversible (Millán-Zambrano et al., 2022).

Each nucleosome core has a histone globular domain which mediates interaction with other histones in the nucleosome and interaction with the DNA that is wrapped around it at its lateral surface. Histones also have a tail region that protrudes from the nucleosome core and is enriched in positively charged amino acids. The tail of each of the core histones have a unique amino acid sequence (as shown in the grey shading **figure 6**).

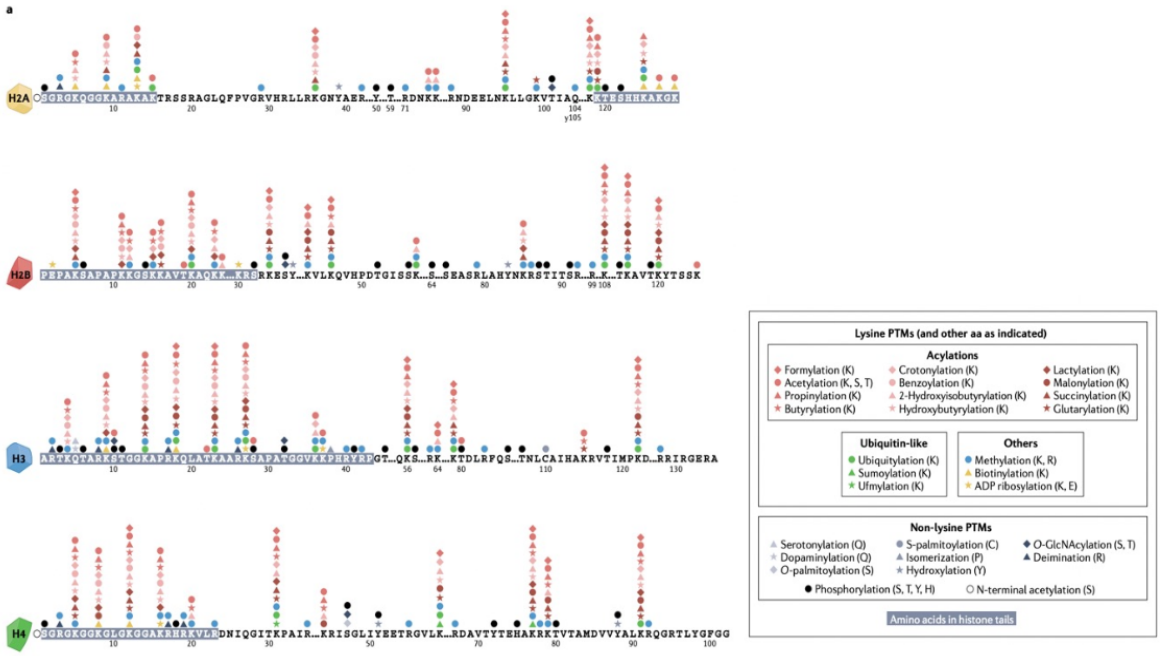


Figure 6: Selected sites for post translational modifications in the tail of core histone proteins H2A, H2B, H3, H4. The unique amino acids in each of this histone proteins are marked inside the blue box, together with position numbers of the amino acid. Amino acid within histone tails are indicated in grey shading. On the right are representation of symbols corresponding to the chemical modifications (Millán-Zambrano et al., 2022).

Histone tails have previously been reported to interact with nucleosomal DNA and affect DNA wrapping (Zheng & Hayes, 2003). It was seen that histone H3 and H4 tails affected the dynamics of DNA unwinding and re-wrapping around the nucleosome, also known as “DNA breathing” (Andresen et al., 2013) (Ghoneim et al., 2021). In fact, some PTMs on histone tails which alter their charge, have been shown to influence the conformational dynamics of histone tails. The PTMs here are recognized by the reader proteins and in turn affect the accessibility to chromatin associated proteins and their activities. Nuclear Magnetic Resonance (NMR) studies revealed that the phosphorylation of serine or threonine residues or acetylation of lysine on H3 tail have been associated with its weakened interaction with nucleosome and linker DNA. This leads to enhanced binding of histone reader PHD finger domains which further recruits other transcription factors (Morrison et al., 2018). PHD fingers are one of the histone effector domains, other than bromodomains or chromodomains which are known to be other histone readers. Additionally, substitution of lysine 16 in H4 (H4K16) to glutamine in its tail results in tail compaction and weakens its interaction with DNA. Another study confirmed the direct effect of acetylation of H4 tail at lysine 16 (H4K16Ac) on higher order chromatin structure. The incorporation of H4K16Ac into the nucleosome array disrupted the assembly of the nucleosomes into 30 nm like fibers. It can also prevent the chromatin remodeling enzyme ACF to slide nucleosomes in *cis* along DNA; this was studied through an *in vitro* reconstitution of end-positioned mono-nucleosome and through gel shift assay (Shogren-Knaak et al., 2006).

Methylation of histone tails like histone 3 lysine 4 (H3K4me3) have also been associated with transcription as they are found enriched at the promoters and TSS. While H3K4me3 has been implicated in recruitment of transcription initiation factor TFIID to express p53 target genes, there are reports that H3K4me3 only slightly enhances expression in a context based on the status of DNA methylation as H3K4me3 occurs in mutually exclusive way with DNA methylation. Therefore, H3K4me3 can act to protect genomic regions from improper DNA methylation during development as seen in oocytes of mouse, xenopus and zebrafish, signifying crosstalks between histone PTMs and DNA epigenetic modifications (Millán-Zambrano et al., 2022).

Further, di or tri methylation of histone 3 lysine 36 (H3K36me) has been tightly related to actively transcribed regions, though they do not directly cause transcriptional elongation (Millán-Zambrano et al., 2022). Both H3K36me2 and H3K36me3 have been known to direct the functioning of Rpd3S. Rpd3S is a histone deacetylase complex recruited by elongating RNA polymerase II, to remove histone acetylation and thereby inhibit transcription initiation from non-promoter regions within a gene body, which is also called cryptic transcription (Lee et al., 2013). Another study found that SET2 methyltransferase dependent H3K36 methylation facilitates Non-Homologous End Joining (NHEJ) mediated repair over Homologous Recombination (HR) which occurs when H3K36 is acetylated in fission yeast (Pai et al., 2014). When observing its cross

talk with DNA epigenetic modifications, H3K36me2 accumulates in transcriptionally active regions and also serves to act as a mark to attract DNA methyltransferase DNMT3A to induce DNA methylation patterns. H3K36me3 attracts DNMT3B and the histone deacetylase complex to prevent cryptic transcription. Further, H3K36me2/3 has been also associated to repressed satellite transcription in fission yeast, mouse, and human pericentromeric heterochromatin (PCH) (Goto et al., 2024).

During development, some genes may be contained in regions orchestrated by (super)enhancers (regions that mediate transcription of genes associated with them) or orchestrated by (super)silencers (regions in the genome that mediate silencing of gene expression). Two repressive histone marks associated with genome silencing through histone methylation are H3K27me3 and H3K9me3.

H3K27 is methylated by a methyltransferase complex called PRC 2 (Polycomb repressive complex 2) and represents the facultative heterochromatin (where developmental genes are repressed based on cell-type or in time-dependent manner during cell development). Though the mechanisms causing this repression are being explored and not completely understood, there are investigations on how H3K27me3 may act as silencers (regions in the genome that mediate silencing of gene expression). H3K27me3 can mechanistically compact associated DNA to form clusters of H3K27me3 peaks in the genome and facilitate chromatin interaction to silence target genes. It was shown that removal of H3K27me3 silencer led to changes in long-range chromatin interaction with the IGF2 gene thereby upregulating IGF2 expression, hinting at the repressive capability of H3K27me3 (Cai et al. 2021).

H3K9 is di/trimethylated by the methyltransferase SUV39h1/h2 or SETDB1 or G9A in human/mouse. Binding of the reader heterochromatin protein HP1 to the methylated H3K9 causes HP1 oligomerization and spreading, forming the constitutive heterochromatin (where regions stay silenced all the time). This primarily occurs in the repetitive DNA clusters like centromeric and telomeric satellites and other interspersed repeats like transposable elements (TEs) scattered in the euchromatic regions, but also in silence lineage-specific genes (Padeken et al., 2022). There are also reports on how H3K9me3 established by either SUV39 or SETDB1 are different. SUV39 forms heterochromatin at pericentromere and SETDB1 acts to heterochromatinise telomeres, promote recombination for alternate lengthening of telomeres (ALT), loss of SETDB1 suppresses ALT. Generally, heterochromatin is associated with repressed transcription, but it was seen that such heterochromatin formation by SETDB1 led to increased telomere transcription possibly due to H3K9me3 ’s coexistence with H3K36me3 (Gauchier et al., 2019). Further, cross-talks between H3K9me3 and DNMT1 mediated DNA methylation with the help of UHRF1 (Ubiquitin like, containing PHD and RING Finger domains) were previously observed, as UHRF1 binds to hemimethylated DNA and H3K9me3 at replicated heterochromatin, and this catalyzes the ubiquitylation of H3 at some lysine residues. This ubiquitylated site is recognized by DNMT1 and then causes allosteric stimulation of DNMT1 (Ren et al., 2020).

Overall, the literature suggests that most of the modifications in the histone tail do not have a direct effect on the stability or structure of chromatin/nucleosome with the exception for H4K16Ac, but that they rather have an outcome through binding of effector proteins. On the other hand, core histone modifications at the lateral surface can directly affect the binding of DNA around the nucleosome and affect nucleosome mobility on/from the DNA (Millán-Zambrano et al., 2022).

iii. Nucleosome positioning through histone sliding, ejection and eviction:

As chromatin is a hub of dynamics in terms of chemical modifications or spatial orientation of DNA or histones, the nucleosome undergoes constant remodeling in an ATP-independent or ATP-dependent manner. The alteration in the nucleosome wrapping, sliding, assembly and deposition of histone variants is termed chromatin remodeling, and it is associated to changes in nucleosome occupancy, positioning and composition. This occurs in two ways: First, histone tails or core undergo PTM affecting the interaction at DNA-histone or histone-histone interface. This causes a shift in the free energy difference between fully wrapped nucleosomes and altered nucleosome structures thereby changing the energy landscape of the nucleosome. Second, some PTMs can also lead to recruitment of histone chaperones or chromatin remodelers that utilize ATP to make changes in nucleosomes (Bowman & Poirier, 2015).

One distinguished histone PTM is the poly (ADP-ribosylation) carried out by ADP-ribose polymerase (PARPs), hence termed as PARylation. This PTM is unique compared to other PTMs because of its two phosphates for every single ADP-ribose unit, giving it a stronger electrostatic character, which strongly competes with DNA. PARylation was seen to occur in H1 and the core histone particularly H2B. During DNA damage, in addition to phosphorylation of H2AX, histones are also PARylated by PARPs in single and double stranded breaks. Inhibition of PARPs suppressed eviction of histones from the site of DNA lesions. In fact, at DNA lesions following damage, PARylated histones were recognized by the FACT complex, a histone chaperone, and facilitated the removal of histones (Yang et al., 2020).

Generally, transcription or repair occurs in regions devoid of nucleosomes to avoid any roadblock and easy accessibility, however for genes whose promoters still have nucleosomes, for example genes present at the facultative heterochromatin, the transcription machinery can also be accompanied by chromatin remodelers that alter the chromatin structure in an ATP-dependent manner. Chromatin remodelers in eukaryotes function distinctively by nucleosome sliding along the DNA, histone eviction, histone variant deposition into the octamer or nucleosome exchange as shown in **figure 7** (Clapier et al., 2017), and are classified into four families:

a. ISWI (Imitation switch): They mostly allow linear translation of nucleosomes along DNA without any histone ejection and are mostly associated with DNA replication, repair and expression. As histones like H2A-H2B dimers and H3-H4 tetramers are deposited following DNA replication, the ISWI remodeler subfamily complex like ACF helps nucleosomes to assemble into mature fully wrapped octameric nucleosomes followed by sliding for proper spacing between them to form a nucleosome array (Clapier et al., 2017). ACF accumulates in the mouse pericentromeric heterochromatin (PCH) and its depletion delays the progression of S phase (Goto et al., 2024). The WICH complex that recruits methyltransferase in PCH is involved in modulating H4K20me3 and H3K9me3 in the major satellite and rDNA and regulating genome instability. Most ISWI subfamily complexes are involved in repressing access to chromatin except the NURF complex (Hasan & Ahuja, 2019).
b. SWI/SNF (Switch/Sucrose Non-fermentable): They mostly let the nucleosome slide along the DNA or can even eject histones making the DNA more accessible to different proteins. They were the first ATP-dependent remodelers to be discovered in yeast. They are known for regulating transcription by remodeling promoters and enhancers as their inactivation led to reduced nucleosome occupancy at many promoters and TSS. BAF, a complex of one subfamily, can directly eject H3K27me3 marked nucleosomes or Polycomb repressive complexes (Kadoch et al., 2017) (Stanton et al., 2017). The subunits of this family are found mutated in many cancers.
c. CHD (Chromodomain Helicase DNA-binding): These complexes have chromodomains to bind to methylated histones or DNA. They also act similarly to ISWI in maturation and spacing between nucleosomes.
d. INO80: The remodelers of this family help ejection of particular histones independent of replication and replace them with other canonical histones or histone variants.

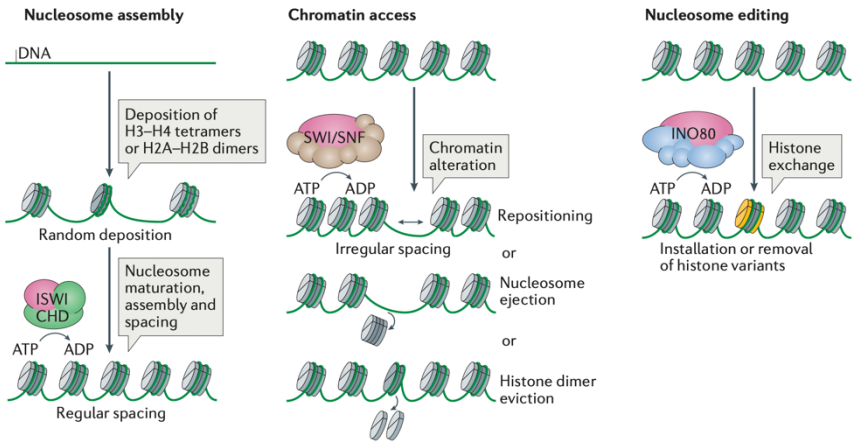


Figure 7: Functional classification of different ATP dependent chromatin remodelers. Nucleosome assembly: ISWI and CHD family remodelers. Chromatin access: SWI/SNF family remodelers. Nucleosome editing: INO80 remodeler (Clapier et al., 2017).

The histone modifications are set-up, maintained and reset via enzymatic and non-enzymatic mechanisms. Enzymes that catalyze the formation of a particular PTM are called writers; proteins that identify and recognize specific PTMs on histones are called readers; erasers are enzymes that remove PTMs (Millán-Zambrano et al., 2022). The enzymes involved depend on the nutrient (substrates) availability and presence of co-factors which are by-products of many interconnected signaling pathways. For example, metabolite like butyrate obtained during fatty acid metabolism inhibits histone deacetylase (HDACs). Butyrate produced during fasting forms β-hydroxybutyrylate histones that in turn can switch on genes responsive during starvation. Another example is the alteration in methionine metabolism, that causes alteration in DNA methylation or histone methylation including H3K4me3 or H3K9me1/2/3 in human colon cancer cells (Dai et al., 2020) (S. Zhao et al., 2021). In mouse, methylase SUV39H1 at ribosomal DNA repeats is regulated by the energy status, particularly NAD+ and HDAC SIRT1 to allow deacetylation and subsequent methylation of histones H3 and H4 (Padeken et al., 2022). Further, lactate formation primarily occurring in cancer cells lead to histone lactylation which has been found to induce homeostatic genes associated to wound healing (Zhang et al., 2019).
Therefore, factors other than DNA sequence, like the environment, diet, or conditions like senescence with dysregulated metabolism (Crouch et al., 2022), hugely influence the expression of genes through histone modifications and chromatin compaction and can therefore be included as epigenetic controllers of cell behavior.

1.2 Cell Cycle and checkpoints

An individual cell and its fate through its life cycle is determined by signals it receives from its surroundings, such as availability of growth factors, proper cell attachment mediated by integrins, proper cell metabolism and genome integrity. Based on these elements, a cell would decide whether it should proliferate into generating more daughter cells or halt its journey. In addition, some cells depending on the extracellular signal decide to enter a state of post-mitosis, a terminally differentiated state from which cells don't resume their proliferation and perform specialized functions.
All these signals (external and internal) are integrated and processed through a network of interacting proteins where the decision is made about cell proliferation and transition through various phases. This results in exponential multiplication of mammalian cells and generation of two genetically identical daughter cells through the process called mitotic cell cycle. This is however different from meiosis which is a special type of cell division where four daughter cells are generated, and they occur in germ cells.

While the stages of the cell cycle leading to cell division (mitosis) were previously studied and described by Walter Flemming in 1882, understanding of the complete cell cycle began around mid 1900s by Stephan R. Pelc, a pioneer in autoradiography and Alma Howard when they observed incorporation of phosphorous-32 into dividing meristematic cells of *Vicia faba*. They were also able to time the incorporation into the nuclear DNA by removing all P-32 and DNA by acid hydrolysis and they named it as S (synthesis) phase. They saw that the synthesis did not occur all throughout the cell cycle. The gaps they saw that occurred before and after S phase was thought to have other metabolic processes and hence, they termed it as G1 (Gap 1) and G2 (Gap 2) phases ("From the Archive," 2023). Later in the 1960s, Laszlo Lajtha, a leading stem cell biologist, observed that hematopoietic stem cells escaped the toxic effects of radiations and chemicals, and predictively attributed this to the quiescent state of cells, which was termed as G0 (Orlic & Sharkis, 1997). The four steps of a complete cell mitosis are:

i. <u>G1 Phase</u>:

In the mitotic cell cycle, new cells formed after the process of cytokinesis advance to the next cycle of growth and cell division. In this phase termed as G1, within a discreet window time (initial 80-90% of G1), the cell must decide based on its extracellular environment and growth signals (mitogens) whether it will proceed to enter the new cycle of growth, stay in G1 or to retract to a specialized resting state called G0 (quiescent phase) for a specific period with or without terminal differentiation. This critical decision-making point is called the restriction point or R point, beyond which the cell would proliferate even in the absence of mitogens. However, a very recent study (Cornwell et al., 2024) challenged this long-standing textbook-fact as they found that upon removing mitogens from the media before the R point, around 15% cells still proceeded to replicate DNA, and this was evident as subsequent G1 cells had 4N number of chromosomes.

Once it has decided to advance into a new cycle, the cell needs to have enough cellular constituents sufficient for distribution to the upcoming two new daughter cells and this is ensured by doubling all the macromolecular components, including the genome inside the cell. Almost immediately after cytokinesis, preparation for doubling the genome starts by accumulation of necessary proteins and building blocks of DNA which typically lasts up to 11-12 hrs.

The progression to cell cycle is dictated by the signal broadcast which is transmitted into the cell by downstream responders (enzymes) like protein kinases. They phosphorylate multiple substrates acting as switch for the activities of these substrates and prepares the cell for S or M phase. For example, phosphorylation of Lamin A/B by CDK1 (cyclin dependent kinase) which is required for breaking the nuclear envelope during early mitosis (Kochin et al., 2014) or CDK2 mediated phosphorylation of linker histone H1 prevents its interaction with HP1-α and disassembles higher order chromatin structures. Phosphorylation of H2B at serine 6 by CDK1 at early mitosis at inner centromere and pericentromeric heterochromatin is required for mitotic chromosome segregation (Seibert et al., 2019).

Cyclin dependent kinases are serine/threonine kinases and, as their name suggests, they are dependent on associated regulatory subunits called cyclin proteins. Most of G1 is driven by the complex of newly synthesized cyclin D with CDK 4/6 until the R point and beyond this, a complex of cyclin E-CDK2 takes over to prepare substrates necessary for entering the S phase (**figure 8 A**).

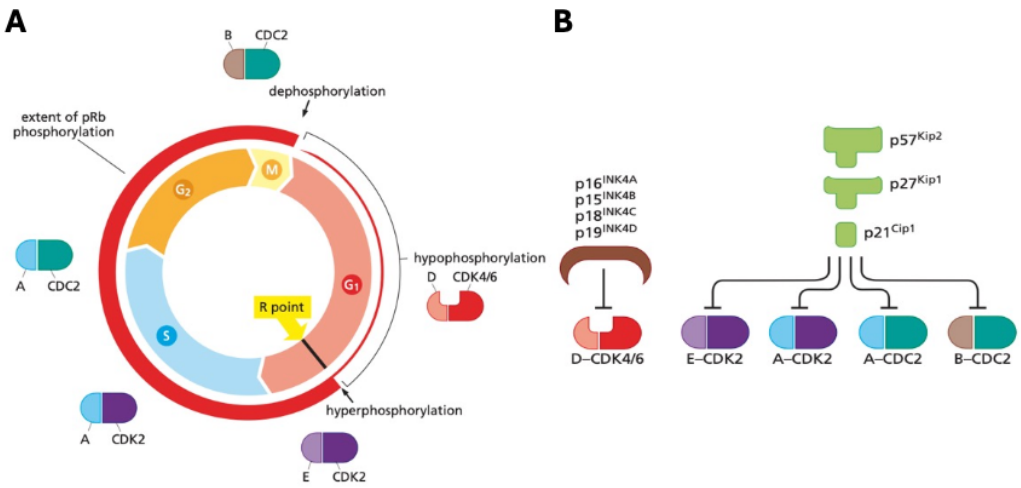


Figure 8: Regulation of cyclin-CDK complex governing the progression of cells cycle (Weinberg, 2013).

A) Schematic of different phases of eukaryotic cell cycle with different cyclin-CDKs regulating the cell cycle progression.

B) Inhibition of cyclin-CDKs by a number of CDK inhibitors or tumor suppressors.

It has long been thought that this CDK 4/6 complex phosphorylates tumor suppressor retinoblastoma (Rb) that in turn leads to Rb's dissociation from transcription factors E2F 1, 2, 3 and subsequently activates them and 100s of genes. For example, E2F bound to a promoter can attract histone acetylases and remodel the chromatin to induce gene expression. In fact, E2F mediated promoters are also remodeled by SWI/SNF (Nagl et al., 2007). However, during cell cycle pause/arrest when Rb remains hypo-phosphorylated, it blocks the transactivation domain of E2F to repress its ability to transcribe genes and Rb can independently recruit proteins like HDAC (histone deacetylase) to remodel chromatin and repress gene transcription (Blais & Dynlacht, 2007). Therefore, these cell-cycle regulatory proteins serve to remodel the architecture of chromatin that influences the progression of the cell cycle. Other genes expressed in late G1 are genes involved in synthesizing DNA nucleotide precursors (dihydro folate synthase, thymidine kinase).
However, when a cell encounters severe stress like DNA damage, hypoxia, etc. there is the possibility of reversing phosphorylation of Rb mediated by phosphorylases and this brings up the growth inhibitory effect of tumor suppressor protein Rb. Even a CDK inhibitor like p16 through its inhibitory effect on CDK 4/6 can prevent phosphorylation of pRb and prevent cell cycle progression (**figure 8 B**). These cellular stresses can also activate cell cycle arrest through the DNA damage response mediated by ATM (ataxia-telangiectasia mutated) or ATR (ATM- and Rad3-Related) and/or p53 which will be discussed in later sections. E2F also activates cyclin E and A for progression to subsequent phases. Complex of cyclin E-CDK2 further hyperphosphorylates pRb and inactivates it. This complex also inactivates CDK2 inhibitor like $p27^{Kip1}$ operating as a positive-feedback loop. The cell then proceeds to S phase.

As mentioned above, the mitogenic signals or growth factors can drive cells into G1 and this can happen though distinct signalling pathways. For example, increase in cyclin D through signalling induced by small GTP binding and hydrolysing protein called Ras, located close to the cell membrane. While in the nucleus, a growth promoting TF called Myc along with its complex partner Max, drives the signal cascade to express cyclin D2 and also CDK4 by inhibiting the action of CDK inhibitors p21 or p27 as shown in **figure 9**.

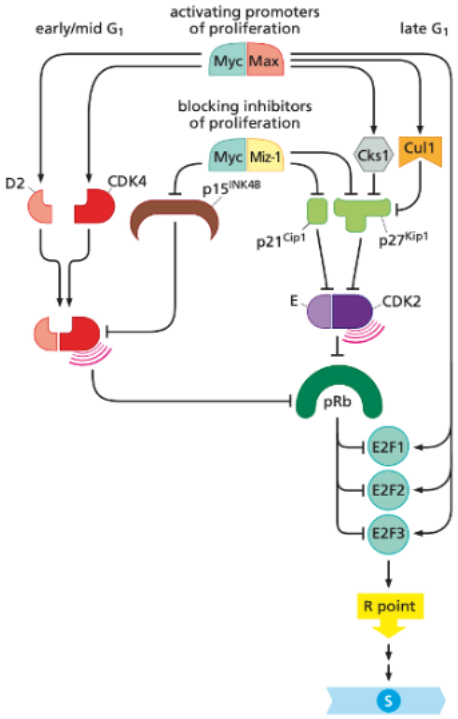


Figure 9: Myc together with Max modulates positive and negative regulators for cell cycle advance: First, it is able to induce expression of growth promoting cyclin D2 and CDK4 and advance through early G1. Simultaneously, it can also increase expression of Cul1 and Cks1 (degragdes CDK inhibitor p27). Moreover, Myc together with its partner Miz-1 can induce repression of CDK inhibitors $p21^{Cip1}$, $p15^{INK4b}$ and $p27^{Kip1}$. Overall Myc leads to advancement to S phase (Weinberg, 2013).

ii. S phase:

As the cell transitions from G1 to S phase, cyclin A starts building up in the cell and complexes with CDK2 and now phosphorylates E2F, switching off its transcriptional activity. During S phase, the genome of the cell gets replicated requiring 6-8 hours. The double-stranded DNA (dsDNA) is unwound, and the parental strand acts as a template for a complex replication machinery to form duplicated daughter strands. The details of this replication machinery will not be explored here; however, this is achieved by a coordination of key proteins at the replication fork: helicase, single stranded binding proteins, primase, replicative polymerases. Pol α is required for the initiation of the synthesis of both the lagging and leading strand. At leading strand, Pol ε takes over Pol α to continue the synthesis, and at the lagging strand, Pol δ takes over pol α to generate 100-200 bp fragment called Okazaki fragment (Koyanagi et al., 2022).

To start replicating, the helicase unwinds the double-stranded DNA (dsDNA) and the single-strand DNA (ssDNA) binding protein like Replication Protein A (RPA) stabilizes the ssDNA. The primase then builds a short RNA primer, and the DNA Polymerase begins its processivity. To achieve this, there must be a coordination between DNA unwinding and Pol-primase activity as DNA must be unwound for the polymerase to act. This link ensures that the helicase doesn't just run off leaving the Pol-primase far behind or else it might lead to generation of long stretches ssDNA (bound by RPA). Persistent binding of RPA to long stretches of ssDNA is a signal for the cell that there is a problem in replication. Indeed, RPA-bound ssDNA recruits (ATR) kinase and activated ATR leads to triggering of S-phase checkpoint surveillance mechanism (Segurado & Tercero, 2009) (Toledo et al., 2013).

Unwound DNA is more susceptible to replication stress, especially the endogenous replication stress which the cell intrinsically faces as shown in **figure 10** and this can lead to ssDNA formation (Panagopoulos & Altmeyer, 2021). Exogenous DNA damaging agents can also stall replication forks as DNA polymerase can encounter bases. It is during the S phase when much of DNA repair occurs inside the cell (Li et al., 2023).

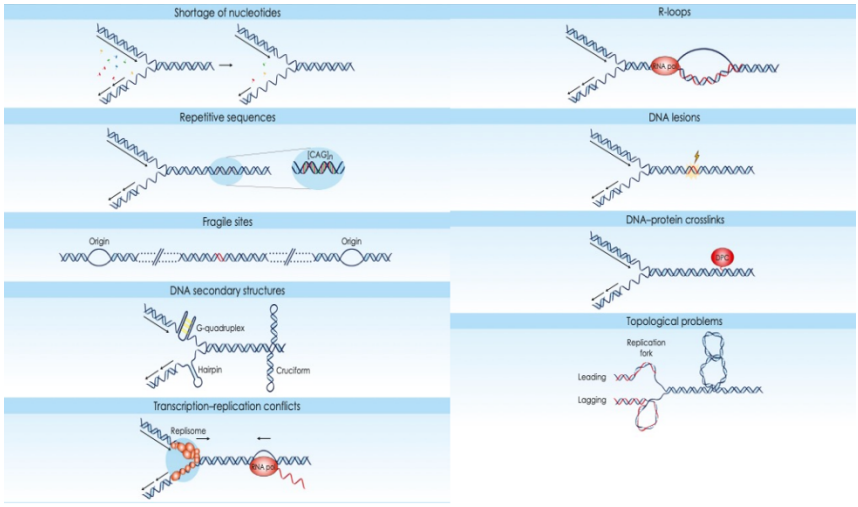


Figure 10: Endogenous sources of replication stress. The sources are nucleotide shortage, repetitive DNA sequences, chromosomal fragile sites, DNA secondary structures, transcription–replication conflicts, co-transcriptional R-loops, replication fork-stalling DNA lesions, and DNA–protein crosslinks, as well as topological constraints that affect replication fork progression (Panagopoulos & Altmeyer, 2021).

Activated ATR at stalled replication forks ultimately have been shown to delay progression of cell cycle, stabilize replication forks and prevent late origins from firing (Leung et al., 2023). At RPA-coated ssDNA, ATR in complex with its interacting protein ATRIP is activated by TOPBP1 (topoisomerase II binding protein 1) or ETAA1 (Erwing tumor associated antigen 1). The activated ATR kinase can then either directly activate tumor suppressor p53 by phosphorylation or via phosphorylating downstream Checkpoint kinase1 (CHK1) which then in turn phosphorylates p53 (also known as the guardian of the cell) to activate it resulting in cell cycle arrest (**figure 11 A**). CHK1 also phosphorylates Cdc25, a phosphatase which when phosphorylated becomes inactive. Active Cdc25 is known to activate CDKs.

ATR is also known to play an important role in unstressed cells, for example, ATR regulates the speed of origin firing and prevent excess origin firing during early S phase to maintain the right amounts of dNTPs (Menolfi et al., 2023). Further ATR is also known to inhibit the activity nuclease Mus81 and protect replication forks. ATR also protects the nascent DNA from ssDNA gaps generated by Primase-Polymerase during DNA replication (Leung et al., 2023).

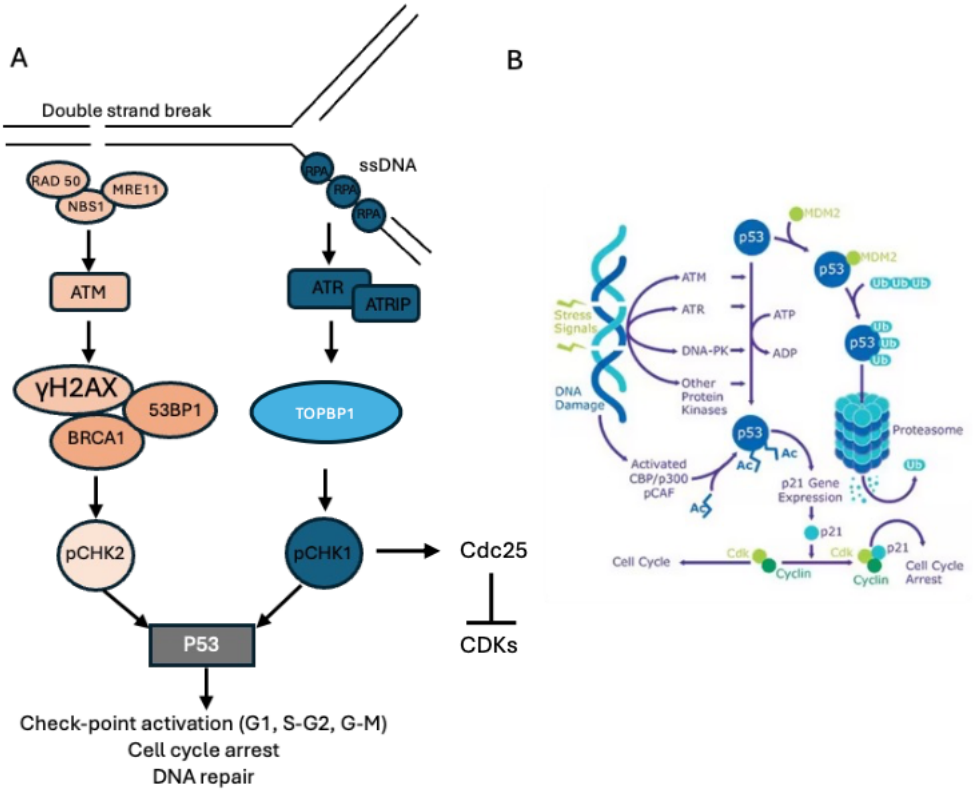


Figure 11: A) dsDNA break and ssDNA activating the DNA damage response leading to cell-cycle checkpoint activation and cell cycle arrest.
B) In unstressed cells, MDM2 binds to p53 and continuously ubiquitylates it for proteasomal degradation. When p53 is phosphorylated by ATM/ATR pathway, it becomes resistant to degradation. Additionally, p53 can also be acetylated by p300 that makes p53 transcriptionally active and induces p21 expression.

In absence of ATR signaling, a persistent ssDNA gets converted into dsDNA breaks (DSB), especially at replication forks. This break is detected by MRN complex (MRE11-RAD50-NBS1) and leads to recruitment and activation of the ATM kinase. ATM recruited at dsDNA breaks then either directly phosphorylates p53 or through phosphorylation of CHK2. Activated p53 increases p21 expression which inhibits cyclinE-CDK2 activity (**figure 11 B**) and leads to cell cycle arrest.
ATM also phosphorylates the histone H2AX also known as γH2AX, at the site of insult and spreads to megasized flanking regions, within TADs, allowing a megabase scale signalling event (Arnould et al., 2021). γH2AX acts as a docking site for protein complexes like mediator of DNA damage checkpoint protein 1 (MDC1) which in turn, recruits proteins like 53BP1 and BRCA for Non-homologous end joining (NHEJ) and Homologous Recombination (HR) mediated DNA damage repair pathways, respectively (Noubissi et al., 2021). The choice of HR versus NHEJ is a matter of investigation but multiple factors affect the choice of repair, for example, the DNA end resection after DSB creates 3’

overhangs and favors HR, while blocking end resection will favor NHEJ (F. Zhao et al., 2020). In another study it was shown that removal of 53BP1 from DSB is mediated by phosphorylation of SP1 by cyclin A-CDK2 and this leads to SP1's eviction exclusively in S/G2. Failure to evict leads to BRCA1 binding and HR initiation (Swift et al., 2021). While accurate repair by HR is preferred, in conditions when the DNA overhangs formed by resection fail to base pair completely, but rather short DNA tails make base pairings by microhomologies, then the microhomology mediated end joining repair pathway (MMEJ) comes to play (Daley & Sung, 2014).

iii. G2 Phase:

At the end of S phase after successful DNA replication and repair, the ATR mediated S phase checkpoint is turned off and mitotic transcriptional network is turned on. ATR inhibition deregulates S/G2 transition causing early mitosis, under-replicated DNA and DNA damage (Saldivar et al., 2018).

Under normal conditions, as the cell makes the S/G2 transition, it starts accumulating cyclin B and prepares itself for mitosis. This phase bears accumulation of pro-mitotic regulators like Bora together with Aurora A which later leads to activation of Polo like kinase (Plk1). They are important for activation of CDK1 (also called CDC2), centrosome maturation and mitotic entry (Seki et al., 2008). At the end of G2, CDK1 activity rises and cyclinB-CDK1 translocate from cytoplasm to nucleus before the nuclear envelop breakdown (Lockhead et al., 2020). It has also been seen that the p38 MAPK (Mitogen activated protein kinase) also mediates the initiation of G2/M checkpoint. p38 inhibits and phosphorylates Cdc25B. Cdc25B dephosphorylation is known to activate CDC2 (CDK1) and promote G2-S transition. Further, p38 MAPK can phosphorylate p53 and lead to G2/M checkpoint (**figure 12**) (Thornton & Rincon, 2009) (Lockhead et al., 2020).

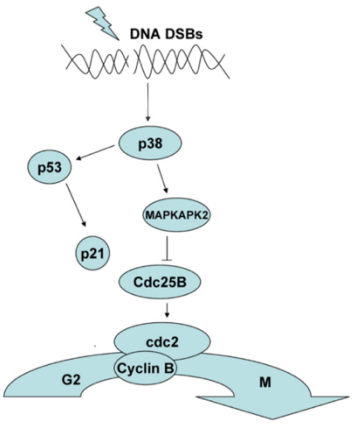


Figure 12: Role of p38 MAPK in G2/M checkpoint

iv. Mitosis:

After all the duplication of cellular components including the genome, the cell begins to proceed to mitosis for equal distribution of these components to each daughter cell. This is usually the shortest phase of the cell cycle and comprises five stages: prophase, metaphase, anaphase, telophase and cytokinesis.

Prophase:

The newly replicated sister chromatids have to be held together until the anaphase when sister chromatids are segregated to migrate to the opposite poles in the cell. During S phase as the DNA undergoes replication, this cohesion is achieved by protein cohesin that was deposited along the length of the chromosome before the S phase (Srinivasan et al., 2020). Cohesins are members of structural maintenance of chromosome (SMC) proteins that are DNA binding proteins modulating DNA architecture. The cohesion along the chromosome arms starts to be removed with the activity of kinases Aurora B, Plk1, but not the cohesion at centromeres (Stevens et al., 2011). The centromere is the region in the chromosome that holds the two sister chromatids together. Later cohesin removal activity is taken over by separase which we will see later. The initiation of prophase begins with condensation of DNA by assembly of condensin complex achieved by phosphorylation activity of cyclinB-CDK1 (Wang, 2021) as shown in **figure 13** (Skibbens, 2019).

Metaphase:

The nuclear envelope starts to breakdown at the beginning of metaphase and the chromosome starts to migrate to align at the equator of the cell. The components of the outer kinetochore build on the chromosome. The kinetochore is a structure formed on centromeres and is comprised of an assembly of proteins on which the spindle microtubules attach for sister chromatids to undergo separation. This occurs during the prometaphase when all the chromosomes start to get aligned at the equator, mediated by microtubules (MTs) nucleating out of the spindles poles towards the chromosomes and aligning them at the center (from Microtubule organizing center in the centrosome at both poles) (Navarro & Cheeseman, 2021). Here, the Spindle Assembly checkpoint (SAC) ensures that all the chromosomes are attached to the microtubules from the opposite poles (microtubule biorientation), otherwise it prevents the progression from metaphase to anaphase and acts as a mitotic block. Moreover, the strength of binding is sensed as tension created across the sister chromatid upon pulling by the MTs. As a result, the tension generated across sister chromatids (which are cohesed at the centromere until anaphase (Hauf et al., 2005)) because of pulling acts like a confirmation of proper attachment of MTs to the kinetochore. Insufficient tension leading to destabilization of weakly attached MTs

activates Aurora B which is able to sense tension and promote error correction by modifying kinetochore proteins (Bunning & Gupta Jr., 2023). Therefore, attachment of MTs to the kinetochore and the strength of binding (tension) are the two ways to ensure proper attachment of MTs to the kinetochore.

Once properly attached, separation of sister chromatids is mediated by E3 ubiquitin ligase Anaphase promoting complex/cyclosome together with its coactivator Cdc20 (APC/C^{Cdc20}). This then ubiquinates and degrades securin releasing the enzyme separase to allow segregation of sister chromatids; it also degrades cyclin B to promote transition to anaphase (Schrock et al., 2020). However, unattached kinetochore promotes SAC signaling by catalyzing the formation of Mitotic checkpoint complex (Mad2, Cdc20, BubR1) on kinetochores, a potent inhibitor of APC/C (Lara-Gonzalez et al., 2021).

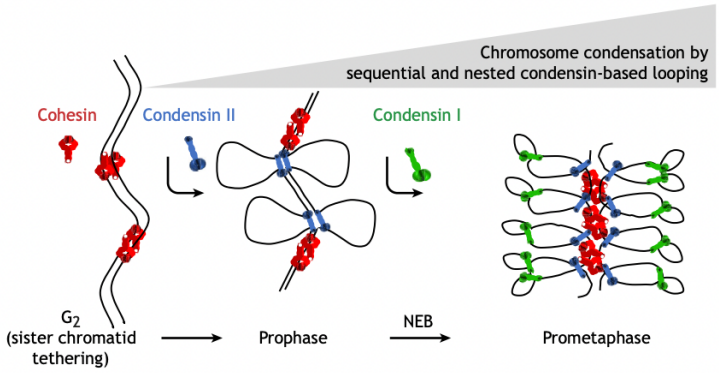


Figure 13: Cell-cycle-regulated chromosome compaction by sequential recruitment of condensins II and I promote the formation of nested loops. Sister chromatids are tethered together by cohesins (red). During prophase, condensin II (blue) binds DNA and extrudes loops. After nuclear envelope breakdown (NEB), condensin I (green) binds the looped DNA and forms new loops that are nested within the condensin II-generated loops. As cells progress into late prometaphase, DNA loop extrusion and compression of the helical scaffold continues. Not shown is the role for cohesin in generating cis-based compaction, which also promotes chromosome condensation (Adapted from Skibbens, 2019).

Anaphase:

After successfully ensuring that all the chromosomes have biorientation microtubule attachment, there is initiation of separation and migrating of sister chromatids to opposite poles. This is achieved in two steps: first, the depolymerization of the microtubules (MTs) that initially originated from spindle pole and attach to the kinetochore (called kinetochore-attached microtubules), leading each chromatid to move towards the respective poles. This phase is called Anaphase A (mediated by k-fiber explained below); the second is the spindle elongation at midzone (by interpolar MTs) that further separates the disjointed sister chromatids and is called as Anaphase B. Anaphase A and B occur almost simultaneously.

Microtubules have the growing end called 'plus' end that attaches to the kinetochore and the 'minus' which is towards the pole. Each spindle is comprised of many filaments of microtubules bundled together to form the kinetochore fiber (k-fiber), terminating at each kinetochore.

There are two types of forces that act on the chromosomes to align them and separate them to proceed the poles: a) Pulling forces by the MTs that act on the kinetochore (k-fiber) and b) Polar ejection forces by interpolar MTs that push and act on the arms of the chromosomes. These forces were shown to be responsible for aligning the chromatids at the equator (Brouhard & Hunt, 2005). The pulling of the chromatids towards the poles and the force required are generated by depolymerization of the microtubules attached to the kinetochore (plus end) or depolymerization at the minus end. Polar ejection forces lead to pushing the chromatid, driven by polymerization of MT at its plus end (as shown in **figure 14**) (Vukušić & Tolić, 2021), with pulling forces exceeding the pushing forces. The first study to measure force required to impede the movement of chromatids to the pole was carried out in the spermatocyte of grasshopper and found that 700 pN was the force generated on the kinetochore to exert movement towards the pole. Indeed, it closely corresponds to the force calculated in a recent study where each kinetochore MT exerted 15pN force (considering each spindle comprise of 17 microtubules in human) (McVey et al., 2021). As anaphase A progresses, the centromeric constrictions and the kinetochore are the major sites where the force of the MTs is transmitted as they lead the movement when the chromosomes are pulled towards the poles (as shown in **figure 14** (Asbury, 2017)). Therefore, structures like the centromere-kinetochore which act as a load bearing element should be stable enough to withstand and exert forces to act against the pulling forces of MT bundles.

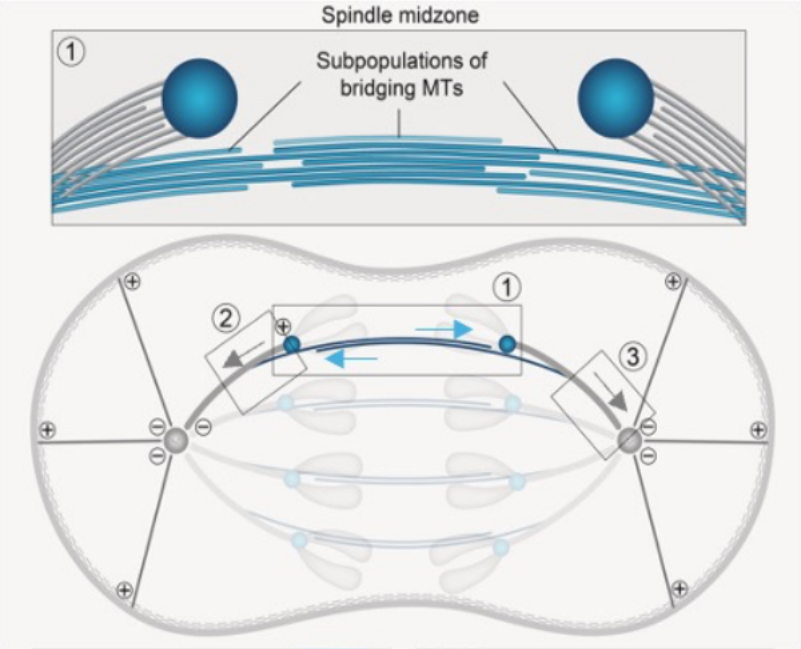


Figure 14: Structure of mitotic spindle in human cells during anaphase. The + and – sign denote the microtubule ends. Blue circles represent kinetochores attached at the centromere of the chromosome. The microtubules in grey represent k-fiber and the ones in blue represent the interpolar MTs. The grey arrows represent the pulling forces by k-fibers and the blue arrows represent pushing forces by interpolar MTs (Vukušić & Tolić, 2021).

2. The Centromere

Centromeres were first described in 1882 by German anatomist Walther Flemming as primary constrictions on distinct mitotic chromosomes connected to the fibers arising from the spindles (Flemming, 1882). After almost 50 years, the term centromere was first coined by Cyril Darlington in 1936 which emanated from Greek meaning centro (center) and meros (part). Centromeres show wide variation in their structure and organization among different organisms. The spectrum begins from the presence of monocentric chromosomes in some eukaryotes where the centromeres are restricted at a single locus to holocentric chromosomes present in some nematodes, insects and plants where the centromeres are diffused along the entire length of chromosome. The monocentric loci can occur as regional centromere or point centromere. Regional centromeres range from kilobase-megabase of DNA, where the centromere form multiple microtubule attachment per chromosome, and they mostly occur in eukaryotes including human. On the other hand point centromeres in budding yeast span 125 bp of DNA forming a single nucleosome unit, capable of forming functional kinetochore and is found in budding yeast (Furuyama & Biggins, 2007).

In human, the centromere is a constricted region in the chromosome that holds the two sister chromatids together, as originally described. Unlike the term, centromeres do not necessarily occur at the center of the chromosome. (Sub)metacentric chromosomes are those where both the arms of the chromosome on either side of the centromere are approximately of same length, acrocentric chromosomes are those whose arms on one side of the centromere is quite short than the other and telocentric where the centromeres are present close to the tip of chromosomes. Despite the variation in their structure and organization across different species, centromeres uniquely carry out one function: to attach the chromosomes to the dynamic microtubules arising from the spindle poles. They do that by assembling a load-bearing protein complex called the kinetochore during mitosis and meiosis (Cleveland et al., 2003). The entire process of segregation of sister chromatids into the daughter cells during each stage mitosis has been described in detail previously in section 1.2. iv.
For a long time centromeres were not well differentiated from kinetochores but evolution of better fixatives and electron microscopy led to a revelation of a multilayered structure (kinetochore) assembled on the surface of chromatin structure (centromeres) and bound to microtubules (Fukagawa & Earnshaw, 2014). The kinetochore is assembled on a series of centromeric proteins which form the constitutive centromere associated network (CCAN) (Hoffmann et al., 2016a). Through discovery of patients with CREST syndrome (Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, Telangiectasia), it was found that their sera consisted of anti-centromere auto-antibodies (ACA) which stained the nuclei of rat liver in patterned speckles (Moroi et al., 1980). Later, upon characterization of this sera, it was found that it could recognize the

three antigens in human cells, which was designated as centromeric proteins (CENPs), namely CENP-A, CENP-B and CENP-C (W. C. Earnshaw & Rothfield, 1985). Over the years many other centromeric proteins were discovered but we will majorly discuss about CENP-A/B/C built on centromeric satellite DNA.

2.1 Centromeric DNA:

The human genome sequencing surprisingly accounted less than 5% regions coding for protein compared to 95% of non-coding genome of which at least half corresponds to repetitive DNA sequences (International Human Genome Sequencing Consortium et al., 2001). Based on structure, repetitive DNA sequences can be classified into two groups: Interspersed repeats, where repeats are scattered throughout the genome and lack higher order structure, and Tandem repeats, where clusters of individual sequence units are adjacent to each other and organized as direct repeats (head-to-tail) or inverted repeats (head-to-head and tail-to-tail) (McNulty & Sullivan, 2018).

One of the many types of tandem repeats is the Satellite repeats which was originally discovered as a low density band obtained after separating mouse genomic DNA along the cesium chloride density gradient (Yasmineh & Yunis, 1974). The advent of density gradient separation through addition of mercury or silver led to identification of more separate bands in the human male placental DNA corresponding to human satellite I, II, III and IV (Mitchell et al., 1979) (Corneo et al., 1968) (Corneo et al., 1970) (Corneo et al., 1971). The separation of these bands was obtained based on the average base composition of DNA as low molecular weight like 'A' and 'T' tend to have lower buoyancy and separate from the relatively bulky 'G' and 'C'. Further analysis through the action of restriction endonuclease revealed repeated DNA families which are defined by regularly spaced enzyme recognition sites. By the use of restriction endonuclease EcoRI, one of the family called alpha satellite was identified with tandem repeats of 340 dimer or 680 bps tetramer which also later hybridized to centromeres of human (Manuelidis, 1978) (Willard, 1985). Moreover, through sequencing it was revealed that these repeats consisted of adjacent but diverged copies of AT rich 171 bp unit that showed homology to African green monkey (Manuelidis & Wu, 1978) (Willard, 1985). fIt was found that these alpha satellite sequences are different among different chromosomes leading to the notion that α-satellite DNA did not have a consensus sequence (Willard, 1985).

The Satellite DNA occurring in the human centromeric and pericentromeric region accounts to nearly 6.2% of the recently-assembled telomere-to-telomere genome (T2T genome sequencing), of which ~2.8% are alpha satellite (in the human CHM13 cell line), being the most abundant satellite repeat family of the genome. In human, centromeres are defined by a complex of various proteins classically built on the alpha satellite DNA which consist of AT-rich repeats of 171 bp monomer to form a higher order repeat (HOR)

that can further occur in tandem arrays (head-to-tail) to form HOR arrays (Altemose, Logsdon, et al., 2022). Each HOR can be composed of 2 to 34 α-satellite monomers (each monomer with 60-80% homology) to a length of 5.8 Kbp that can further occur as hundred to thousand repeats to form homogenous HOR arrays (each HOR being 97-100% identical) spanning upto 5 Mbp long (**figure 15**).

In fact, Altemose and Logsdon reported over thousands of different α-satellite monomers and grouped these monomers into 20 suprachromosomal families forming over 80 different HOR arrays (Altemose, Logsdon, et al., 2022). 18 out of 23 chromosomes may contain upto 9 distinct HOR arrays. Kinetochore proteins form on only a subset of these HOR arrays, which are generally the largest array and form the active HORs. Active HORs (ranging from 4.8 Mb on chromosome 18 to 340 kb on chromosome 21) has strong CENP-A enrichment on 7-24% of the total length of active HOR and reduced CENP-A enrichment beyond. Jansen's lab observed in human RPE-1 cells that for every 25 nucleosome, there is deposition of one CENP-A nucleosome and represents a 50 fold enrichment of CENP-A at centromeres than compared to the entire genome (Bodor et al., 2014). With the latest advent of DiMeLo-Seq, it is now known that for every 4 nucleosomes there is one CENP-A nucleosome, as shown for chromosome X of HG002 cell line. DiMeLo-Seq is a technique to map DNA-Protein interaction with high resolution on single molecule long-read DNA sequence, together with analyzing DNA sequence modifications (Altemose, Maslan, et al., 2022).

A point to note here is that the same HOR is not necessarily active in both the chromatids of a chromosome pair giving rise to centromeric epialleles (Maloney et al., 2012). Around 1/3rd of the α-satellite DNA contains CENP-B boxes, a 17 bp sequence that are binding sites for CENP-B protein and are present in varying quantities between chromosomes (Rosandić et al., 2006) and is absent in the Y chromosome (W. Earnshaw et al., 1987). The HOR regions are further flanked on both sides by inactive pericentromeric regions which also bear satellite sequences, especially short α-sat monomers lacking HORs and non-alpha satellite repeats occurring in contigs (Altemose, Logsdon, et al., 2022) as well as intermingled with transposable elements like LINES or SINES (Schueler et al., 2001). As previously mentioned about other satellite sequences, pericentromeric repeats are known to have Satellite I (chromosome 3,4 and acrocentric 13, 14, 15, 21, 22), satellite II (chromosome 1, 2, 7, 10, 16, 22) and satellite III (chromosome 1, 9, 10, Y, and acrocentric 13, 14, 15, 21, 22).

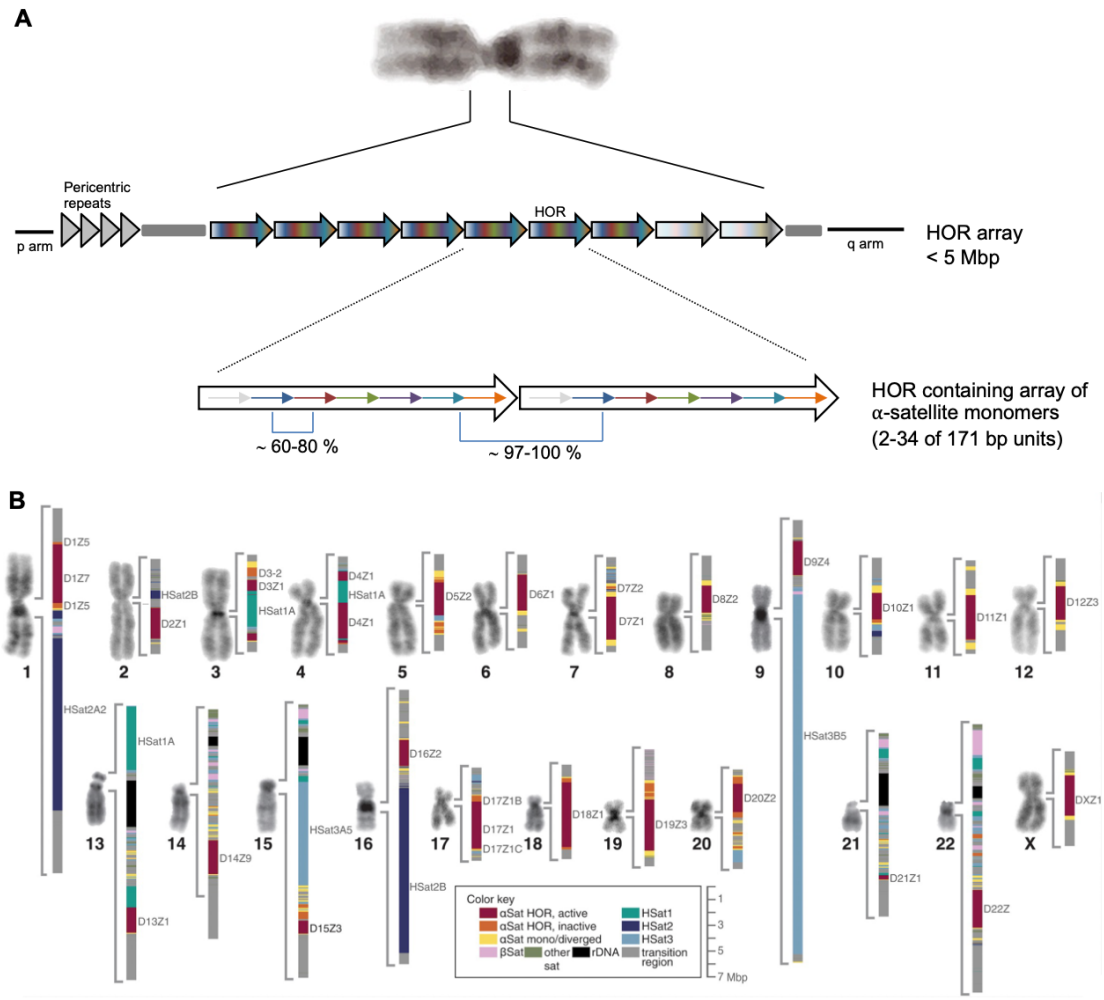


Figure 15: A) Schematic of the organization of centromeric DNA in humans. Centromeres organize themselves with upto 9 HOR arrays spanning upto 5Mb which are 97-100% similar with few sites of sequence variation, HOR structural rearrangement, inversions/or sites of insertions of transposable elements. Each HOR which can be of upto 5.8Kbp long contains 2-34 monomers of α-satellite repeat units organized as head-to-tail tandem repeats. At the adjacent on the right is another HOR array. Flanking regions in grey show monomeric and divergent α-sat monomers, followed by pericentromeric sequences that are found on a subset of chromosomes.
B) Micrographs of CHM13 chromosomes showing the length of HORs at the centromeric and pericentromeric regions across different chromosomes (Altemose, Logsdon, et al., 2022).

2.2 Centromeric Proteins

2.2.1 CENP-A

After the initial discovery of CENP-A by (Guldner et al., 1984) and (Earnshaw & Rothfield, 1985), its further characterization by immunofluorescence using anti-CENP-A in human cells revealed that CENP-A localized at the centromeres. Upon extraction from the nucleus, it co-purified with the other core histone proteins H3 and H4 and nucleosome core particles. This led to the conclusion that CENP-A behaved as a histone like component at the centromeric region (D. Palmer et al., 1987). Further, through peptide sequencing analysis some of the segments of CENP-A was found to have similarity with that of histone H3, while other segments were not related to any histone, giving it a centromere specific-distinct H3 variant like characteristic (D. K. Palmer et al., 1991). The histone-fold domain (HFD) of CENP-A mediate the interaction with histone H4 and shares almost 62% sequence homology with canonical H3, whereas its N-terminal domain remain divergent (**figure 16**) (Sullivan et al., 1994). This study together with Cleveland's lab (Black et al., 2004) found that a sequence in the first loop and second α-helix in the HFD region of the C-terminus was responsible to direct CENP-A to the centromeres and hence named CENP-A targeting domain (CATD), which was absent in the HFD of histone H3. Using deuterium exchange/mass spectrometry coupled with hydrodynamic measures, it was also found that the CATD domain confers compaction and conformation rigidity to the subnucleosomal tetramer built with histone H4 compared to the tetramers of histone H3 and H4 (Black et al., 2004). The CATD domain of CENP-A also mediates its interaction with other centromeric proteins, like CENP-N (Carroll et al., 2009). Further, specific sequence of both terminal tails of CENP-A, which differ from H3, were shown to be crucial for the interaction with other centromeric proteins. For example, CENP-A interacts directly with another centromeric protein CENP-B through it N-terminal tail and favors CENP-B loading at the centromeres (Fachinetti et al., 2013) (Fujita et al., 2015a). Another example is the C-terminal domain that together with the CATD of CENP-A enables its interaction with CENP-C (Fachinetti et al., 2013) (Logsdon et al., 2015).

Using deuterium exchange/mass spectrometry coupled with hydrodynamic measures, it was also found that CATD domain of CENP-A confers compaction and conformation rigidity to the subnucleosomal tetramer with histone H4 compared to the tetramers of histone H3 and H4 (Black et al., 2004). CENP-A has been implicated to create a dynamic chromatin structure as cryo-EM study have shown that CENP-A nucleosomes compared to canonical H3 have increased flexibility and this might allow formation of dynamic higher order chromatin structure (Takizawa et al., 2020) (Nagpal et al., 2023). Conversely, CENP-A has also been shown to increase compaction of chromatin architecture by relieving conformational stress as CENP-A containing nucleosomes were found to be more condensed than canonical H3 and to induce genomic constraints at the

nucleosome DNA entry/exit sites to bring neighboring nucleosome to close proximity (Panchenko et al., 2011) (Geiss et al., 2014).

CENP-A is a key centromeric protein for cell viability. Removal of CENP-A causes missegregation of chromosome, formation of micronuclei as well of nuclear blebbing. CENP-A null mice beyond 6.5 days were not viable. The absence of CENP-A leads to loss of CENP-C and loss of discrete immunofluorescence signal of CENP-B to form diffused CENP-B signal in the nucleus indicating decompaction in local centromeric chromatin (Howman et al., 2000). Moreover, CENP-A recruits kinetochore components to the centromere in many species including mouse, Drosophila, *S.cerevisiae*, as well as in humans (Howman et al., 2000) (Blower & Karpen, 2001) (Collins et al., 2005) (Foltz et al., 2006). CENP-A always localize at centromeres that are active, and can promote kinetochore assembly even when targeted to ectopic loci (Heun et al., 2006) (Logsdon et al., 2015a). Therefore this concludes that CENP-A acts as an epigenetic mark to define, maintain and as well to propagate centromere function, irrespective of the DNA sequence on which CENP-A is incorporated.

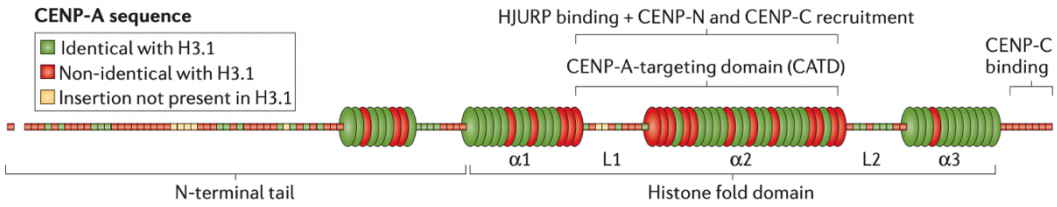


Figure 16: Schematic of human CENP-A primary and secondary structure with H3.1. Each segment corresponds to a single amino acid. α-helices are represented by barrels (designated by α1, α2, α3) and loops (designated by L1, L2, L3) are represented by loops. Adapted from (McKinley & Cheeseman, 2016).

Deposition of CENP-A at centromere:

Unlike canonical histones which are highly expressed and deposited at the centromere during DNA replication, CENP-A is highly expressed during G2 and is deposited at the centromere following mitotic exit (Jansen et al., 2007) which is mediated by its chaperone protein called Holliday Junction Recognition Protein (HJURP). CENP-A first forms a pre-nucleosomal complex with H4 and HJURP (Dunleavy et al., 2009) (Foltz et al., 2009) to be recruited at the centromere by Mis18 complex (Y. Fujita et al., 2007) following late telophase and early G1 phase. Super resolution microscopy has revealed that CENP-A forms rosette-like clusters around HJURP forming 250-300 nm large globular structures and with progressing G1, HJURP is lost from the rosette leading to compaction of the CENP-A nucleosome (Andronov et al., 2019). In G1, CENP-A is incorporated at the centromere as well as in transcriptionally active sites on the arms of chromosome (Nechemia-Arbely et al., 2019). During S phase, the ectopically deposited non-centromeric CENP-A are removed from those sites due to lack of centromere protein to stabilize it. For example, CENP-C by direct interaction with CENP-A plays an important role in the incorporation and stabilization of CENP-A nucleosome (Falk et al., 2015).

As the cells proceed to S phase, newly deposited CENP-A in the parental DNA strand are redistributed to the daughter centromeres. While centromeres are replicated, canonical H3 variants H3.3 and H3.1 (as mentioned above) are deposited in the new daughter strand, the old CENP-A are diluted from the parental strands and are distributed to the new daughter strands. The resulting gaps generated in the new daughter strand are replaced and filled-in by 'place-holder' H3.3 until the next G1. In the subsequent G1, the CENP-A expressed during the G2 of the previous cell cycle, are deposited at the centromeres by replacing those place-holder H3.3 (Dunleavy et al., 2011). With again CENP-A dilution and distribution during S phase, the cells pass through mitosis with half of the total amount of CENP-A loaded initially in G1. In fact, CENP-A is important for the nucleation of kinetochore components. Studies have shown that targeted rapid degradation of CENP-A before initial steps of centromere assembly, prior to kinetochore assembly, leads to blocking the deposition of CENP-C and CENP-N, causing defective kinetochore and failure in chromosome segregation. However after the first steps of centromere assembly in G1/S phase, when CENP-A is removed, CENP-B bound to centromeric DNA is sufficient to preserve the anchoring of CENP-C and kinetochore at the centromeres (Hoffmann et al., 2016).

2.2.2 CENP-B

Another important protein that is associated with centromeres is CENP-B, which is the only known protein that binds to centromere in sequence-dependent manner. (Masumoto and his colleagues first identified the DNA sequence-specific binding property of CENP-B (Masumoto et al., 1989). They observed that cellular nuclear extracts incubated with a cloned fragment of centromeric DNA, was immunoprecipitated by serum from CREST patient. This led to identification of CENP-B as the antigen that specifically bound to 17 bp motif consisting of non-palindromic AT rich sequence.
CENP-B is an approximately 80 kDa molecular weight protein comprising of 599 amino acids. Through its N-terminal CENP-B can bind to a 17 bp specific sequence called the CENP-B box that are present in roughly every other α-sat monomer (Gamba & Fachinetti, 2020). Micrococcal nuclease (Mnase) mapping found that CENP-B box occur at the entry/exit site of CENP-A nucleosome *invitro*. Moreover, it is seen that CENP-B is more stable when bound to the proximal region of CENP-A nucleosome than when it is bound to the distal linker region. Also in *in vivo*, binding of CENP-B to CENP-B box enhanced the retention of pre-assembled CENP-A nucleosome (R. Fujita et al., 2015a). At its C-terminus, is the domain imparting CENP-B its ability to dimerize with another CENP-B via their hydrophobic surfaces (Tawaramoto et al., 2003), connecting two distant CENP-B boxes. The DDE domain is derived from pogo-like transposases family but is evolved by losing its transposase ability but retaining its capacity to bind DNA (Kipling, 1997). The acidic domains of CENP-B have functions that are still unclear. The two acidic domains are rich in glutamic and aspartic acid residues (79% of 1st domain contain acidic residues and 85% of second domain) (W. Earnshaw et al., 1987) with a potential to interact with diverse proteins. Indeed, domain I has been reported to interact with CENP-C (Suzuki et al., 2004) leading to stabilization of CENP-C on centromeric DNA to favor the assembly of kinetochore (Fachinetti et al., 2015). Further, according to Uniprot, human CENP-B also comprise of multiple disordered regions 143-184, 387-475, 495-544.

There have been several studies to determine the role of CENP-B in centromere function and cell viability and shed the paradoxical behavior of CENP-B. The importance of CENP-B in centromere function has been rejected by some studies for example, CENP-B is absent in the Y-chromosome of human (W. Earnshaw et al., 1987) (W. C. Earnshaw et al., 1989). Additionally, it has been shown that CENP-B null mice remain viable (Kapoor et al., 1998 , Perez-Castro et al., 1998 and Hudson et al., 1998). However, other studies have shown that CENP-B ensures chromosome segregation fidelity by ensuring interaction with CENP-C, since CENP-C recruited by CENP-A and mediates the assembly of kinetochore components (Fachinetti et al., 2015) (Hoffmann et al., 2016). CENP-B also provides memory for maintenance of centromeres by promoting *de novo* CENP-A deposition by interacting with and preserving a critical level of CENP-C (Hoffmann et al., 2020). Also, CENP-B box as well as α-sat sequences is also required for the *de novo*

formation of mammalian artificial chromosome (MAC) and assembly of functional centromere (Ohzeki et al., 2002).

The repetitive AT rich satellite DNA bears dyad symmetries as observed by spectroscopic studies as well as *in silico* prediction, that lead to formation of several non-B form of DNA (Kasinathan & Henikoff, 2018). However, CENP-B binding sites which are deficient of any dyad symmetry are enriched with non-B form of DNA implying that CENP-B may be directly responsible for forming these non-B structures (Kasinathan & Henikoff, 2018). CENP-B through its DBD is known to induce sharp twists (kinks) at two protein contact sites on the CENP-B box leading to an overall DNA bending by ~ 60° (Tanaka, 2001) and affects nucleosomal positioning in cis in the centromeric DNA (Tanaka et al., 2005). It is assumed that this property of CENP-B may contribute to phasing of bulk and CENP-A nucleosome in the α-satellite DNA (Hasson et al., 2013). Additionally, in the work by Chardon et al., 2022, where I have also participated, the ability of CENP-B to homodimerize was found to mediate the compaction of centromeric DNA by forming loop-like structures and promoting centromere clustering, and such higher order structure is important for centromere positioning and integrity during mitosis in the cells (**figure 17**).

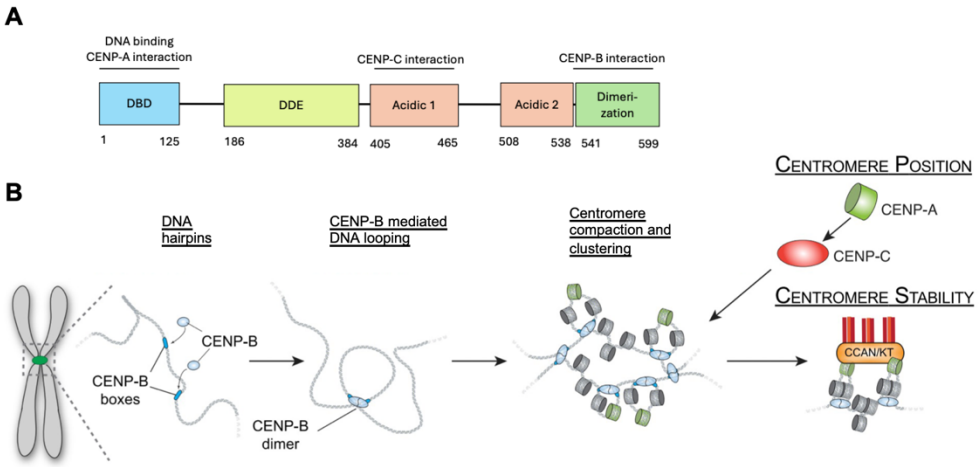


Figure 17: A) Schematic of human CENP-B domain organization: CENP-A consists of 599 amino acids. N-terminus consists of DNA binding domain in 1-125 amino acid, DDE (a homologue of DDE superfamily endonuclease with homology to pogo-like transposases) in 186-384 amino acid, two acidic domains in 405-465 and 508-538 amino acid and dimerization domain 541-599 amino acid in the C-terminus.

B) Model of centromeric DNA structure and function via CENP-B mediated DNA loops (Chardon et al., 2022).

2.2.3 CENP-C

Centromeric protein CENP-C, a 140 KDa protein (W. C. Earnshaw & Rothfield, 1985) is an important component of the CCAN that directly interacts with CENP-A and CENP-B and subsequently recruits other CCAN components. The CCAN is a subcomplex of kinetochore associates with other centromeric proteins and renders a platform for the assembly of kinetochore (Hara et al., 2023). CENP-C comprises of four functional domains: The N-terminal, the central region, CENP-C motif and homodimerization domain. The N-terminal domain of CENP-C serves to recruit other CCAN subcomplex like the CENP-H/I/K/M, and Mis12, an outer kinetochore complex which in turn is associated with microtubule binding Ndc80 complex (Klare et al., 2015). The central domain (amino acid 426-537) of CENP-C as well as its CENP-C motif (amino acid 735-755) are required for binding to CENP-A. However CENP-C motif is not sufficient for targeting to centromere without the presence of dimerization domain of CENP-C (Kato et al., 2013) (Pesenti et al., 2022). CENP-C has been known to affect nucleosome shape and dynamics. Through its central domain CENP-C can reshape the CENP-A nucleosomes and stabilize them at the centromeres (Falk et al., 2015). CENP-C is known to interact with CATD domain and carboxy-terminal tail of CENP-A (Fachinetti et al., 2013) (Logsdon et al., 2015).

The HJURP-H4-CENP-A soluble complex is directed to the centromere by Mis18 complex, but how Mis18 complex recognizes the centromere is not yet clear. It was earlier proposed that CENP-C interacts with Mis18 via its C-terminus and facilitates the recruitment of Mis18 to the centromeres (Dambacher et al., 2012). The C-terminus of CENP-C that also includes the Cupin region, has been implicated for its ability to homodimerize. In fact, it has shown that CENP-C through its Cupin domain can self-oligomerize which is important for gathering CCAN and this is presumably linked with CENP-A nucleosome for their stable association with centromeric chromatin (Hara et al., 2023). Structural and biochemical studies have established the multivalency of CENP-C on CENP-A nucleosomes. They further attributed the homodimerization of CENP-C to crosslink the dispersed CENP-A nucleosomes in the centromeric chromatin (Ali-Ahmad et al., 2019). Alternatively, CENP-C can be recruited to centromeres independent of CENP-A via the involvement of the acidic and DNA binding domain of CENP-B (Hoffmann et al., 2020) which stabilizes CENP-C (Fachinetti et al., 2015).

3. Senescence

In previous sections, we explored how a normal cell goes through different phases of its lifetime, in the cell cycle that is precisely controlled and regulated by a network of key regulators and checkpoints. In the course of its life, a cell faces a variety of biotic stresses like infections by pathogens, external abiotic stresses like exposure to toxic chemicals such as crosslinking agents, or ionizing radiations, extreme weather conditions or endogenous stresses that are usually formed by metabolizing exogenous agents like generation of free radicals inside the body, or endogenous replication stress. All these stresses have been shown to affect the DNA, and it is reported that on an average our cells may be subjected to 70,000 instances of DNA damages per day (Huang & Zhou, 2021). In response to such adversities, cells trigger a series of events that arrest the cell cycle progression, buying themselves some time, space and resources to promote recovery, activate their DNA repair mechanisms and if these mechanisms are ineffective (due to severity of DNA damage or inefficient repair mechanism like in cancer cells), the cells die or commit apoptosis. If the damage is not severe enough for the cells to die (sublethal), the cells may enter a state where they do not divide anymore, also known as Senescence.

Senescence is a phenomenon where a cell exits from the cell cycle irreversibly but remains metabolically active as long as it is supplied with essential nutrients and growth factors. This is different from quiescent G0 cells which undergo reversible cell cycle arrest mainly in response to deficiency of growth factors. Cellular senescence was first observed in the mid 1960s where human diploid cell lines exhibited limited replicative cycles before stopping to proliferate after a certain number of divisions. This came to be known as the Hayflick limit that drives what is known as replicative senescence.

3.1 Types of Senescence:

Senescence can be induced under multiple scenarios:

a. Replicative stress (RS): Telomeres are repeats of the TTAGGG sequence at the terminal ends of chromosomes and are maintained thanks to a specific structure comprising a T-loop (telomere loop) and D loop as shown in **figure 18 A** (Weinberg, 2013), stabilized by the telomere-specific protein complex Shelterin. While synthesizing the lagging strand of the telomere, the RNA primers that are deposited at the 5' end of the newly synthesized lagging strand have to be removed and replaced with dNTPs by a DNA polymerase that requires a 3'OH group to initiate synthesis. At the very tip of the chromosomes, due to unavailability of a preceding OH group to mediate the dNTP deposition and polymerization by DNA polymerase, there remains a gap approximately equal to the size of the displaced RNA primer (~10bp) at the newly formed lagging strand thereby creating a G-rich 3' overhang on the template strand **(figure 18 B).** Other than this end-replication problem, possibly other factors like action of exonucleases may influence telomere erosion since it is seen that many normal human cells shed 50-100 bp of telomeric DNA in each cell cycle (Weinberg, 2013).

   The Shelterin complex sequesters the telomeric DNA to protect it from being recognized as DNA damage. After a certain, limited number of cellular cycles, the telomeres shorten so much that they become uncapped from the shielding proteins and start to be recognized as DNA damage, thereby signaling the DNA damage response mediated by ATM/p53/p21, leading to cell cycle arrest. However, these telomeres still retain minimum protection to avoid DNA repair and telomere fusions (Rossiello et al., 2022). Persistent DNA damage response signaling arising from short telomeres therefore can induce cellular senescence in proliferating cells that is implicated in ageing. Replicative senescence does not occur in telomerase positive cells like germ cells. However, in post mitotic cells like neurons, cardiomyocytes, osteocytes, telomere dysfunction can be driven by DNA damage within the telomere.

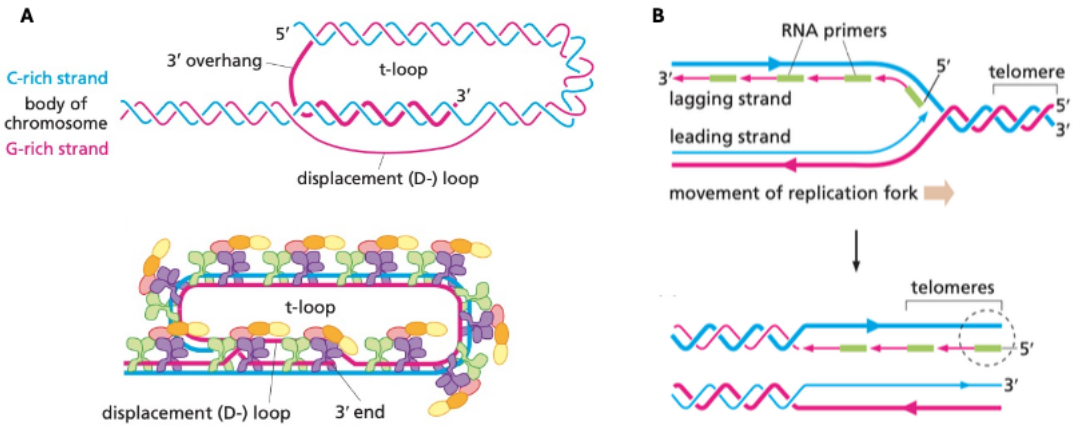


Figure 18: A) Top: Organization of telomeric DNA to form three stranded structure t-loop where the 3' overhang is complimentary to small stretch of C-rich region thereby forming the displacement d-loop. Bottom: t-loop and d-loop bound by multiple shelterin complex protein like TRF1, TRF2.
B) Schematic of telomeric DNA replication. Top: Normal DNA replication where the synthesis of lagging strand happens by RNA primers shown in green. The 3'OH ends of these RNA primers serve as sites to initiate new daughter strand DNA synthesis. Later RNA primers are removed and replaced with DNA that also requires a 3'OH initiation site which is obtained from the newly synthesized DNA fragments (in pink). Bottom: The RNA primer removed at end at telomeres (shown inside dotted circle), is removed but cannot be replaced creating a shorter lagging-daughter strand (Weinberg, 2013).

b. Oncogene induced Senescence (OIS):

In a normal scenario, proto-oncogenes regulate physiological functions important for cellular functioning. However, when an oncogene is activated, it might drive initiation and progression of cancer by stimulating cell proliferation. Oncogene induced senescence acts as a defensive mechanism against cancer by halting the proliferation of cells which have oncogenic signal activation. Indeed, after the discovery of Ras as an oncogene in immortalized mouse cells, studies in 1987 showed that in wildtype cells, Ras initially triggered an initial level of high cell proliferation, but then irreversible growth arrest was observed with accumulation of p53 and p16 (Courtois-Cox et al., 2008).

OIS can be induced by many oncogenes like Ras, Myc or absence of tumor suppressors like PTEN. Senescence progression through oncogene activation can occur either through DNA damage response (DDR) or independent of DDR.

i. <u>OIS through DDR</u>:

Activation of oncogenic signals leads to unbalanced stimulation of genes that promote cell growth resulting in a high amount of DNA replicative stress, thereby causing a lot of dsDNA breaks. This was evident as it was seen that oncogene activation in cells led to increased number of active replicons and partly replicated DNA. Additionally, oncogenes expression could trigger DNA hyper replication causing breaks and activating the DDR (Di Micco et al., 2006). The breaks are subject to activating the DNA damage response primarily through p53/p21 pathway and/or p16/Rb pathway and causing cell cycle arrest within few cell generations after oncogene overexpression.

Role of Ras:

Oncogenic signals activate the p16/Rb pathway through a variety of ways. As a reminder (as shown in **figure 8**), p16 inhibits CDK4/6 mediated phosphorylation of Rb. Hypophosphorylated Rb binds transcription factor E2Fand inhibits expression of cell proliferating genes. The regulation of expression of $p16^{INK4}$ is not very clearly established but there are few reports about it.

It was seen that Ras oncogenic signal induces epigenetic depression of $p16^{INK4}$. As oncogene signaling like Ras induce hyperproliferation (causing DNA damage) by activating the Mitogen activated protein kinase pathways (MAPK) and activation of transcription factor E2F takes place, it results in increased expression of DNMT1 (a DNA methyl transferase that can interact with histone methyltransferase like G9a) consistent with increased levels of H3K9me2 in p16 promoter and vicinity in early papilloma, causing repression of p16. However, with continued oncogenic signaling, the highly proliferating cells accumulate DNA damage and so inhibited E2F can no longer induce DNMT1 expression, therefore resulting in reduced H3K9me2 and derepression pf p16 (Yamakoshi et al., 2009). Indeed, it was seen that senescent cells accumulate H3K9 methylation to form heterochromatic foci, mediated by p16, on certain areas of the genome which we will explore in later section 3.3.E (Narita et al., 2003).

Further, activation of the MAPK pathway through Ras activates Erk kinase (see **figure 19**) (Weinberg, 2013), that often phosphorylates and activates TFs Ets. Ets 1 and 2 are responsible for increased p16 transcription (Rayess et al., 2012). Another report says that HMG box containing protein (HBP1) is involved in Ras induced senescence. HBP1 can bind to p16 promoter and regulate the expression of p16 (H. Li et al., 2010).
Thus, Ras signaling induced senescence causes replication stress and DNA damage, that can lead to activation of p53/p21 and /or p16 and impact Rb.

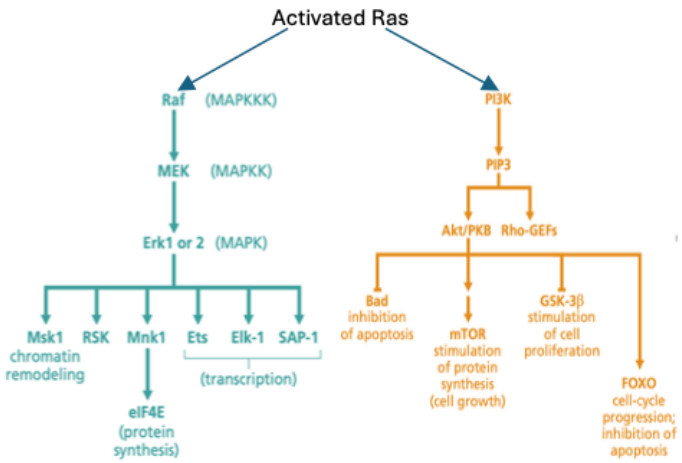


Figure 19: In blue: The MAP Kinase signaling cascade activated by Ras. Ras activates Raf kinase (a MAPKKK) to phosphorylate MEK which then phosphorylates and activates Erk. Erk then phosphorylates kinases in the nucleus that regulates TFs and translation factors in the nucleus. In orange: 2nd effector pathway of Ras where it phosphorylates and actives phosphatidylinositol 3 kinase (PI3K) leading to tethering of Akt (aka PKB) to plasma membrane (PM). Such association of Akt to PM leads to activation of Akt kinase, which can aid cell survival, inactivate GSK-3B (antagonist of cyclin D1, Myc) and activate mTOR (a kinase stimulating protein synthesis and cell growth) (Weinberg, 2013).

ii. <u>OIS independent of DDR</u>:

Ras oncogenic activation can also have a second effect and lead to hyperactivation of the Akt pathway as shown in **figure 19**. It is seen that the Akt pathway is hyperactivated in 30% of cancers in human (with mutated tumor suppressor gene PTEN) (Astle et al., 2012). Akt is a serine/threonine kinase protein encoded by retrovirus-associated oncogene and bears a pelckstrin homology (PH) domain. Ras can bind PI3K and bring PI3K close to plasma membrane. PI3K gets phosphorylated to form PIP3K. PIP3K generally recruits proteins that carry a PH domain and hence attracts Akt. Thus, the Akt now associated to the plasma membrane is functionally activated as a kinase and functions in cell growth and proliferation (Weinberg, 2013).

It is seen that this pathway when overly active triggers cellular senescence by enhancing p53 expression via mTORC1. Akt signaling causes MDM2 to be localized to the nucleolus thereby increasing the stabilization and accumulation of p53. MDM2 is a E3 ubiquitin ligase protein that ubiquitinates p53 for proteosomal degradation. Senescence caused due to Akt signaling doesn't bear any DNA damage and is mediated by p53 response (Astle et al., 2012) (Zhu et al., 2020).

Role of Myc:

While the OIS through Ras and its mutants has been studied extensively, there are reports on how Myc oncogene can play a role in senescence. MYC codes for a transcription factor that is essential for genes involved in cell proliferation and differentiation and apoptosis. Its misregulation has been associated with cell hyperproliferation and tumorigenesis, but its function in senescence is not very entirely clear. Activated Ras and Myc like other oncogenes limit its tumorigenic potential by promoting cellular senescence or apoptosis respectively. Myc, which has been mainly associated with apoptosis, operates in part via p53 stabilization as Myc can induce expression of p19 ARF. ARF is a protein derived from **a**lternate **r**eading **f**rame of the $p16^{INK4A}$ locus and it sequesters/inhibits MDM2, thereby accumulating p53. The decision afterwards on whether to proceed to apoptosis or senescence is not very well understood and is being currently explored. A study

saw that c-Myc is able to promote senescence by inducing transcription of ubiquitin specific protease 10 (USP10) that mediated de-ubiquitination of p14ARF and therefore stabilizing p53 to promote senescence (Ko et al., 2018).

However, there is another opposite report that Myc suppresses cellular senescence mediated by CDK2's activity of phosphorylating Myc. In other words, inhibition of CDK2 can inhibit Myc driven tumorigenesis by inducing cellular senescence. When mice (transplanted with hematopoietic stem cells bearing anti-apoptotic Myc) were lethally irradiated, they developed Acute Myeloid leukemia (AML) and died within 17 days. However, in these mice, when CDK2 was inhibited, the mice accumulated senescence, lived longer and the disease onset was delayed (Bazzar et al., 2021).

Along similar lines, the importance of Myc loss in maintaining the irreversibility of senescence was recently shown (Afifi et al., 2023). It was described that after persistent duration of stress in conditions of senescence, the cells lowered their level of Myc to enter and maintain replicative senescence and oncogene induced senescence, and if the senescent cells expressed non-degradable MYC, they reverted to cycling. Therefore, the study highlights that the initiation of senescence is based on the duration of cellular stress and MYC loss is important for both entry to senescence as well as its maintenance.

3.2 Role of senescent cells

Senescence is an outcome of the physiological conditions and stimuli that a cell is exposed to. With the increased incidence of diseases like cancer and inflammatory diseases, and the ever -increasing interest in understanding aging, age-related disorders, and strategies of prolonged healthy life span, studying cellular senescence is becoming more important than ever to the global community. Many studies are shedding light on the importance of cellular senescence in different pathological as well as normal physiological functions:

A. Tumorigenesis: We just saw how DNA damage and oncogene activation like Ras can produce cellular senescence. Also, inactivation of tumor suppressor genes like PTEN cause cellular senescence through the Akt-mTOR-p53 pathway and p19ARF (analogous to p14 ARF in human)-MDM2-p53 pathway (Alimonti et al., 2010). Senescence is actually a preventive mechanism for the cells to stop passing on the carcinogenic mutation or DNA damage to the daughter cells. Senescent cells have a secretory phenotype called Senescence associated secretory phenotype (SASP), which comprises inflammatory cytokines and chemokines to facilitate their removal and immuno clearance. Cytokines can signal in autocrine or paracrine manner and dictate growth, differentiation, proliferation and migration (Kartikasari et al., 2021). However, a persistent and chronic inflammatory microenvironment may promote tumorigenesis. It was seen that inhibiting SASP could avoid tumorigenesis. It was also shown that inhibition of metalloproteinase (MMP) can alter SASP composition that otherwise causes metastasis in prostate cancer. Inhibiting the MMP inhibitor protein TIMP1 caused metastasis (Guccini et al., 2021). Additionally, OIS through Ras activated growth regulated chemokine and promoted tumorigenesis in ovarian cells (W. Huang et al., 2022). Further, colon tumors were seen to have overexpressed and secreted phospholipase D2 that induced senescence in surrounding fibroblasts. These senescent cells secreted SASP leading to increased stemness of cancer cells (Muñoz-Galván et al., 2019).

B. Tissue structure and developmental process: Apart from the tumor-suppressive or tumor-promoting functions of senescent cells, they have also been implicated in developmental processes and investigations are still underway to understand this better. Presence of senescent cells was observed in the mouse embryo during its development and was responsible for patterning of tissue growth (Storer et al., 2013). Similarly, senescent cells were found to be present in the lungs of young mice and disrupting senescence affected lung development (Yao et al., 2023). Such programmed-senescence related to development is different from DNA damage induced senescence (De Magalhães, 2024). In another study, (Grosse et al., 2020) senescence build-up in mice liver and removal of these senescent cells have been shown to affect the blood-tissue barrier and cause perivascular fibrosis. There is also

evidence that senescence enhances cellular function, as it was seen that p16 mediated senescence prompted pancreatic beta cells to secrete insulin and led to better glucose metabolism (Helman et al., 2016).

C. Wound healing and repair: The process to undergo repair of tissue injury involves inflammation, replacement with new tissue and remodeling of the tissue. The role of senescence in wound healing is possibly through SASP. At the site of amputation of Cnidaria (metazoan), senescent cells disrupted the differentiated state of the surrounding somatic cells and induced stemness in them to drive regeneration (Salinas-Saavedra et al., 2023). Also, in zebrafish senescent cells were shown to be important for regeneration of tissues at the site of injury after fin amputation. In mammals, few studies like the one carried out by (Cheng et al., 2022) showed that within 2 days after partial hepatectomy in mice of 2-3 months old, the hepatic stellate cells (HSC) beared senescence which released SASP like interleukin 6 (IL6), and CXCL2. Together they activate Erk1/2 signaling to induce proliferation of hepatocyte cells.

D. Aging: With aging of an individual, telomere shortening gives rise to senescence, increasing the accumulation of senescent cells. With all the current buzz on aging and senescence in the media and industry, it is important to note here that senescence is a process of adaptation. Indeed, senescent cells are not aged cells. They are functional cells for several physiological processes. SASP may disrupt tissue homeostasis, enhance inflammation and age-related disorders. A study where p16-positive senescent cells were eliminated in mice led to a delayed tumorigenesis, a diminished age-related deterioration of organs like kidney, heart, and adipocytes tissues, and it extended the lifespan of the mice by 24-27% (Baker et al., 2016). Therefore, there is a high demand for targeted removal of senescent cells by using senolytics, i.e., a class of drugs that clear senescent cells.

3.3 Hallmarks of senescence

Senescence is an important physiological process that determines the fate of a cell either during its natural developmental process or when it faces adversities. In particular, we saw how senescent cells elevate tumorigenesis and age-related disorders. Therefore, in the interest of being able to selectively target senescent cells, it is important to accurately characterize these cells. However, there is not just one single hallmark of a senescence, making it very subjective and context-dependent, senescent cells show certain phenotypes (**figure 20**).

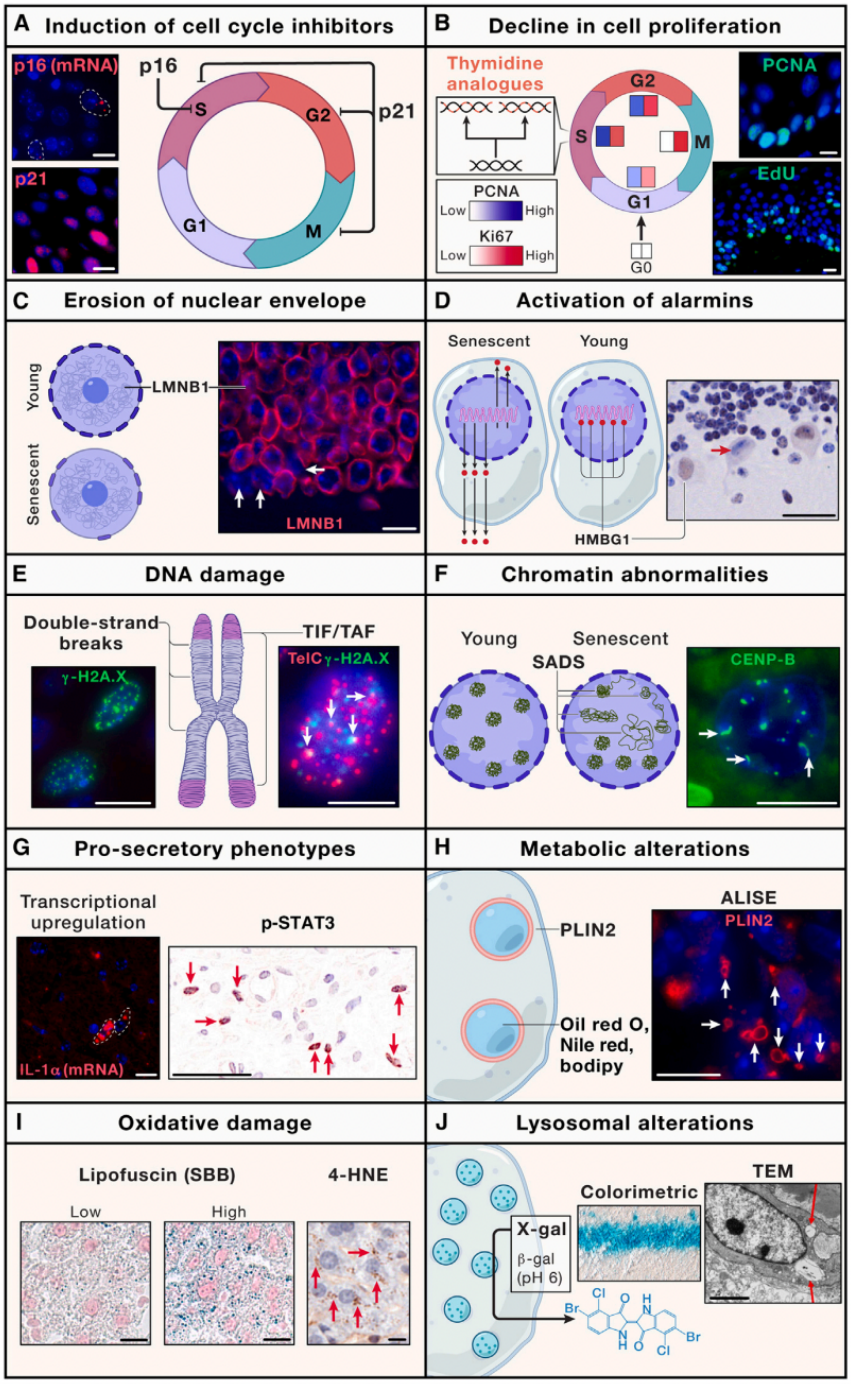


Figure 20: Hallmarks of cellular senescence (from Ogrodnik et al., 2024)*:*

A) *Expression of cell cycle inhibitors to arrest cell cycle: p16 ^{INK4A} during G1→S and p21$^{Cip/Waf}$ during G1→S, S→G2 and G2→M. Images showing RNA-FISH against p16 in aged mice brain and p21 in wounded epidermis*

B) *Decline in cell proliferation as visualized by reduced PCNA or Thymidine analog Edu incorporation over 6 hours by wounded skin samples*

C) *Erosion in nuclear envelop by reduction in Lamin B1. White arrows show neurons negative for Lamin B1 in dentate gyrus of aged mouse.*

D) *Senescent cells rapidly release endogenous proteins called Alarmins into the cytoplasm and to the extracellular space upon tissue damage. Red arrow shows neuron with no intranuclear HMGB1 in the cerebellum of aged mice.*

E) *DNA damage foci through γH2AX occurring at the chromosome.*

F) *Alteration in chromatin re-organization can give rise to the formation of SADS for example (white arrows) in the hepatocyte of aged liver.*

G) *Production of Senescence Associated Secretory Phenotype (SASP) visualized by RNA-FISH (against IL-α) or by histological detection of the pro-inflammatory pathways like p-STAT3 in wounded skin.*

H) *Alteration in the metabolome of the cell. Image shows increased accumulation of lipid droplets (staining against Perilipin (PLIN2) in the aged mouse brain cells.*

I) *Senescent cells show increased production of reactive oxygen species (ROS) leading to oxidative damage as visualized by the presence of Lipofuscin and 4-hydroxynonenal in the aged livers*

J) *Increased lysosomal activity visualized by increased of β-galactosidase enzymatic activity in the hippocampus of aged mouse.*

A. Altered Metabolism

As said earlier, senescent cells are metabolically active but have an altered metabolism. Due to limited studies on the metabolome of senescent cells, there is however no single direct metabolic marker of senescence and additionally, there is still a controversy on whether metabolic changes are a cause or a consequence of senescence. However, the metabolism of senescent cells is dysregulated, such that it reprograms degradation pathways of proteins, or mitochondrial metabolism. Senescent cells have a large cell size but are not dividing, hinting about altered metabolic state and protein production in the cell. It is seen that senescent cells adopt a more glycolytic state (anaerobic respiration in the cytoplasm) but also have higher levels of AMP and ADP compared to ATP. This leads to increased AMP-activated kinase (an energy sensor of the cell) which in turn activates p53 and downstream p21, which is crucial in promoting and maintaining senescence especially associated to DNA damage. Further, p53 inhibits glucose uptake and glycolysis by affecting the expression of glucose transporters like GLUT1 & 4 and promotes oxidative phosphorylation (mitochondrial respiration), and fatty acid oxidation which acts like a compensation to reduced energy production (Wiley & Campisi, 2016). Rb or retinoblastoma, another key tumor suppressor gene playing an important role in senescence can also induce metabolic changes. It was shown to increase oxidative

phosphorylation and also lower the formation of dNTPs by regulating transcription factor E2F (Hernandez-Segura et al., 2018).
There are reports (albeit not entirely consistent) of mitochondrial dysfunction during OIS in human fibroblasts. While some reports attribute the loss of ATP to mitochondrial dysfunctionality, others reported high ATP production during OIS.
The next questions can be: what dictates the energy production? Where is this energy used in non-proliferative senescent cells? It is understood that in order to maintain the senescent state, for example, for production of proteins required for senescence like SASP and processes promoting senescence like autophagy, cells utilize the energy in maintaining senescence.

B. Altered proteostasis

Additionally, senescent cells have altered protein metabolism and loss of protein homeostasis, also called proteostasis. Alteration in homeostasis of any macromolecule including proteins can be in two ways: degradation and translation.
Degradation of protein or any other macromolecule can occur via autophagy. Autophagy is a process in which proteins and other cellular components are engulfed by membrane-bound autophagosomes and fused with a lysosome (a membrane bound organelle for mass biodegradation). Autophagy is important for overall turnover of macromolecules as they mediate elimination of unwanted or damaged proteins, lipids, organelles and maintain bioenergetic homeostasis in a cell. However, autophagy can also be activated when cells are stressed. Autophagy was originally thought to remove just damaged cellular components, and to be beneficial for cellular homeostasis. However, a new positive co-relation between autophagy and cellular senescence progression (shown by Young et al., 2009) was identified that went beyond the classical definition of autophagy function: the maintenance of cellular homeostasis during stressful conditions. The group found that during OIS in human fibroblast, autophagy is activated and knockdown of few autophagy regulators delayed SASP (effecting IL-6 and IL-8 at post transcriptional level) and OIS. To coordinate the simultaneous observation of increased expression of SASP proteins and increased degradation through autophagy during senescence seemed paradoxical but they hypothesized that the mediation between these two processes occurs by mTOR (mammalian target of rapamycin), a master regulator of protein synthesis and a negative regulator of autophagy. Then, Narita's lab found that a distinct cellular compartment called TOR-autophagy spatial coupling compartment (TASSC) released amino acids as a result of autophagy to supply building blocks for mTOR and activate it, thereby stimulating SASP (IL6 and IL8), coupling of catabolic process by autophagy to the anabolic process of protein synthesis (Narita et al., 2011) (Salama et al., 2014). Since then, there have been reports of autophagy promoting senescence (Capparelli et al., 2012) (Slobodnyuk et al., 2019).
Conversely, there are also reports that senescence is promoted by inhibition of autophagy. For example, TF GATA4 accumulates during senescence by autophagy

inhibition and regulates SASP by GATA4-NF-kB signaling (Kang et al., 2015), whereas in normal conditions, GATA4 is degraded by 'selective autophagy' where only specific types of substrates are degraded. Therefore, the mode of autophagy, whether selective or general, dictates whether the autophagic response is promoting or inhibiting senescence (Kang & Elledge, 2016) (Kwon et al., 2017).

C. Accumulation of Beta galactosidase

We already saw that general autophagy plays a role in cellular senescence and that the autophagosomes fuse with lysosomes for degradation of macromolecules. Lysosomes are organelles that are acidic in nature and have hydrolytic enzymes in them that carry out degradation of macromolecular components during autophagy. During senescence the increase in lysosomal mass reflects increase in autophagy and therefore the activity of lysosomal Beta galactosidase enzyme increases significantly. Beta galactosidase is a very widely used biomarker of senescence which is detectable at pH 6.0, but it is also detected in cells when they are very confluent.

D. Loss of Lamin B1

Senescent cells also favour 'selective autophagy' which we already saw above. A number of proteins are selectively degraded during senescence. One another example is the autophagic degradation of nuclear lamina protein Lamin B (Dou et al., 2015). Lamin B1 is one of the major structural components of the nuclear lamina which is primarily associated with the inner membrane of the nuclear envelope and is integral in maintaining the mechanical stability of the nucleus. During senescence, there is a global loss of Lamin B1. Loss of lamin B1 was evident in senescent cells induced by DNA damage, replicative or oncogene induced senescence in both human and mice and this loss of Lamin B1 can also be at the level of mRNA as reported by (Freund et al., 2012). Accordingly, depleting Lamin B1 slowed down cell proliferation and induced senescence in human fibroblasts as well as mice fibroblasts (Shimi et al., 2011). Further they also showed that Lamin B1 degradation occurs just by activation of p53 or pRb pathway but did not require p38-MAPK, NF-kB or ATM.

Mapping of Lamin B1 led to identification of its association with large regions of lamin associated domains (LADs) of chromatin. These regions were found to be enriched with repressive marks with more prominence of H3K27me3 and less abundance of H3K9me3 (Guelen et al., 2008). During senescence, Sadaie et al. (2013) have shown that though Lamin B1 expression is depleted globally, the depletion isn't uniform and there is a redistribution of Lamin B1. Lamin B1 binding reduced mainly from H3K9me3 rich regions and accumulated in small regions (~2% of the genome) corresponding to cell cycle genes

which are normally expressed during cell cycle and now had H3K27me3 due to senescence.
This detachment of Lamin B1 from H3K9me3 rich regions causes spatial repositioning of the peripheral heterochromatin and leads to formation of Senescence-associated heterochromatin Foci (SAHF).
Along the same lines, Shah et al., (2013) found depletion of H3K27me3 from regions that correlated with down-regulated cell cycle genes and up-regulated SASP genes. Therefore, downregulation of Lamin B1 implicated senescence by altering chromatin landscape and (in)directly influencing gene expression.
Additionally, compromised lamin B1 integrity leads to extrusion of the lamin-associated domains of chromatin, which in turn leads to the formation of cytoplasmic chromatin fragments (CCFs). These CCFs cross through the deformed nuclear lamina to go into the cytoplasm either to get degraded by autophagy or get picked up-up by cGAS-STING to initiate the SASP expression (Roger et al., 2021).

E. Chromatin changes: SAHF and SADS

Senescence Associated Heterochromatin Foci (SAHF)

Senescent cells also undergo changes in their chromatin structure, one of them is the compaction of chromatin to form dense heterochromatin structures also called Senescence associated heterochromatin foci (SAHF) which was first described by (Narita et al., 2003) in OIS in human fibroblast cells. These structures are silent facultative heterochromatin and the level to which SAHF is formed depends on the type of senescence and cell types. For example, human fibroblasts highly exhibit SAHF when faced with oncogene induced senescence but less propensity upon DNA damage, and BJ fibroblast cells are not able to form SAHF (Kosar et al., 2011). Moreover, cells from the Hutchinson Guilford progeria syndrome (HGPS) patients and from aged individuals show loss of visible heterochromatin blocks. SAHF is also absent is all senescent mice cells (Swanson et al., 2013a). Also SAHF contain repressive epigenetic marks like H3K9me3 and have heterochromatin associated proteins like Hp1, rich in histone variant macroH2A. MacroH2A histones are involved in silencing of genes and they are also resistant to ATP-dependent chromatin remodelers and TF binding (Angelov et al., 2003). There are studies which show the loss of linker DNA-binding protein histone H1 and implications of chromatin structural proteins like HMGA accumulation in SAHF formation (Funayama et al., 2006) (Narita et al., 2006).
When Narita's lab first described SAHF, they showed that SAHF formation requires in part, p16/Rb pathways where Rb accumulates on and represses E2F-responsive promoters and genes involved in cell proliferation, for example Cyclin A (Narita et al. 2003). In their study they proposed that, since Rb is known to bind proteins like HP1 and some histone methyltransferases like SUV39H1 (Trimarchi & Lees, 2002), Rb might

control the seeding of SAHF by directing the recruitment of methylases on E2F promoters leading to methylation of H3. Methylated histones create a binding site for HP1, which in turn can dimerize and form higher order chromatin structures.

Several studies show that SAHF not only promotes gene silencing but also protects from DNA-damage replication stress that occurs by oncogene activation. SAHF restrains (inactivates) the DDR, thereby preventing DDR signaling to cause apoptosis which would occur if SAHF is perturbed as shown in human fibroblast cells (Di Micco et al., 2011).

We saw before that Lamin B1 loss can be one of the contributing factors for formation of SAHF by redistribution of peripheral heterochromatin. It is proposed that SAHF formation is a two-step process: mobilization of existing heterochromatin and condensation of heterochromatin by chromatin-binding factors (Olan et al., 2023). Studies show that histones chaperons HIRA and ASF1A and High Mobility group A (HMGA) proteins are also implicated in SAHF formation (R. Zhang et al., 2005) (Narita et al., 2006). Heterochromatin, for example the inactive X chromosome, is generally nucleosome rich as compared to euchromatin which have depleted nucleosome. Similarly, it is seen that SAHF contains more nucleosome density. It has been seen that HIRA relocates to the PML bodies, mediated by the Wnt signaling pathway and this is important for SAHF formation (Ye et al., 2007). SAHF has been proposed to be a re-arrangement of already-existing repressive chromatin (Chandra et al., 2012) through genome wide analysis of H3K27me3 and H3K9me3 that revealed that each focus of SAHF has a core region comprised of H3K9me3 followed by H3K27me3 at the periphery. Thus the exclusive layers of constitutive and facultative heterochromatin and euchromatin is possibly a way to coordinate the repression or activation of genes required for senescence progression (Chandra & Narita, 2013). However, it is also seen that SAHF are still retained in cells which have bypassed OIS through depletion of p53 or ATM in p16-low backgrounds. Therefore the speculation is that probably SAHF mediates other context-dependent downstream effects, similar to how SASP does despite p53 inactivation in p16 low cells, which is to foster a tumorigenic microenvironment (Di Micco et al., 2011) (Salama et al., 2014).

Senescence associated distension of satellite DNA (SADS)

Another alteration in the chromatin rearrangement during senescence is the senescence associated distension of satellite DNA (SADS). In order to map genome-wide open-chromatin conformations, De Cecco et al., (2013) used formaldehyde-assisted isolation of regulatory elements (FAIRE) in early and late passage of human diploid fibroblasts. In FAIRE, the chromatin is first crosslinked with formaldehyde and after chromatin extraction, the regions which are excessively bound with histones (irrespective of histone modification) remain separated into the organic phase from the regions that are relatively histone deficient into the aqueous phase. They found that while growing cells have distinct open and closed chromatin profiles, senescent cells had a flat FAIRE profile. There was a loss of FAIRE signal from open and active promoters and FAIRE signal gain from closed heterochromatic regions (**figure 21**). Usually, open regions became more closed with exception of few genes and regions that are usually heterochromatinised including retrotransposon elements like Alu, SVA and L1, that become more open together with the satellites of the centromeric and pericentromeric regions. Additionally, these regions also had increased transcription. Additionally, transposition of retrotransposon elements was observed only in late senescence.

Soon after, Swanson's lab demonstrated that α-satellite DNA and Satellite II from centromere and pericentromere undergo distension in the early times of when cells commit to senescence. They have shown that within 48 hours of the last DNA replication, late passaged Tig 1 diploid fibroblast cells (from fetal lung) exhibit SADS as they enter senescence. Moreover, SADS occur even before SAHF formation but not vice versa, as SMURF2 overexpression led to accumulation of 39% SADS by day 2 and 5% ASHF by day 5. SMURF2 (Smad ubiquitin regulatory factor 2) is an E3 ubiquitin ligase that is known to induce senescence as characterized by presence of SADS, SAHF and β-gal staining (Swanson et al., 2013).

Unlike SAHF, the occurrence of SADS is more consistent across different senescent types and cell types. Senescence induced by DNA damage is independent of SMURF2 overexpression (OE) (H. Zhang & Cohen, 2004), oncogenic Ras signaling or oxidative stress exhibited SADS. Additionally, senescent murine cells and HGPS cells which typically lack SAHF can still accumulate SADS.

Cells undergo senescence through either p53/21 or p16/Rb pathways. Swanson and his team tested whether SADS is specific to these pathways. LF1 cells with p21 KO as well as p16 low human BJ cells, both had SADS when they underwent SMURF2 induced senescence, implying that SADS occur independent of p21 or p16. Though this study reflects that SADS is not restricted to p21 or p16 pathways, what factors cause SADS is still an open question. Especially with respect to p21, in my study I am investigating the factors upstream of p21.

Furthermore, it was observed that cycling and senescent cells had similar levels of heterochromatin marks (H3K9me3 and H3K27me3) despite distension of satellites in senescent cells (Swanson et al., 2013). Swanson et al proposed that SADS cause

changes in higher order chromatin involving chromatin loops rather than local changes in DNA wrapping due to histone modification. While they investigated the role of nuclear structural protein lamina lamin B1 in higher order folding of DNA, they found that not all cells with reduced Lamin B1 levels had SADS, and SADS did not occur in cells with high Lamin B1. These results imply that lamin B1 may be only one of the many factors that might influence satellite architecture. However, till today it is not fully known as to what is the mechanism that leads to SADS and whether they have any implication in cellular senescence and centromere function.

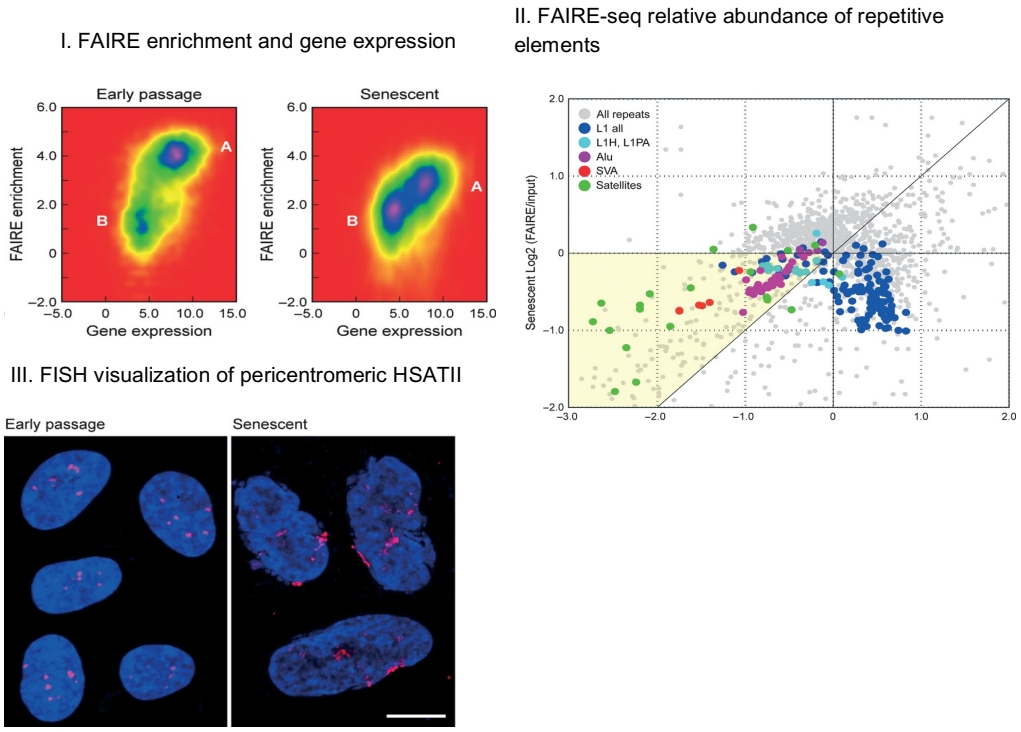


Figure 21: (I) FAIRE enrichment (reads per gene) early passage has 2 well-separated gene clusters (A) with higher FAIRE and gene expression signal (B) lower values for both (II) Abundance of repetitive elements Alu, L1, SVA and Satellite in FAIRE-Seq datasets. Quadrant 4 in yellow represents the de-repression in senescent cells where most of the active Alu, SVA and satellites are located. (III) FISH against pericentromeric satellite II (in red) in early passage vs senescent human diploid fibroblast. Scale bar 10 μm. (De Cecco et al., 2013).

4. cGAS in the cytoplasm and nucleus of cells

4.1 cGAS: A DNA sensor

The cyclic GMP-AMP synthase (cGAS) that belongs to the nucleotidyltransferase family, was first identified by Sun et al., 2013. When cells contain DNA in the cytoplasm other than being normally present in the nucleus (or in mitochondria), the DNA is sensed as a foreign substance invading the cell, thereby triggering a cascade of events to evoke the innate immunity. cGAS phase-separate, recognizes and binds to cytosolic DNA. It catalyzes the synthesis of cyclic guanosine monophosphate-adenosine monophosphate (cyclic GMP-AMP → cGAMP) (Zhou et al., 2021). cGAMP then binds to and activates STING (Stimulator of Interferon Genes), an adaptor protein to coordinate signal transduction, in the endoplasmic reticulum (ER). The activated STING is translocated to the Golgi bodies where it recruits Tank binding kinase 1 (TBK) and IkB kinase (IKK). TBK1 phosphorylates Interferon Response Factor 3 (IRF3). IRF3 then is translocated to the nucleus and acts as a transcription factor to induce the expression of Interferon (IFN) and Interferon Stimulated Genes (ISGs). Further, activated IKK phosphorylates and degrades IkB family proteins, the inhibitors of NF-kB. NF-kB then is exported to the nucleus and cause the expression of inflammatory cytokines and chemokines like interleukins to activate the innate immune response (**figure 22 A**) (Lu et al., 2024).

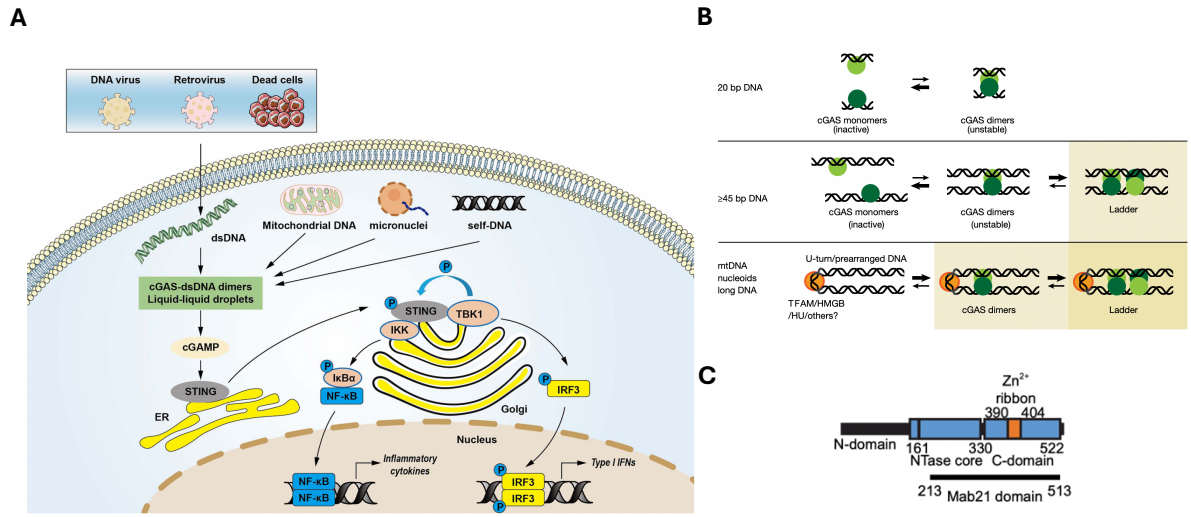


Figure 22: A) Invading pathogen or self-DNA in the cytoplasm activate the innate immune response through the cGAS-STING pathway (Xu et al., 2021). *B) cGAS binding to small DNA of 20 bp and forms unstable cGAS dimers; When bound to DNA of at least 45 bp, cGAS-DNA can dimerize to form ladder like structure leading to stable activation of cGAS; DNA bending and U turns induced by other proteins like TFAM, HMGB pre-arrange the DNA to be nucleated by cGAS* (Andreeva et al., 2017). *C) Schematic of the human cGAS structure and its domains* (Gentili et al., 2019).

cGAS preferentially binds to double stranded DNA (Kd 87.6 nM), though it can bind to single stranded DNA as well with a lower affinity (Kd 1.5 μM) (Kranzusch et al., 2013). cGAS dimer can bind to dsDNA as short as 15-20 bp, but its activation requires additional dimerization. Therefore, longer DNA >45 bp facilitates the cooperative assembly and binding of several cGAS dimers in a head-head conformation forming ladder-like structure, leading to its robust activation in a DNA length and structure dependent manner. In fact, pre-structuring the DNA by inducing bends by other proteins was seen to be preferential for cGAS binding (Andreeva et al., 2017) (**figure 22 B**).

cGAS is composed of 522 amino acid (molecular weight = 60 kDa) (Pathare et al., 2020). It comprises of a non-enzymatic positively charged region, a disordered N-terminal and a positively charged C terminal region, both of which can interact with dsDNA (**figure 22 C**). The C terminus comprise of the catalytic Nucleotidyltransferase (NTase) core domain and Mab21 (Male abnormal 21) domain. cGAS when bound to dsDNA undergoes conformation changes in the C terminal domain leading to its catalytic activity to transform GTP or ATP to cGAMP. The disordered N terminal domain has been implicated in liquid-liquid phase separating cGAS-DNA, which is hypothesized to be important for concentrating cGAS/DNA, thereby enhancing cGAMP production. Phase separation also protects cGAS from degradation by exonucleases (Zhou et al., 2021).

4.2 Regulation of cGAS activity

Other than the cytoplasm, cGAS is also found not only in nucleus but is also chromatinized, with limited enzymatic activity (Lahaye et al., 2018). cGAS binds to the negative acidic patch of nucleosome H2A-H2B and has stronger binding affinity than with naked DNA. Binding of cGAS to nucleosome core particle (NCP) sterically hinders the direct interaction of two cGAS preventing cGAS dimerization and its catalytic activation (Kujirai et al., 2020). Therefore, after nuclear membrane breakdown, cytoplasmic cGAS associates with chromatin during mitosis and stays inactive. Within the micronuclei, chromatinized DNA has been reported to elicit low cGAS-STING mediated immune signaling until the nucleosome in the micronuclei loses its integrity, loses its supercoiled DNA structure and disrupts micronuclei membrane (De Oliveira Mann & Hopfner, 2021). It has been demonstrated that, during oncogene induced DNA damage, MRE11-RAD50-NBN (MRN) complex bound nucleosome causes cGAS eviction from the nucleosome acidic path sequestration resulting in cGAS mobilization to the cytosol and its activation by dsDNA (Cho et al., 2024).

cGAS is also inactivated in a nucleosome-independent manner. cGAS is selectively hyperphosphorylated at its N-terminus by mitotic kinases like Aurora B thereby blocking its DNA binding and phase separation as well as preventing its oligomerization and activation (T. Li et al., 2021). Further, during mitotic entry, kinase CDK1-cyclin B phosphorylates cGAS at S305 preventing its ability of cGAMP synthesis (Zhong et al., 2020). Moreover, methylation of cGAS by methyltransferase SUV39H1 facilitates tethering of cGAS to chromatin and limits its enzymatic activity, (Fang et al., 2023). Additionally, DNA structural proteins like barrier to autointegration factor 1 (BAF) compete with cGAS to bind to self-DNA. When cells encounter an transient loss of membrane integrity, BAF outcompetes the cytosolic cGAS binding for self-DNA thereby preventing the formation of cGAS-DNA complex and inhibiting cGAS activity (Guey et al., 2020).

cGAS translocation in the cytoplasm and nucleus:
The cytosolic and nuclear transport and localization of cGAS have been under study to understand the factors influencing its localization and function. Gentili et al., (2019) reported that once cGAS is in the nucleus, its localization, preferentially at long interspersed nuclear elements (LINES) and at centromeres, is determined through its N terminal (amino acids 1-160) domain and remain catalytically active (though 200-fold less than cytosolic cGAS). The other domain of cGAS 161-212 was seen to be important for cytosolic retention or nuclear export of cGAS. H. Sun et al., (2021) have shown that cGAS is translocated from nucleus to cytoplasm in response to presence of cytosolic DNA, mediated by nuclear export receptor (CRM1) and Nuclear Export Signal (NES) region (169-174). Another report suggested when cells undergo DNA damage, cGAS undergoes dephosphorylation at Y215 and is translocated from cytoplasm to the site of

DNA damage in the nucleus, mediated by nuclear transport receptor importin α, to impair homologous recombination (HR) (Liu et al., 2018).

4.3 Functions of cGAS:

cGAS in senescence and cancer:

The role of cGAS-STING pathway and cellular senescence has been closely studied. Presence of cytoplasmic DNA fragments in senescent cells, or cytosolic DNA arising from DNA damage, oxidative stress, highly proliferative oncogene signaling, chemotherapies, is recognized by the cytosolic DNA sensor cGAS, thereby activating the STING mediated signaling and induction of SASP. Dou et al., 2017 showed that in spontaneous or induced senescence, SASP induction primarily occurred by NF-kB pathway and not through interferon pathway (IFN-β) as p38 MAPK inhibited the induction of STING mediated interferon in primary human cells and mice. But other studies have focused on the role of interferon pathway (IFN1) in cGAS-STING activation (Frisch & MacFawn, 2020). Activating cGAS-STING pathway acts as a barrier to neoplastic transformation upregulating inflammatory genes like IFN-1 as well as SASP containing pro-inflammatory cytokines, chemokines, proteases and growth factors. In a cell-autonomous manner, this serves to recruit immune cells for clearance of the tumor cell or senescent cell, thereby cGAS-STING pathway serve to have tumor-suppressive roles (J. Kwon & Bakhoum, 2020). However, chronic activation of cGAS-STING can lead to persistent inflammatory signaling and cancer in the surrounding cells. An interesting phenomenon speculated by J. Kwon & Bakhoum, (2020) is that chromosome instability (CIN), generated through chromosome segregation defects, leads to micronuclei generation and is a source of cytosolic DNA activating the cGAS-STING signaling. This possibly explains why during early tumorigenesis, altered aneuploidy is rare but as the tumor develops tolerance for chronic inflammation, occurrence of complex aneuploidy expands. In some context, STING activation can occur independently of cGAS. DNA binding protein IFI16, which is also involved in canonical cytosolic DNA sensing, can trigger the alternate activation of STING in cGAS independent manner in a etoposide-induced DNA damage condition (Unterholzner & Dunphy, 2019).

Taken together, the cGAS-STING pathway can lead to different consequences as acute STING activation reinforce senescence progression or immune clearance of senescent/damaged/tumor cells through SASP. Chronic cGAS-STING signaling on the other hand, functions in a cell non-autonomous manner to elicit tumorigenic properties to neighboring normal cells. As cancer cells develops tolerance for chronic inflammation and lose downstream cell-cycle regulators, the inflammation becomes tumorigenic and enable senescence evasion (J. Kwon & Bakhoum, 2020).

Over last few years, studies have focused to identify the non-canonical function of cGAS in the nucleus, beyond its role in innate immune activation through cytosolic DNA sensing. Its role in the nucleus has been mostly shed in but not limited to DNA replication and DNA damage repair.

DNA replication:

Cancer cells often have repressed STING expression (offering resistance to tumor immunity) but cGAS expression is rarely affected indicating that cGAS may have STING independent functions. Tumor cells generally have replication stress as a result of hyperproliferation and uncontrolled DNA replication giving rise to genetic instability. Chen et al. found that cGAS can bind to DNA and act as a roadblock for replication forks progression without affecting the replisome. This will slow down the replication fork movement and reduce the susceptibility of cells to DNA damage. Deficiency of cGAS induced fast replication fork movement, hyperproliferation and replicative stress (Chen et al., 2020).

DNA damage repair (HR):

Cells when faced with DNA damage can undergo various types of DNA damage repair phenomenon, for example, non-homologous end joining (NHEJ), Homologous recombination (HR), Microhomology mediated End joining (MMEJ), NER (Nucleotide Excision Repair). DNA repair by HR is important for genome integrity but if it goes uncontrolled, it can lead to abnormal chromosomal re-arrangements.

As mentioned above, when there is DNA damage, cGAS is hyperphosphorylated at Y215 and translocated inside the nucleus at the site of DNA damage by interaction of C-terminus (213-522) of cGAS to the phospho epitope of H2AX. At the damage site, cGAS interacts with PARP1 via PAR poly (ADP-ribose) and inhibits the formation of PARP1-Timeless complex. The interaction of PARP1 and Timeless is crucial to promote homologous recombination (HR) based DNA repair. Therefore, depleting cGAS suppressed DNA damage and impaired the growth of tumor (Liu et al., 2018).

HR DNA repair was also found impaired by cGAS mediated compaction of dsDNA. The ability of cGAS to self-oligomerize cause dsDNA compaction to form a higher ordered state restrains the RAD51-coated ssDNA filaments to undergo strand invasion for homologous recombination, although not by being specifically recruited to dsDNA breaks, thereby accelerating genome instability (H. Jiang et al., 2019a). Another study focused on the effect of cGAS-STING mediated signaling on the DDR. TBK1 activation through STING leads to the autophosphorylation of ATM kinase and activation of p53-p21 leading to cell cycle arrest following DNA damage. Loss of cGAS negatively affected the DDR induced by genotoxic stress. Despite cGAS stimulate ATM, there is inhibitory effect of stimulated cGAMP on PARylation which then inhibits HD repair after genotoxic insults on human (Banerjee et al., 2021).

DNA damage repair (NHEJ):

Broken chromosome tips, telomere attrition, or absence of telomeric proteins can cause telomere vulnerability which are identified as double strand breaks. This can activate the DNA damage response and repair mechanisms causing recombinations, chromosome end-to-end fusions or DNA degradations. It is reported that the telomere associated protein TRF1/2 and POT1 (of the shelterin complex) is removed from the telomeres during mitosis. Though this activates the DDR during metaphase but there is no subsequent mitotic DNA repair. Li et al., (2022) reported that cGAS generally occupies TRF2-deficient telomeres that protects the telomere ends during mitosis. Following DNA damage, as ATM phosphorylates H2AX at serine 139 it creates a binding site for MDC1 recruitment. MDC1 interacts with E3 ubiquitin ligase RNF8 which regulates the DDR (Tan et al., 2021; Tsai et al., 2020). At critically short TRF2 deficient telomeres, the DDR is blocked as cGAS present at those sites, recruit CDK1 through its NTase domain. CDK1 phosphorylates 53BP1 and RNF8 thereby disrupting the interaction of RNF8 with MDC1 and inhibiting DDR and NHEJ mediated DNA repair (X. Li et al., 2022). Therefore, during mitosis, cGAS acts as a safeguard for the critically short telomeres to prevent them from undergoing chromosome fusions and enhance genome stability.

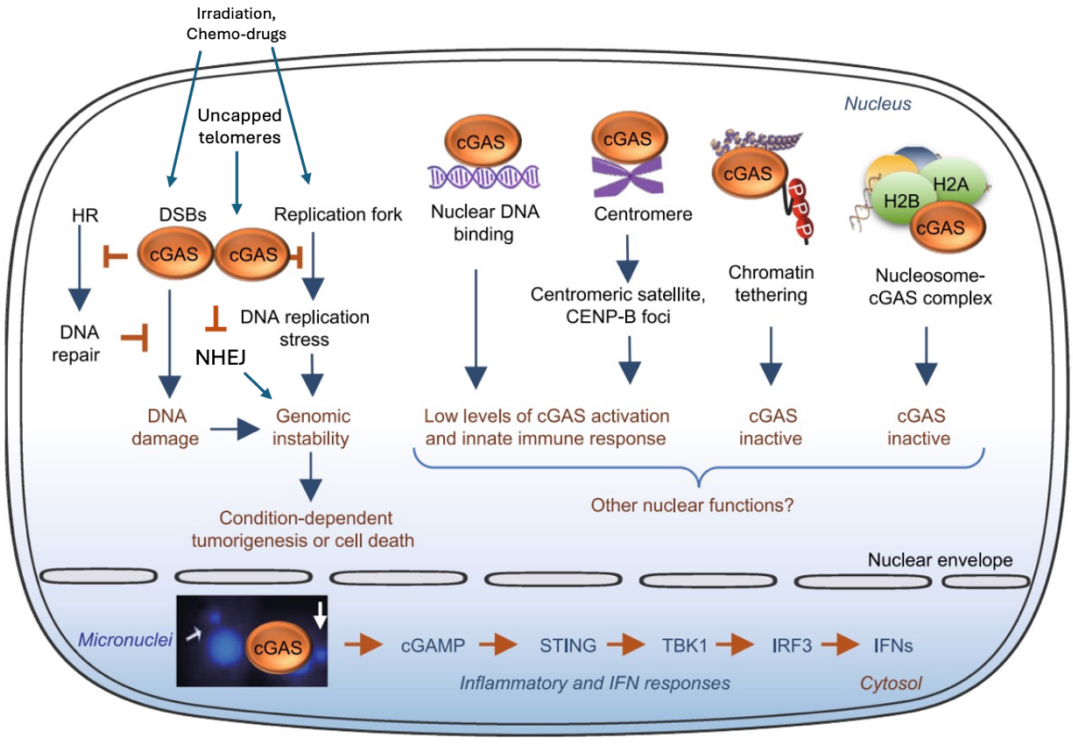


Figure 23: Nuclear functions of cGAS: DNA damage through irradiation or chemo-drugs leads to genome instability in the cell. cGAS then binds to DSB and inhibits HR-mediated DNA repair, leading to enhanced genome instability and tumorigenesis. On the other hand, cGAS suppress replication-stress induced DNA damage by slowing replication forks through directly binding DNA and DNA replication component Proliferation Cell Nuclear Antigen (PCNA), without interfering with the replisome, leading to resistance to radiation and chemotherapy in cancer cells. In the uncapped telomeres, cGAS prevents NHEJ mediated DNA repair by inhibiting chromosome fusions and prevent genome instability. Binding of cGAS to centromeric satellites have been associated with low levels of

cGAS activation whereas tethering of cGAS to nucleosome and hyper-phosphorylation of its N-terminal leads to cGAS inactivation. However, the role of cGAS in the centromere and its roles in the nucleus are not completely understood. In the cytoplasm, cGAS serves to recognize the cytoplasmic DNA leading to innate immune activation and pro-inflammatory and interferon response (modified from Bai & Liu, 2022*).*

Objectives of the PhD project

In the introduction, we looked in detail about how different cellular stress activate the sensors and checkpoints and thereby arrest the cell cycle. Whether it is p16/pRb or p53/p21 pathway, senescence occurs mainly through these key players. Previous research have focused on the occurrence of different senescent hallmarks, for example: the hallmark of chromatin re-organization SAHF has been found to be dependent on key factors (Narita et al., 2003) and to occur during senescence in a context and cell line dependent manner. SADS is another type of chromatin re-organization (Swanson et al., 2013) and it is seen to occur universally across all cell and senescence types. However, its mechanism of formation and its implication in senescence progression remains unknown. Therefore, it becomes important to explore the role of the cell cycle regulators and the pathways that may influence SADS.

Satellite DNA in the human centromeric and pericentromeric region accounts to nearly 6.2% of the recently-assembled telomere-to-telomere genome, of which ~2.8% are alpha satellite (in the CHM13 cell line) (Altemose et al., 2022). During mitosis, proper segregation of sister chromatids requires a functional centromere for proper kinetochore assembly and microtubule attachment. CENP-A nucleosome needs to be accessible to allow loading of kinetochore while the underneath cenDNA offer a balanced mechanical rigidity and elasticity to withstand the tension developed during sister chromatid segregation by microtubules pulling. In this view, the structure of centromere and pericentromere becomes crucial to ensure the faithful segregation of sister chromatid. In my study I have focused primarily on the centromeric α-satellite DNA, known to contain a subset of active HORs on which kinetochore proteins assemble.

Both centromere and flanking pericentromere adopt secondary structure due to its sequence repetitiveness and is further mediated by other factors, for example, as revealed by super-resolution microscopy, CENP-A nucleosomes are arranged in a rosette-like manner (Andronov et al., 2019). Moreover, CENP-B is capable to mediate centromere compaction besides hairpin-like structure that are formed by centromere sequence, indicating that CENP-B can modulate the centromeric topology in the cell (Chardon et al., 2022). These data suggest that the status of centromeric proteins might change during early stage of senescence and may be a direct cause in promoting SADS formation.

Compaction of the centromere is seen to be important for centromere assembly and maintenance and this might also be influenced by epigenetic marks. Pericentromeres are typically associated with repressive heterochromatin marks both H3K9me3 and H3K27me3 (Martins et al., 2016). While centromeres and pericentromeres have heterochromatin marks, it is seen that centromeres also bear open chromatin marks. In mouse embryonic fibroblasts harboring an artificial chromosome (HAC), CENP-B has been known to promote heterochromatin in the alphoid DNA by recruitment of SUV39H1

and HP1α (Otake et al., 2020). CENP-B is also known to recruit H3K36 methylase ASHL1 which is known to promote open chromatin for CENP-A assembly (Otake et al., 2020). The centromere core has also been associated with active histone modifications indicative of transcription. Transcription of centromere has been further associated to maintenance of centromere (Naughton et al., 2022). Therefore, it becomes imperative to understand the epigenetics and transcription at centromere during senescence. To this end, we are also investigating for some specific modifications in the histone at centromere along with the nucleosome occupancy during senescence.

Another hallmark of senescence is its secretory phenotype which is partly mediated by cytosolic DNA sensor and innate immune activator cGAS. Since cGAS is also seen to be enriched at the centromere (Gentili et al., 2019), I have also investigated how cGAS is recruited at the centromere, whether it affects the centromere structure and if it has any impacts in senescence through centromere.

In summary, I present my PhD work into 3 main parts: In the first part, I explore the factors that are involved in influencing/regulating SADS by focusing on the role of p53 and DNA damage response, the role of centromeric proteins. In the second part, I present the epigenetic alterations at centromere when cells undergo SADS during senescence. In the third part, I focus on the role of cGAS in centromere compaction during senescence and factor that recruits cGAS to centromere.

Aims and Methodologies:

1. Explore the factors regulating SADS during senescence

To study the phenomenon of SADS, I first established senescence in immortalized, non-transformed diploid human foreskin fibroblast BJ cells and in immortalized, diploid retinal pigment epithelial RPE-1 cells.

To interrogate which factors affect distension of centromeric DNA during senescence (a.k.a SADS), I looked into the role of p53 mediating centromere unfolding. For this, I used the cellular tool developed by our lab previously, which comprises of short hairpin RNA based depletion of p53 in immortalized non-transformed human RPE-1 cells. I further looked into the role of DNA damage response in SADS formation by using chemical inhibitors of ATM and ATR.

I also investigated how depletion of centromeric proteins CENP-A, CENP-B and CENP-C affect centromeric DNA compaction by using an auxin-induced degron tagged with centromeric proteins which was already prepared in our lab (Hoffmann et al., 2016).

Further we also investigated the role of CENP-B and its domains in regulating the centromeric DNA distension during senescence. Immortalized BJ cells were transduced with lentivirus carrying doxycycline-inducible CENP-B (FL). DLD-1 cell lines (pseudodiploid colorectal adenocarcinoma cell line) were prepared by inserting dox-inducible CENP-B (FL and mutants) into the isogenic FRT sites through Flp-In system (as in Hoffmann et al., 2020).

2. Epigenetics at centromere during senescence

The objective here is to explore the epigenetic changes at the centromere when cells go to senescence. We investigated particular histone marks typical of open and closed chromatin confirmation in senescent RPE-1 and DLD-1 cells. I also looked into the functional implication of alpha satellite distension in centromere by the transcription status of centromere during senescence in RPE-1 cells.

3. Insights on cGAS at Centromere

Since cGAS associates with centromere (Gentili et al., 2019b), I investigated what recruits cGAS to centromere. Specifically I used human U-2 OS cells (hypertriploid osteosarcoma cell line) engineered with bacterial LacO array containing 200 copies of 256 tandem repeats of LacO sequence in the subtelomeric region of chromosome 1 (Janicki et al., 2004; Logsdon et al., 2015). Further the effect of cGAS in regulating SADS was carried out through CRISPR-Cas9 genome editing using lentivirus in BJ cells to target and knockout cGAS.

Results

1. Factors regulating SADS during senescence

1.1 Kinetics of Senescence

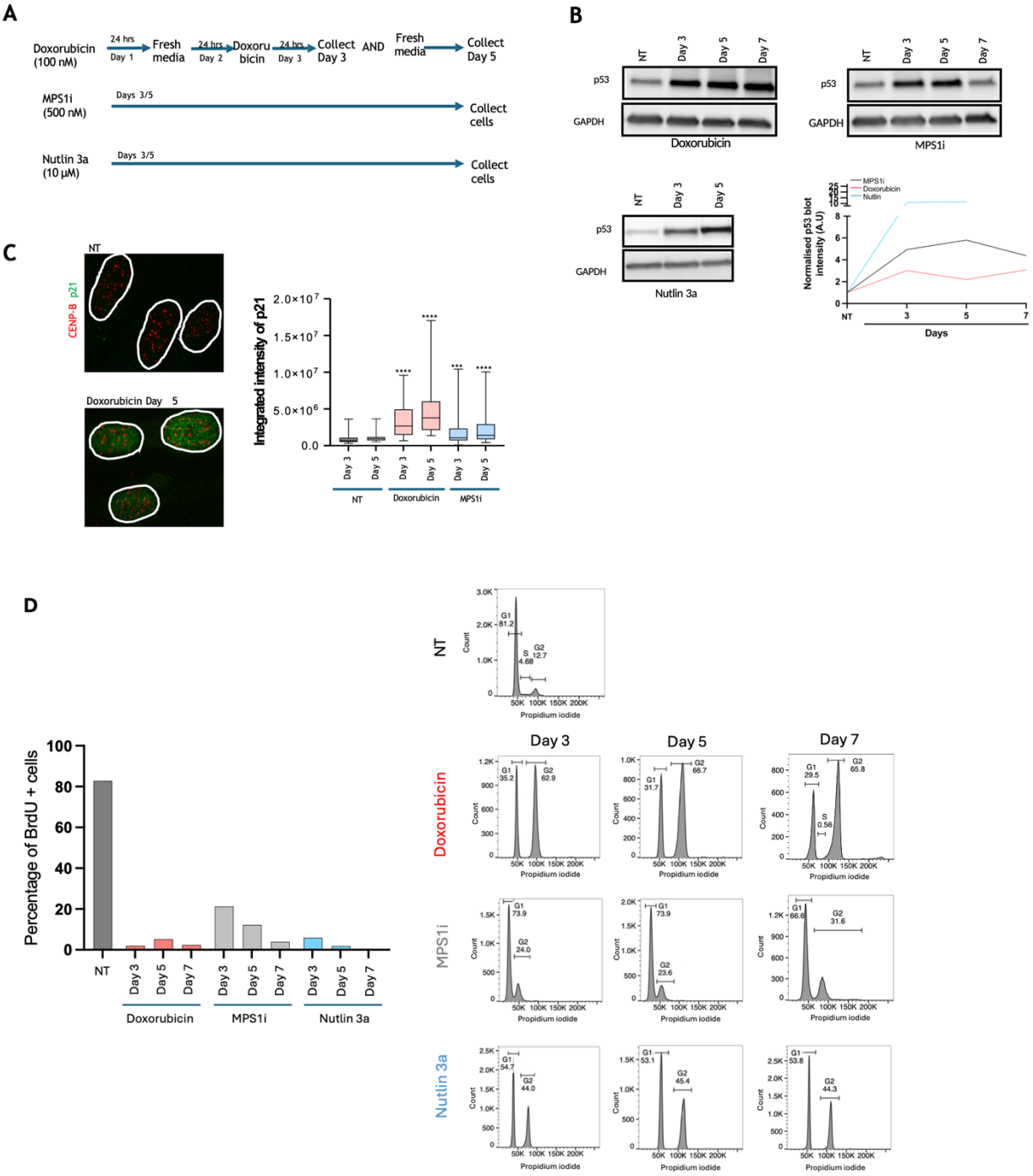
To investigate the factors involved in SADS formation during senescence, I first triggered the formation of senescence by three different methods:
1/ DNA damage using Doxorubicin. Doxorubicin is known to intercalate into DNA and induces torsional stress that might cause nucleosomes destabilization. Further, low doses of doxorubicin is also demonstrated to target and capture primarily topoisomerase II and also upto some extent topoisomerase I and cause single strand and double strand DNA breaks (Kciuk et al., 2023).
2/ Chromosome missegregation using MPS1 inhibitor (MPS1i) which inhibit the spindle assembly checkpoint, leading to the accumulation of aneuploidy (Tardif et al., 2011). In response to aneuploidy cells are known to go to senescence (He et al., 2018) (R. W. Wang et al., 2021).
3/ Nutlin 3a which is known to inhibit MDM2 thereby stabilizing p53.

To trigger senescence, BJ cells were treated with Doxorubicin (2 pulses), MPS1i or Nutlin 3a for 3 and 5 days before collection for further analysis (**figure 24 A**). I looked into markers which are indicators of cellular stress and also senescence. As predicted, p53-positive BJ cells accumulate p53 when treated with Doxorubicin and MPS1i followed by an increase in p21 intensity as seen in **figure 24 B, C**. RPE-1 cells accumulate p53 when treated with nutlin 3a (**figure 24 B**).

To assess the impact of the drugs on cycling capacity of cells, I performed flow cytometry analysis with propidium iodide (PI) intensity and 24 hours of BrdU incorporation in RPE-1 cells to catch the percentage of cells that will pass through replicating phase (including slow) during the doxorubicin, MPS1i and nutlin 3a treatment. Untreated cells had the greatest number of BrdU positive cells with most cells in G1 and some cells in S and G2 phase (**figure 24 D 1st lane)**. With doxorubicin, cells fail to incorporate BrdU, even after 24hr, and they accumulate either in G1 or G2 (**figure 22 D 2nd lane)**. In MPS1i treated cells (**figure 22 D 3rd lane**), most cells are arrested in G1 and slowly reduce BrdU incorporation. Nutlin 3a treated cells stopped cycling completely on day 5 (**figure 24 4th lane**).

A widely used marker of senescence is the β-galactosidase accumulation which is an indication of the lysosomic content of a senescent cell (B. Y. Lee et al., 2006). Upon addition of an external chromogenic substrate for β-galactosidase, for example, X-gal (5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside) at pH 6, β-galactosidase from

senescent cells hydrolyse the substrate to form a blue colored product. By quantifying the accumulation of β-galactosidase in cells following treatment with Doxorubicin, MPS1i or Nutlin after 3 and 5 days I found that Doxorubicin treatment caused the most accumulation of β-galactosidase as seen in **figure 24 E**.



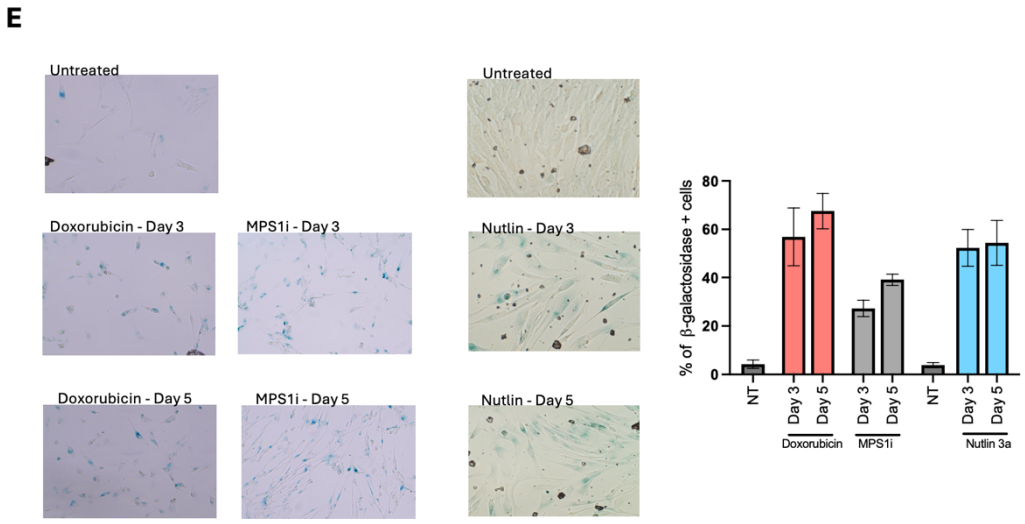


Figure 24: Cells treated with doxorubicin, MPS1i and nutlin 3a show senescence markers like enhanced expression of p53/p21, arrest the cell cycle and increase β-galactosidase expression

A. Schematic of treatment of doxorubicin, MPS1i and nutlin 3a on BJ and RPE-1 cells

B. Western blot showing p53 expression in BJ cells upon treatment with doxorubicin and MPS1i and in RPE-1 cells upon treatment with nutlin 3a for 3, 5 and 7 days. The graph below of p53 band intensity were normalized to house-keeping gene loading control GAPDH intensity. Untreated were normalized to 1. One dot represents one replicate. N=3 replicates per conditions.

*C. Representative projected fluorescence images of p21 in BJ cells treated with doxorubicin and MPS1i. The integrated intensity of p21 was measured in the nucleus of the cell and paired parametric t-test was performed: ****p< 0.0001*

D. Percentage of BrdU positive RPE-1 cells after 24 hours of 30 μM BrdU incorporation in untreated condition and treated with doxorubicin, MPS1i and nutlin 3a for 3, 5 and 7 days. The percentage of cells in G1, S and G2 phase are indicated in the histogram. N=1 replicate.

E. Accumulation of β-galactosidase in BJ cells when treated with doxorubicin, MPS1i and in RPE-1 cells treated with nutlin 3a. Left: Representative images showing the blue coloration of senescent cells following β-galactosidase assay. Right: Quantification of percentage of cells accumulating β-galactosidase after days 3 and 5 of treatment.

1.2 Kinetics of SADS formation

It is known that SADS is an early event of senescence. As one of the aims is to elucidate the factors involved in SADS, it is therefore important to establish that these senescent cells have undergone unfolding of their α-sat DNA upon treatment with Doxorubicin, MPS1i and Nutlin3a. To achieve this, I performed immunofluorescence- coupled with Fluorescence In Situ Hybridization (IF-FISH), where IF was carried out against centromeric proteins and FISH against centromeric DNA using a mixture of six PNA probes of ~50 bp that hybridize to α-satellite DNA ranging from 50 to 1300 kb (Giunta et al., 2021) (**figure 25 A**).

To quantify the distension of centromeric DNA, I measured the circularity = (4π (area/perimeter^2)) and area of each α-sat DNA foci based on its signal intensity through an in-house developed macro in Fiji software, as recently done in our laboratory (Chardon et al., 2022). A value of 1 indicates a perfect circle. A reduction in the value of circularity is indicative of reduction in the compaction of DNA, while an increase in the area of α-sat DNA foci indicates the unfolding and distension of centromeric DNA (**figure 25 B**). As seen in **figure 25 C**, I observed a reduction in the circularity and an increase in the area of α-sat DNA foci at day 3 and 5 when subjected to doxorubicin, MPS1i and nutlin 3a thereby indicating SADS formation.

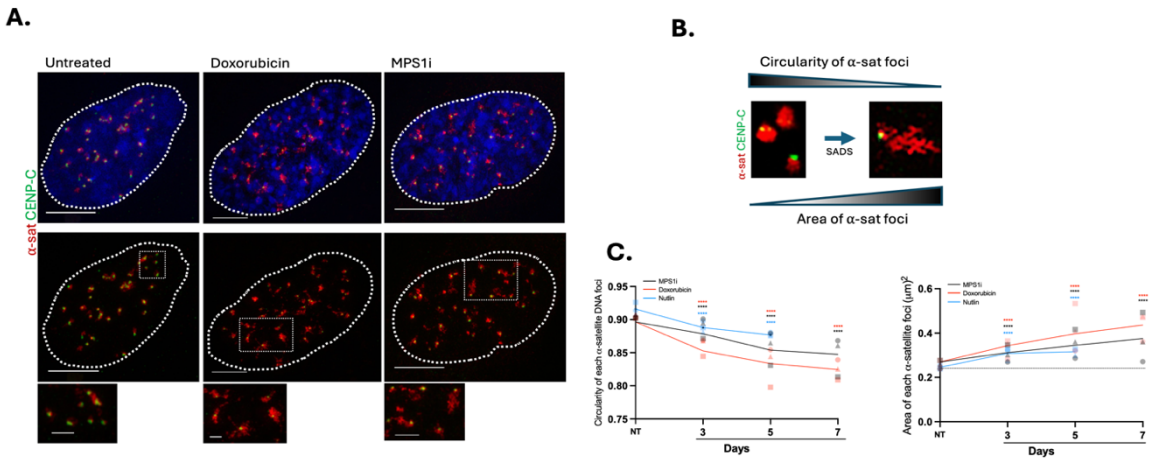


Figure 25: α-satellite DNA undergoes distension when treated with doxorubicin, MPS1i and nutlin 3a.

A) Representative 3D-SIM images of BJ cells treated with doxorubicin or MPS1i. White square marks a zoomed region. Scale bars are 5 μm and in the zoomed images 1 μm.

B) Schematic showing the method of quantification of α-satellite DNA distension characterized by a reduction in circularity and an increase in the area of α-sat DNA foci.

*C) Quantification of α-satellite DNA unfolding after cells were treated with either doxorubicin, MPS1i in BJ cells or nutlin 3a in RPE-1 cells. For statistical analysis, unpaired t-test was performed: ****p<0.0001*

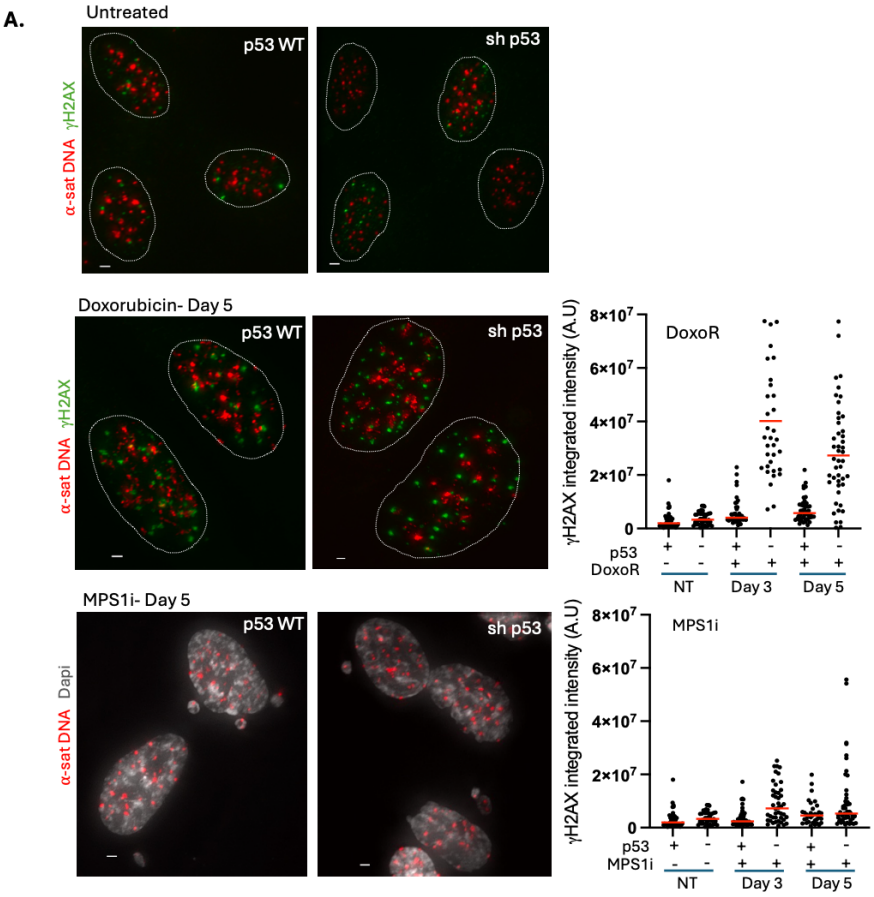
1.3 Role of p53 and DNA damage response in regulating SADS

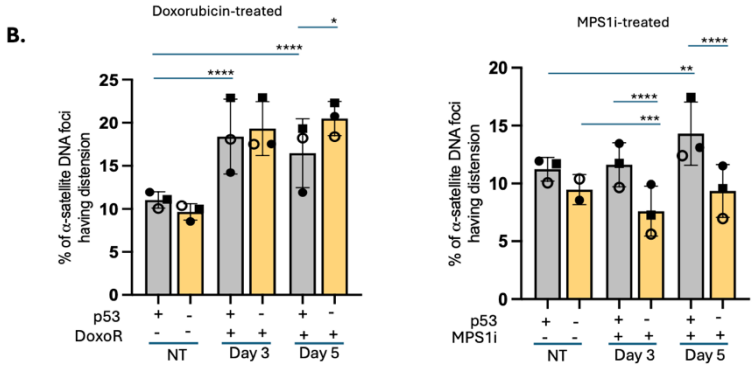
In the previous sections of the introduction, I highlighted how a cycling cell arrests its cell cycle and activates various checkpoints when faced with different cellular adversities that in turn may lead to the activation of p53 and cellular senescence. To understand if SADS formation requires downstream action of p53, I investigated SADS in RPE-1 cells that lack p53 by using short harpin (shRNA) based depletion of p53, stably integrated by lentiviral delivery, in conjunction of doxorubicin or MPS1i treatment. I first quantified the intensity of DNA damage biomarker γH2AX by immunofluorescence staining following application of the drugs. As expected, I found that doxorubicin treated cells accumulated more breaks, interestingly more in the p53 knock down cells (**figure 26 A**). No specific colocalization between γH2AX intensity and α-sat DNA foci in untreated or treated cells was observed excluding the possibility of site of DNA damage directly impacting α-sat distension.

Based on the area and circularity of α-sat signal, I then quantified the percentage of α-satellite DNA foci that got distended. To calculate this percentage, I have first considered a threshold value of alpha-sat foci circularity-area based on the untreated condition and applied this threshold to treated condition. Values above this threshold were considered as distended alpha-satellite DNA (or SADS). I found that depleting p53 had no effect on distension of α-sat DNA when cells underwent DNA damage upon treatment with doxorubicin compared to untreated (**figure 26 B**). In the case of MPS1i, which is known to cause aneuploidy (evident from the presence of micronuclei in grey bearing centromeres in red, **figure 26 A, 3rd lane**), p53 depleted cells partially prevent unfolding of α-sat DNA (**figure 26 B**).

In a single given cell, the unfolding of each α-satellite DNA foci will lead to its spreading, covering more area in the cells as compared to compact α-sat DNA foci. To better quantify the distension of alpha satellites I therefore sum up area of all the α-sat DNA foci in a cell in untreated and doxorubicin treated condition rather than looking into the area of one individual DNA foci, as recently done (Takata et al., 2023). This also enables us to attenuate any bias in segmentation of each α-sat foci signal made by the macro. Therefore, if senescent cells have distended α-sat DNA, then the sum of all α-sat DNA foci per cell will be higher compared to the sum in untreated cells. Accordingly, I found α-sat DNA undergo distension when cells face DNA damage, independently of p53 (**figure 26 B left, 26 C**)**.** However, p53 depleted doxorubicin-treated cells show delayed accumulation of β-galactosidase comparable to cells with wildtype p53 (**figure 26 D**).

Taken together, these results support that DNA damage is sufficient to cause unfolding of α-sat DNA and in this context p53 is dispensable from its formation. However, in aneuploidy-induced senescence via MPS1 inhibition, p53 plays an important role for their formation. The discrepancy between SADS formation and β-galactosidase intensity in doxorubicin-treated cells further suggest that SADS formation is not a direct requirement for senescence activation but requires p53 to activate senescence.


A.
Untreated
p53 WT
sh p53
α-sat DNA γH2AX
Doxorubicin- Day 5
p53 WT
sh p53
α-sat DNA γH2AX
DoxoR
γH2AX integrated intensity (A.U)
8×10^7
6×10^7
4×10^7
2×10^7
0
p53
DoxoR
NT
Day 3
Day 5
MPS1i- Day 5
p53 WT
sh p53
α-sat DNA Dapi
MPS1i
γH2AX integrated intensity (A.U)
p53
MPS1i
NT
Day 3
Day 5


B.
Doxorubicin-treated
% of α-satellite DNA foci having distension
p53
DoxoR
NT
Day 3
Day 5
MPS1i-treated
% of α-satellite DNA foci having distension
p53
MPS1i
NT
Day 3
Day 5

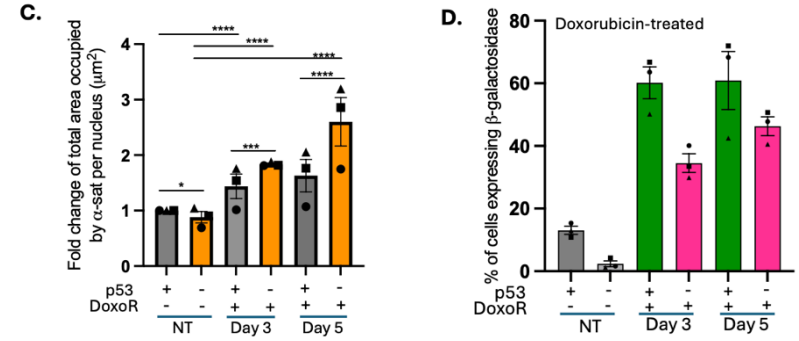

C.
Fold change of total area occupied by α-sat per nucleus (μm²)
p53
DoxoR
NT
Day 3
Day 5
D.
Doxorubicin-treated
% of cells expressing β-galactosidase
p53
DoxoR
NT
Day 3
Day 5

Figure 26: p53 is dispensable for α-satellite DNA unfolding when cells are subjected to DNA damage.

A. IF-FISH (γH2AX - α-sat) images of RPE-1 cells treated with doxorubicin and MPS1i with scale bar of 2 μm. MPS1i treated cells have DAPI signals and show micronuclei formation. On the right are quantification of γH2AX intensity on cells treated with MPS1i and Doxorubicin.

B. Percentage of α-sat having distension upon treatment with MPS1i (left) and doxorubicin (right) in RPE-1 cells. Histogram was plotted based on 3 independent replicates and at least 30 cells in each replicate were analyzed.

*C. Ratio of the sum of area of α-sat foci per nucleus in RPE-1 cells (wt p53 versus sh p53) treated with or without doxorubicin. The ratio was obtained by normalizing with the sum of α-sat foci of untreated sample in WT p53. The statistical analysis is based on ordinary 2-way ANOVA test: ****p <0.0001*

D. Percentage of cells expressing β-galactosidase upon treatment with or without doxorubicin based on 3 independent replicates. Error bars: standard error of mean.

1.4 Role of ATM and ATR in the distension of α-satellite DNA

The dispensability of p53 in α-sat DNA unfolding during DNA damage suggested us to look for the factors upstream of p53 that can have influence on the distension. To this end, I investigated the role of kinases ATM and ATR in regulating SADS. As detailed in the introduction part, ATM is activated upon dsDNA break while ATR upon ssDNA to further phosphorylate p53 (either directly or via phosphorylation of CHK1 and CHK2). To inhibit ATM and ATR, I used chemical inhibitors together with applying cell stressors. Along with DNA damage-based stressor as doxorubicin, I also decided to see the effect of ATR or ATM inhibition on SADS formation via a non-DNA damage-based stressor as nutlin 3a. The experiment was performed as the following: ATM and ATR inhibitors (10 μM) were added 30 minutes prior to application of doxorubicin and nutlin 3a, and the RPE-1 cells were collected after 3 and 5 days of treatment to check for centromeric DNA distension. To visualize α-sat distension, I carried out IF against CENP-B. CENP-B is a DNA sequence-specific binding protein that binds to B-box. B-boxes are present in every other α-sat monomer and spans through the active α-sat DNA. To verify that I can use CENP-B staining as *bona fide* read out for SADS, I performed an IF-FISH with CENP-B and α-sat DNA in stressed condition. Here I see that α-sat DNA and CENP-B perfectly colocalize with each other (**figure 27 A**), confirming that CENP-B can be used as a proxy of α-sat DNA.

Using CENP-B to score for SADS, doxorubicin-treated cells (orange columns) display ~2 to 3 fold increase in α-sat distension (SADS), while either ATM or ATR inhibition (pink and grey columns respectively) led to modest reduction in SADS, with more reduction in ATM inhibition compared to ATR inhibition (**figure 27 B, C**). However, the distension was still significantly higher compared to non-treated (NT). This hints that DNA damage can induce distension of α-sat DNA with only minor effects of DNA damage response.

I confirmed the induction of DNA damage and/or the p53 activation by immunoblotting. In doxorubicin condition, cells accumulate γH2AX while no such accumulation was observed in nutlin 3a-treated cells (**figure 27 D**). p53 was activated in both conditions as expected. ATM or ATR inhibition did not impact neither γH2AX or p53 activation. Indeed, it was previously seen that cells can still undergo phosphorylation of H2AX through alternate DNA-dependent protein kinase (DNA-PK) when ATM or ATR is inhibited (Trakarnphornsombat & Kimura, 2023). This could explain why I did not observe changes in γH2AX accumulation.

In addition, cells treated with p53 stabilizer nutlin 3a, has milder effect compared to doxorubicin but still accumulate almost 1.5 to 1.6 fold increase in distended α-satellite DNA (**figure 27 B, C**). However, inhibition of either ATM or ATR did not affect α-sat distension in this condition. As p53 stabilization does not cause any DNA break, as evident from reduced γH2AX intensity (**figure 27 D**)**,** inhibiting the DNA damage response did not have any effect on SADS (**figure 27 B, C**).

I then monitored the accumulation of β-galactosidase to follow the senescence hallmarks at a later step. Here I observed that inhibition of ATM or ATR delayed their accumulation in both doxorubicin and nutlin 3a treated cells (**figure 27 E**).

In summary, this indicates that p53 stabilization and/or activation can promote unfolding of α-sat DNA independently of the canonical DDR, but both ATM and ATR seems to be necessary for the cells to exhibit later senescence hallmarks such as β-galactosidase accumulation. Stabilization of p53 cause distension of α-sat DNA. But when there is DNA damage, it is sufficient to cause α-sat distension per se, independent of DDR through ATR, but with minor effects of ATM.

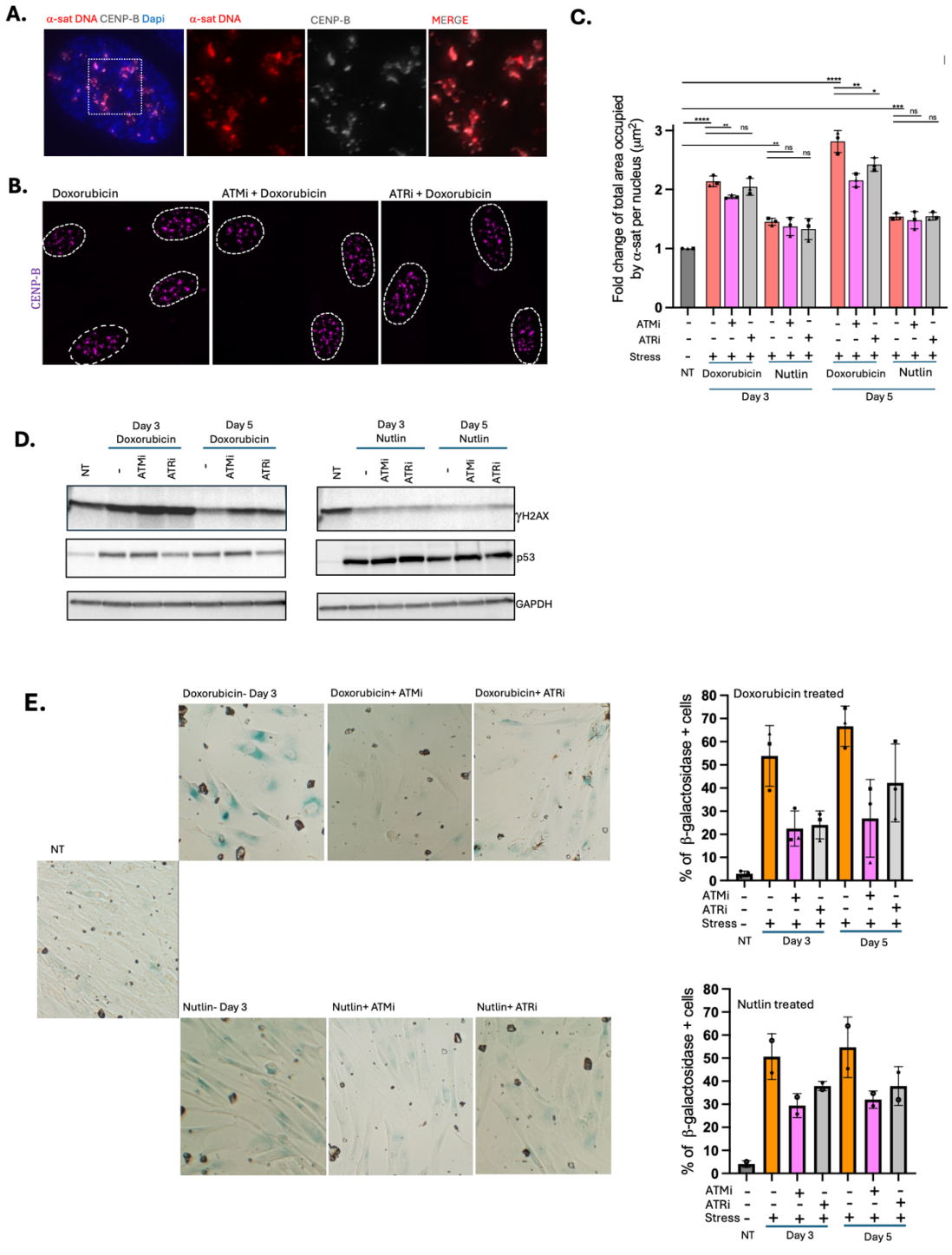


Figure 27: DNA damage is sufficient to cause α-sat distension per se, independent of DDR.

A. Representative image of superimposition of CENP-B and α-sat DNA signals in RPE-1 cells obtained by performing IF-FISH. Dotted square corresponds to the zoomed region on the right.

B. Representative images of IF against CENP-B in cells treated with ATM and ATR inhibitor followed by doxorubicin treatment.

*C. Graph showing the ratio of the total CENP-B foci area per nucleus. (N=3 independent replicates, n >40 cells analyzed per condition in each replicate), and unpaired t-test was performed: ****p<0.0001.*

D. Western blot of cells treated with ATM and ATR inhibitor followed by doxorubicin and nutlin 3a treatment for 3 and 5 days. Blots were obtained for γH2AX, p53 and the loading control GAPDH.

E. Percentage of β-galactosidase positive cells after 3 and 5 days of treatment with ATM/ATR inhibitor, doxorubicin and nutlin 3a. N=3 independent replicates for doxorubicin treatment and N=2 replicates for nutlin 3a treatment, and n> 200 cells per replicate.

1.5 Role of Centromeric protein in regulating SADS

1.5.1 Status of centromeric proteins during senescence

Centromeres are composed of unique repetitive α-satellite DNA and a complex of various centromere associated-proteins whose stability might be altered when the α-satellite distend during senescence. To test this hypothesis, I first measured the abundance of total endogenous centromeric proteins during senescence in the whole cellular lysate. Cells treated with MPS1 inhibitor (MPS1i) and doxorubicin for up to 7 days enter senescence and cease cycling (**figure 28**). This stop in the cell cycle leads to a decrease in the production of centromeric proteins CENP-A/B/C as observed in the Western blot (**Figure 28 A, quantified in B**). However, this reduction in the intensity levels was quite variable across different replicates.

When measured their intensity at the centromeres in the nucleus, CENP-A/C levels remained unchanged while CENP-B showed a very minimal reduction in intensity as seen in the quantification in the graph (**figure 28 C, D**). This falls in a similar line as observed previously (Swanson et al., 2013) where centromeric proteins CENP-A and B were still mainly associated with centromeres in senescent cells. Accordingly, to what described before (**figure 28 A**) CENP-B bound to α-sat appeared distended in doxorubicin and MPS1i treated cells (**figure 28 C**), whereas CENP-A and CENP-C remains visibly undistended.

In summary, the overall abundancy of centromeric proteins decline in senescent cells but their association to centromeres largely remain unchanged with the exception of CENP-B.

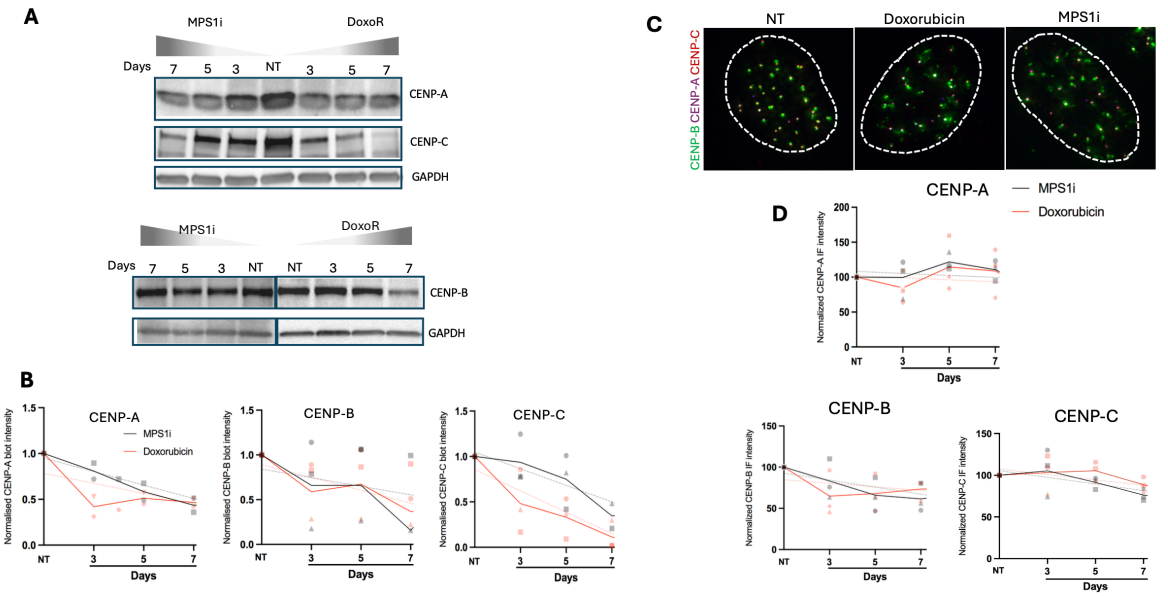


Figure 28: Total level of centromeric proteins reduce in senescent cells but their association to centromeres remain mostly unchanged

- ***A.*** *Western blot showing expression of centromeric proteins CENP-A, CENP-B and CENP-C from the whole cell lysate of BJ cells treated with MPS1i or doxorubicin for 3, 5 and 7 days.*
- ***B.*** *Graph showing the quantification of the expression of CENP-A, CENP-B and CENP-C of 3 independent replicates from A.*
- ***C.*** *Representative projected fluorescence images of BJ cells treated with doxorubicin or MPS1i.*
- ***D.*** *Graph showing the quantification of the intensity of CENP-A, CENP-B and CENP-C at the centromeres. N= 3 independent replicates with n> 40 cells per replicate.*

1.5.2 Role of centromeric proteins in α-satellite compaction

To understand if any changes of CENPs drive the distension of α-sat, I determined the effect of depletion of these centromeric proteins on centromere compaction. Previous works (Andronov et al., 2019) (Melters et al., 2019) showed that the elasticity of centromeric DNA is mediated by the histone variant CENP-A and is further stabilized upon addition of CENP-C. To test their role in centromere compaction I depleted endogenous CENP-A or CENP-C using a rapid auxin-inducible degron system in human non-transformed RPE-1 cells. Specifically: CENP-A^{AID} in WT or CENP-B-/- background to deplete CENP-A, and CENP-C^{AID} to deplete CENP-C (Hoffmann et al., 2016). These cells lines were generated by adding AID tags to both alleles of endogenous CENP-A/CENP-C to induce its rapid and total depletion. A point to note here is that these centromeric proteins are responsible for centromere integrity and maintenance (Giunta et al., 2021) and their absence leads to defect in kinetochore assembly and chromosome missegregation, leading to aneuploidy (Hoffmann et al., 2016). In response to aneuploidy, cells are known to go to senescence (He et al., 2018) (R. W. Wang et al., 2021) through the p53 pathway (M. Li et al., 2010) (Maehara et al., 2010). Therefore, depletion of these centromeric proteins will lead to aneuploidy causing senescence activation and likely leading to SADS formation. To directly test whether depletion of centromeric proteins impact centromere compaction independently of aneuploidy, cells were permanently arrested in G1 through treatment with a CDK4/6 inhibitor (palbociclib) for 24 hours before depletion of the centromeric proteins (**figure 29 A**).

To evaluate the unfolding of centromeric DNA following depletion of centromeric proteins, I performed FISH on α-sat (**figure 29 B**). **Figure 29 C** and **D** shows the circularity of α-sat DNA foci and percentage of α-sat DNA foci that underwent distension, respectively. Accordingly, to aneuploidy response, depletion of both CENP-A and CENP-C in cycling cells for 72 hours had an effect of decompaction on α-sat DNA as visible from reduced circularity and increased percentage of distended α-sat foci (grey columns). Cells treated using palbociclib exhibited also decompaction of α-sat DNA (purple column), accordingly to the fact that prolonged G1 arrest cause also senescence activation (Crozier et al., 2023). Interestingly, removal of CENP-A and CENP-C on G1 arrested cells further enhanced α-satellite distension (green histogram). In condition where CENP-B is knocked out, cells already beared the maximum distension in α-satellite DNA. Accordingly, further removal of CENP-A or addition of palbociclib on these cells did not have a significant additional effect on α-satellite (**figure 29 C** and **D**).
Hence, centromeric proteins CENP-A, CENP-B and CENP-C mediate compaction of centromeric DNA. While arresting the cells using palbociclib had an effect on α-satellite distension, the distension was further promoted upon removal of centromeric protein.

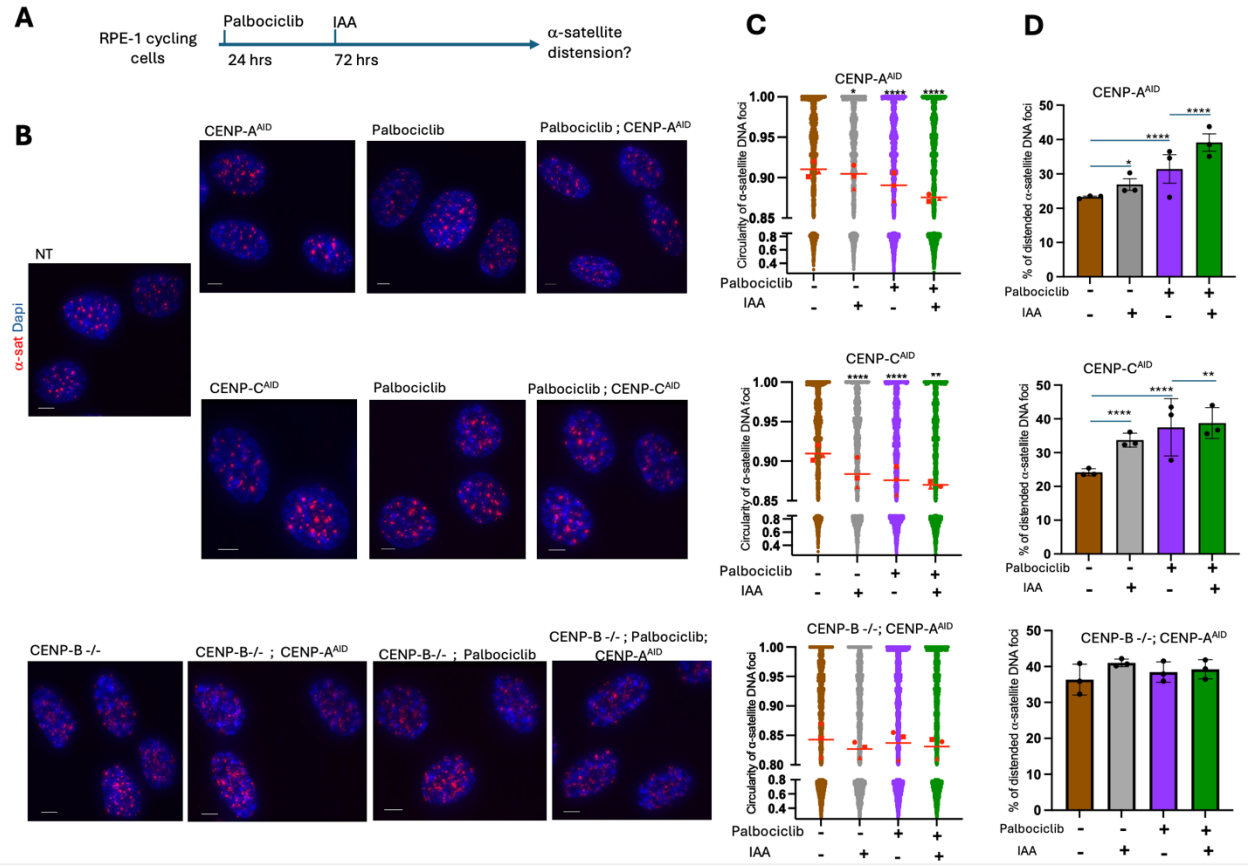


Figure 29: CENP-A, CENP-B and CENP-C depletion further promotes α-satellite distension in cycling or non-cycling cells.

A. *Schematic of the experiment performed to assess the effect of depletion of centromeric proteins on α-satellite distension in G1 arrested cells.*

B. *Representative projected fluorescence images of RPE-1 cells after performing FISH against α-satellite DNA. Top lane in order from left to right represents RPE-1 cells with CENP-A ^{AID} (non-treated, treated with auxin, treated with Palbociclib and co-treated with palbociclib and auxin). Similarly for 2nd lane with CENP-C ^{AID} and last lane with CENP-B -/- with CENP-A ^{AID}. Scale bar 5 μm.*

C. *Circularity of each α-satellite DNA foci for each of the three replicates in three cell types. Mean of each replicate is marked large in red. N= 3 replicates, n >40 cells per replicate.*

D. *Percentage of α-satellite DNA foci undergoing distension following depletion of centromeric proteins. N= 3 replicates, n >40 cells per replicate. Statistical analysis were performed based on Kolmogorov-Smirnov test.*

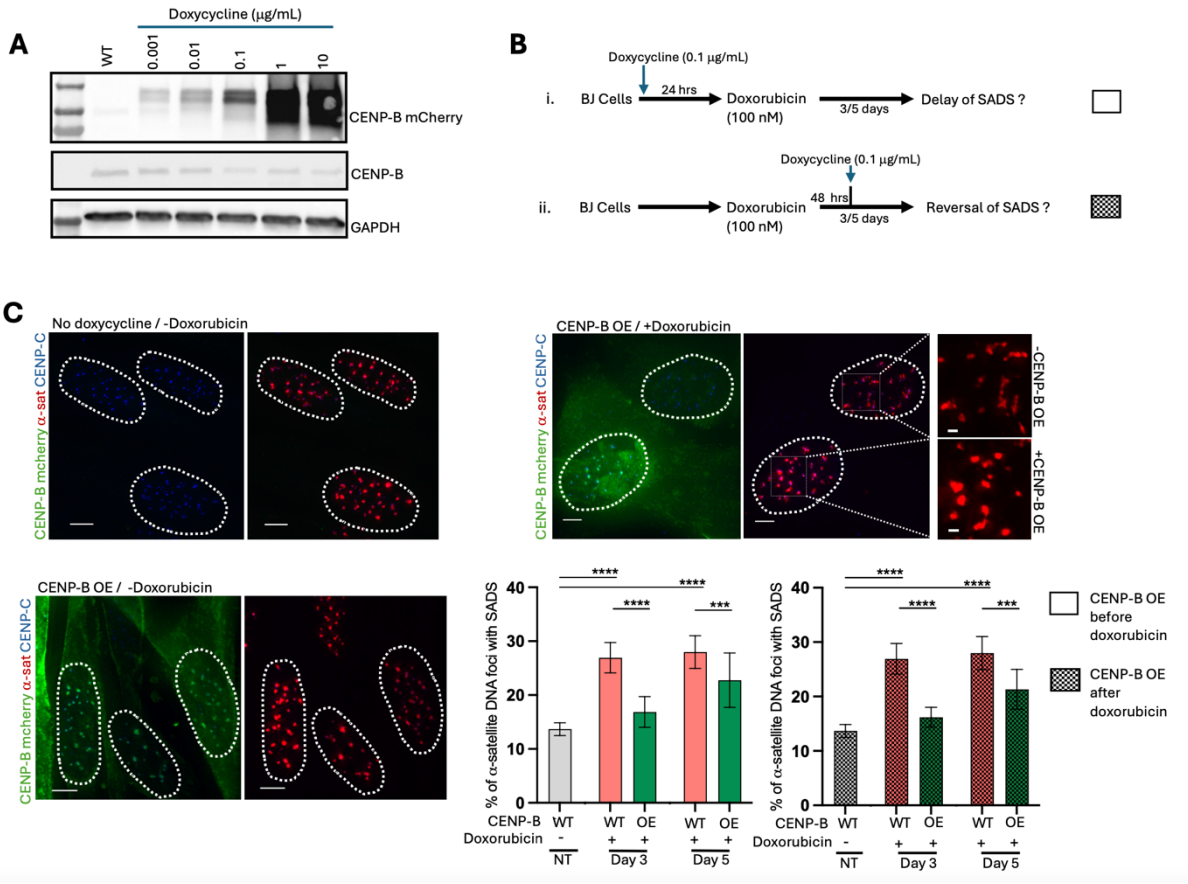
1.5.3 Effect of centromeric protein CENP-B on α-satellite distension and other phenotypes during senescence

Above, I demonstrated how centromeric proteins impact the arrangement of α-satellite in human cells. Especially the depletion of CENP-B had the highest impact on distension of α-satellite. This is supported by a study carried out previously in our lab by (Chardon et al., 2022) in which I show a role for CENP-B in mediating centromere architecture. From this previous work, I now explored the ability of CENP-B in controlling the SADS formation. To perform this, I ectopically overexpressed doxycycline-inducible CENP-B (FL) tagged with mCherry in BJ cells through lentivirus transduction and then confirmed the expression of CENP-B (FL) tagged with mCherry through different doxycycline concentration (**figure 30 A**) using mCherry antibody. I note that upon increasing doxycycline concentration, the level of mCherry tagged CENP-B increased, as expected, while the endogenous CENP-B slightly decreased indicating competition with the endogenous, untagged CENP-B.

Then I explored the ability of CENP-B to mediate centromeric DNA compaction in two scenarios: through CENP-B overexpression either before or after doxorubicin treatment to assess how it will impact on SADS formation as shown in the schematic of **figure 30 B**. In the first scenario, I assessed the ability of CENP-B to delay SADS formation, while in the second if CENP-B can recompact the already formed SADS. We can appreciate in **figure 30 C,** the heterogeneity in the ectopic overexpression of CENP-B mCherry construct on BJ cells treated with doxycycline. A cell which expressed CENP-B mCherry has compacted α-satellite structure upon treatment with doxorubicin, while the other cell which did not express ectopic CENP-B has distended α-sat. Images quantification showed that overexpressing CENP-B either before or after doxorubicin is able to partially delay or reverse SADS in doxorubicin-treated cells compared to cells with WT CENP-B but only at the early time point

However, CENP-B OE during doxorubicin treatment did not delay or prevent p53 or p21 activation (**figure 30 D**)**,** neither prevents the increase in size of the nucleus, another phenotype normally associated to senescent cells (**figure 30 E**). This data also indicates that regulation of centromeric DNA compaction through CENP-B happens independently of the nuclear size since we can uncouple total nucleus size from SADS. Finally, when I tested the effect of CENP-B mediated α-sat DNA compaction on the widely used senescent marker β-galactosidase, I found that overexpressing CENP-B partially delayed the accumulation of β-galactosidase in the early time point, but it was not able to have a reversal effect in the accumulation of β-galactosidase (**figure 30 F**).

Therefore, I conclude that CENP-B plays a role in mediating centromeric DNA organization during senescence. Through our data I can also speculate that SADS might be important to boost the late senescence hallmarks, such as the accumulation of β-galactosidase, but as expected is not linked to other senescence responses like the nuclear size.


A
Doxycycline (μg/mL)
WT
0.001
0.01
0.1
1
10
CENP-B mCherry
CENP-B
GAPDH
B
Doxycycline (0.1 μg/mL)
i.
BJ Cells
24 hrs
Doxorubicin (100 nM)
3/5 days
Delay of SADS ?
ii.
BJ Cells
Doxorubicin (100 nM)
48 hrs
3/5 days
Reversal of SADS ?
C
No doxycycline / -Doxorubicin
CENP-B mcherry α-sat CENP-C
CENP-B OE / +Doxorubicin
-CENP-B OE
+CENP-B OE
CENP-B OE / -Doxorubicin
% of α-satellite DNA foci with SADS
CENP-B OE before doxorubicin
CENP-B OE after doxorubicin
CENP-B WT OE
Doxorubicin - + + +
NT
Day 3
Day 5

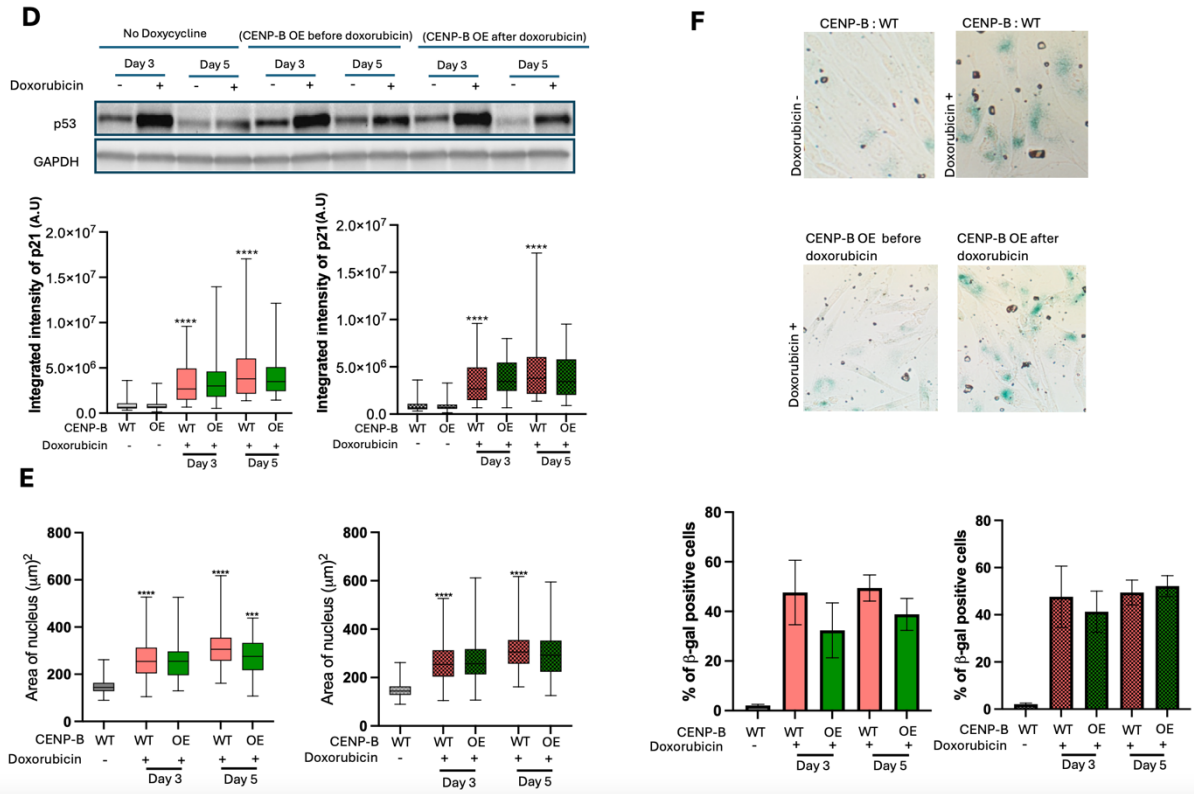

D
No Doxycycline
(CENP-B OE before doxorubicin)
(CENP-B OE after doxorubicin)
Day 3
Day 5
Doxorubicin
p53
GAPDH
Integrated intensity of p21 (A.U)
CENP-B WT OE
Doxorubicin - + + +
Day 3
Day 5
F
CENP-B : WT
Doxorubicin -
Doxorubicin +
CENP-B OE before doxorubicin
CENP-B OE after doxorubicin
E
Area of nucleus (μm)2
% of β-gal positive cells

Figure 30: CENP-B overexpression partially delay key senescence hallmark

- ***A.*** *Ectopic overexpression of CENP-B (FL) mCherry under different concentrations of doxycycline in BJ cells.*
- ***B.*** *Schematic of the experiment performed to assess the CENP-B mediated centromere compaction during senescence. A concentration of 0.1 μg/mL doxycycline was used for all experiments. Doxorubicin treatment was done in 2 pulses with 2 washouts as described in previous section. Cells were collected at day 3 and day 5.*
- ***C.*** *Representative projected fluorescence images of BJ cells transduced with doxycycline inducible CENP-B mCherry. First and second panels on the left correspond to untreated condition without or with ectopic CENP-B mCherry induction respectively. The panel on the right are the cells treated with doxorubicin and doxycycline. Main image: scale bar 5 μm, inset: scale bar of 1 μm. Bar graph shows the quantification of the percentage of α-satellite DNA foci that underwent SADS. n>30 nuclei per replicate, data are the mean of 3 independent biological replicates. Kruskal Wallis test: ****p<0.0001.*
- ***D.*** *Western blot (up) showing expression of p53, and bar graph (down) showing integrated intensity of p21 expression in BJ cells with or without CENP-B mCherry OE by doxycycline, in NT vs doxorubicin treated cells in day 3 and 5. Unpaired t test: ****p<0.0001.*
- ***E.*** *Quantification of the area of nucleus of BJ cells with or without CENP-B mCherry OE in NT vs doxorubicin treated cells in day 3 and 5. For 3 replicates. Unpaired t test: ****p<0.0001*
- ***F.*** *Representative images and quantification of β-galactosidase expressed in BJ cells with or without CENP-B mCherry OE in NT vs doxorubicin treated cells in day 3 and 5 for 3 replicates. n>300 nuclei per replicate.*

1.5.4 Elucidation of the domain(s) of CENP-B involved in α-satellite distension during senescence

As CENP-B contributes to mediate centromeric compaction, we further studied which CENP-B domains are involved in such regulation. To this end, we used various doxycycline-inducible CENP-B constructs: CENP-B Full length (FL), CENP-B devoid of its dimerization domain (CENP-B ΔC) and CENP-B without its acidic domains and dimerization domain (CENP-B ΔAcidic2-ΔC), all in a CENP-B KO background in DLD-1 cell line (**figure 31 A**). To generate these cells, CENP-B KO cells (generated through CRISPR Cas9) were cotransfected with plasmid bearing flp-recombinase to insert the gene of interest (GOI) tagged with mCherry at a single FRT sites integrated in the genome of the cells. We tested the ectopic expression of these GOI through different doxycycline concentration as shown in **figure 31 B,** using Anticentromere antibody (ACA) which binds centromere proteins and in particular the N-Terminal part of CENP-B. The presence of 2 bands for FL and Δacidic2-ΔC could be possibly due to the loss of mCherry tag by alternative splicing (mCherry tagged mutants are indicated by arrows in **figure 31 B**). The loss of a band for CENP-B ΔC is due to silent mutation leading to a stop codon in mCherry.

To perform the experiment, we selected different concentrations of doxycycline to homogenize the expression of the GOI for our experiment: 1 ng/mL for CENP-B (FL), 1000 ng/mL for CENP-B (ΔAcidic2-ΔC) and 100 ng/mL for CENP-B (ΔC). Next, we induced senescence through double pulse of 100 nM doxorubicin for 3 and 5 days in DLD-1 cells expressing ectopic GOI according to the schematic in **figure 31 C**. We confirmed the expression using a CENP-B antibody that recognize and binds to C terminus of CENP-B present in both WT and FL (**figure 31 C first lane**). Expression of CENP-B (ΔC) and (ΔAcidic2-ΔC) was confirmed using ACA antibody (**figure 31 C second lane**)**.**
The DNA damage marker γH2AX was increased after day 3 following doxorubicin treatment while a reduction of the nuclear lamina protein Lamin B1 (used here as a senescence marker as described before) was observed on day 5 **(figure 31 C**)**.** This confirms that similar amount of DNA damage and senescence response was generated across the mutants. Similarly, β-galactosidase activity was confirmed from the increased proportion of blue coloration in senescent cells reaching upto 70-90 % as evident from the widefield images and graph of **figure 31 D.** Different from before (**figure 30 F**) there is no visible delay in β-gal expression in case of CENP-B FL probably due to the fact that we did not induce a high ectopic over-expression of CENP-B (FL) (as evident from the faint bands from **figure 31 C** western blot first lane, indicated by an arrow).

Since SADS is an early event in senescence, DLD-1 cells treated with doxorubicin were collected at day 3 and FISH was performed against α-satellite DNA and imaged. To evaluate formation of SADS, the sum of area of all α-satellite foci per cell was quantified. According to the observed increase of this parameter (**figure 31 E**), all the cells with

CENP-B WT, FL and mutants underwent SADS when treated with doxorubicin. Compared to cells with WT CENP-B, cells with CENP-B KO displayed more SADS when treated with doxorubicin. SADS were slightly reduced when CENP-B FL was expressed, similar to the result in previous section where CENP-B overexpressed in BJ cells (**Figure 30 C**). The CENP-B mutant ΔC had comparable amount of SADS as CENP-B WT, but SADS became significantly higher in cell expressing a CENP-B variant lacking the acidic 2 as well as the dimerization domain. Interestingly, these cells accumulated even more SADS compared to CENP-B KO cells suggesting an additive effect of the depletion acidic 2 region on the distension effect of α-satellite DNA.

Taken altogether, we show that both the acidic 2 and the C-terminal domain of CENP-B are implicated in modulating the architecture of centromeric DNA. The lack of both domains is comparatively higher than the loss of the C-terminal alone in mediating SADS formation, suggesting an important role played by the acidic 2 region that will require further analysis.

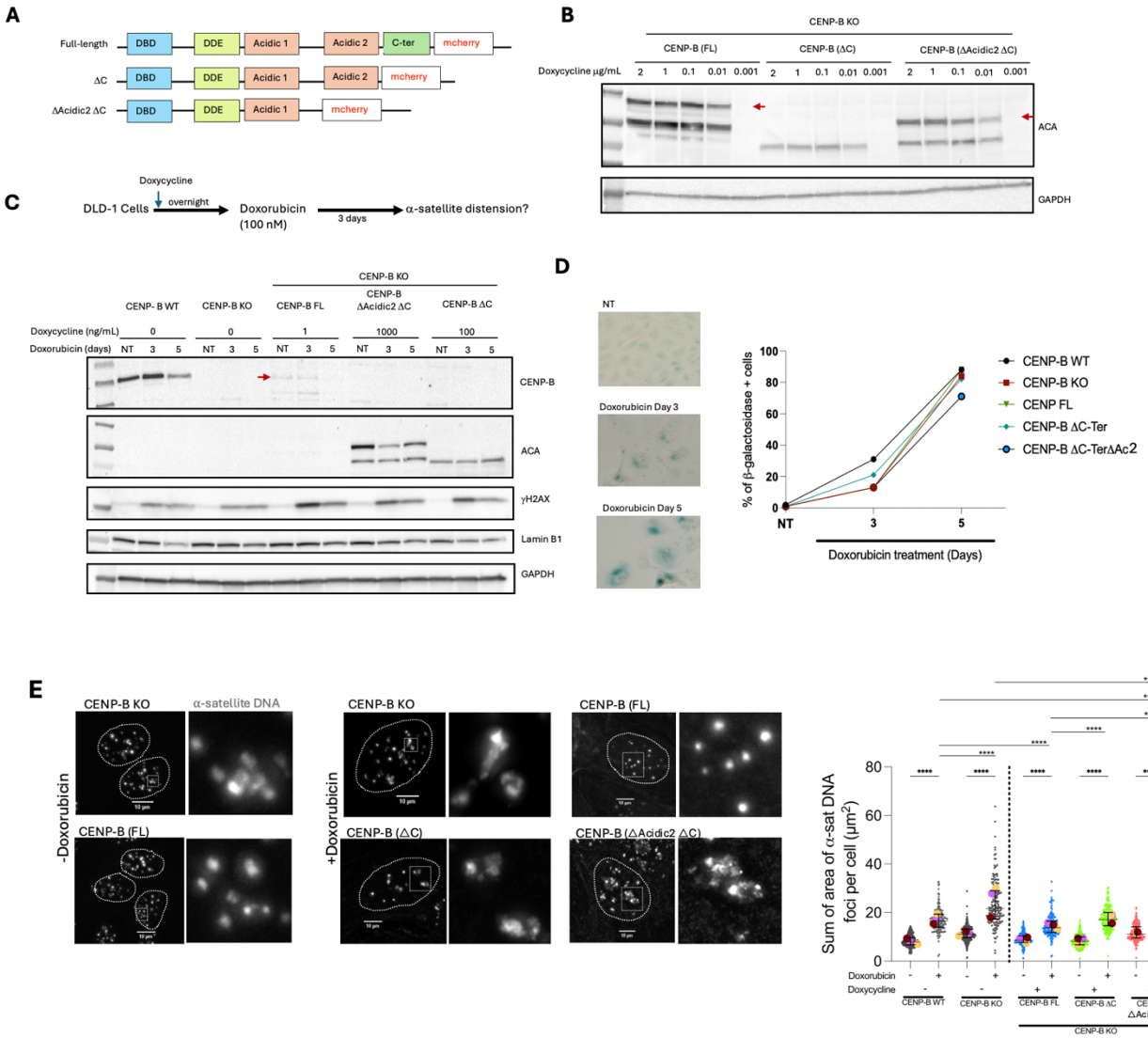


Figure 31: CENP-B acidic 2 and dimerization domain are implicated in α-satellite distension during senescence.

- ***A.*** *Schematic of the domains of CENP-B and its mutants used in the experiment.*
- ***B.*** *Immunoblot showing ectopic overexpression of CENP-B and its mutants with different concentrations of doxycycline in DLD-1 cell line. Red arrows indicate the CENP-B tagged with mCherry.*
- ***C.*** *Top: Schematic of the experiment performed to identify the domain(s) involved in regulating SADS formation. Bottom: Western blot of CENP-B expression, DNA damage marker γH2AX and Lamin B1.*
- ***D.*** *Representative images and analysis of β-galactosidase assay on doxorubicin treatment on DLD-1 CENP-B KO cells expressing either CENP-B FL or mutants.*
- ***E.*** *Representative projected fluorescence images of α-satellite DNA obtained through FISH. Below: Quantification of SADS by summing of all α-satellite DNA foci per cell. N=3 independent replicates with n> 40 cells per condition per replicate. Statistical test: unpaired two-way ANOVA ****p ≤ 0.0001*

2. Epigenetic alterations associated to centromere during senescence onset

2.1 Changes in histone marks at α-satellite DNA during senescence onset

Modifications in histone tail and core has been key to impact DNA compaction around nucleosome impacting the accessibility of DNA to protein machineries further regulating various processes. I therefore assessed whether histones at centromeric DNA underwent post-translational modifications during unfolding of α-satellite DNA at senescence onset. In particular, I investigated epigenetic marks such as H3K9me3, a mark of constitutive heterochromatin that is present at centromeres and it has been shown to be required for accurate mitotic segregation (Martins et al., 2020); H3K27me3, a mark of facultative heterochromatin enriched at centromere in the absence of canonical heterochromatin (Martins et al., 2020); H3K4me3 that is associated to transcriptionally active regions is mostly found in promoters and TSS. H3K36me3, reported to be associated with not just transcribing regions but also associated with heterochromatin, both facultative and constitutive (Chantalat et al., 2011) and it was observed to be present at centromeric DNA (Otake et al., 2020b); H4K5/8/12/16ac, a mark of relaxed chromatin known to induce open conformations of polynucleosome (Lin et al., 2024).

The integrated intensities of each histone epigenetic mark were measured at the centromere (based on the colocalization with the ACA signal) in both RPE-1 and DLD-1 cells following senescence induction via doxorubicin. Since SADS is an early event in senescence, we measured the epigenetic changes associated with SADS at an early time point (day 3). When treated with doxorubicin, open chromatin marks like H4K5/8/12/16ac and H3K36me3 increased almost 1.5 to 2 folds at the centromeres in both RPE-1 cells and DLD-1 cells, while H3K4me3 mostly remain invariable (**figure 32 A and B**)**.** Surprisingly, both RPE-1 and DLD-1 cells exhibited also an increase in H3K9me3 at the centromere, while H3K27me3 also increased but only in DLD-1 cells. The simultaneous increase of both open and closed chromatin marks at the centromere may reflect the dynamics of epigenetic changes at the centromere during senescence. It is also possible that the larger α-sat arrays spanning up to 5 Mb carry different epigenetic status within a given centromere. Furthermore, as DLD-1 cells have higher enrichment of heterochromatin mark than RPE-1, it indicates cell-specific and context-dependent alterations in chromatin homeostasis in cancerous vs non-cancerous cells.

I then investigated histone modifications in RPE-1 cells upon treatment with nutlin 3a. Preliminary data showed almost opposite histone modifications compared to what was observed in doxorubicin (**figure 32 C**). Nutlin 3a treated cells were depleted in open chromatin marks like H4K5/8/12/16ac, H3K36me3 and H3K4me3, and showed an enrichment in facultative H3K27me3 as opposed to constitutive heterochromatin.

Taken together, the post-translational modifications of histones leading to open and closed chromatin marks at the centromere might represent a transitional state of chromatin compaction during senescence. This could pertain to chromatin accessibility either directly or through chromatin remodelers and therefore could have an impact on expression of the underlying DNA.

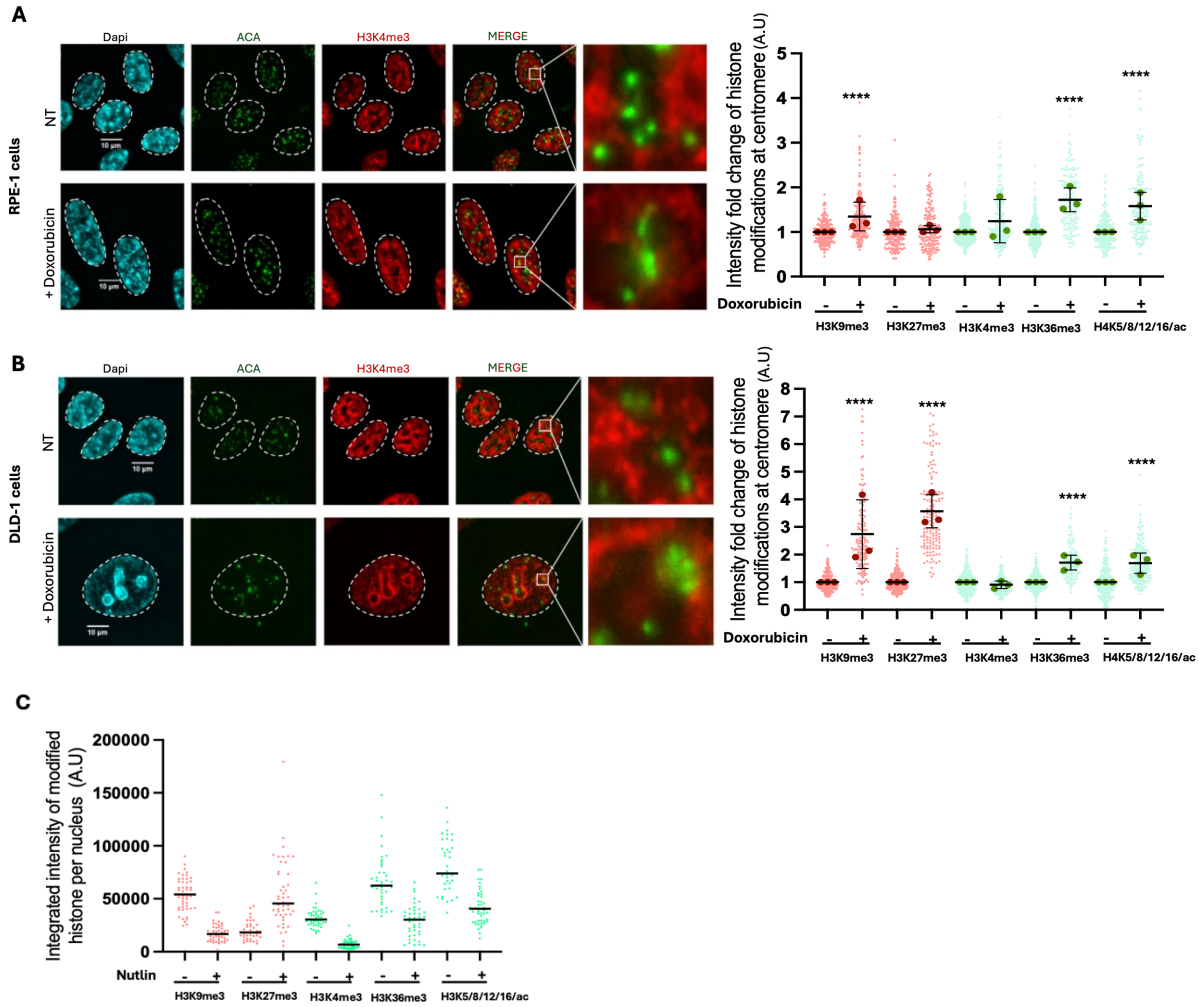


Figure 32: Centromeric DNA is associated to both open and closed chromatin marks during senescence.

- ***A.*** *Representative projected fluorescence images of RPE-1 cells treated with and without doxorubicin. On the right is the graph quantifying the fold change of the intensities of different histone modifications H3K9me3, H3K27me3, H3K4me3, H3K36me3, H3K5/8/12/16ac. N=3 independent replicates, n> 40 cells per condition.*
- ***B.*** *Representative projected fluorescence images of DLD-1 cells treated with and without doxorubicin. On the right is the graph quantifying the fold change of the intensities of different histone modifications H3K9me3, H3K27me3, H3K4me3, H3K36me3, H3K5/8/12/16ac. N=3 independent replicate, n> 40 cells per condition. Statistical test: unpaired ANOVA with Brown-Forsythe and Welch's correction.*
- ***C.*** *Quantification of the integrated intensity of different histone modifications in RPE-1 cells treated with and without nutlin 3a in one replicate, n> 40 cells per replicate.*

2.2 Transcription of centromeric DNA during senescence

Chromatin accessibility allows different machineries to act and regulate genome processing, including transcription. I showed above that during senescence α-satellite DNA is differentially associated with both open and closed chromatin marks in RPE-1 and DLD-1 cells during doxorubicin-induced senescence and likely during nutlin 3a treatment. Hence, I evaluated the transcription status of centromeres in RPE-1 cells as a consequence of SADS and epigenetic changes on day 5 of treatment with doxorubicin or nutlin 3a, by performing RNA-FISH against α-satellite transcript**.** Transcription status was measured by automatically quantifying the number of RNA foci present in a cell, the total area of centromeric RNA (cenRNA) occupancy per nucleus and the integrated intensity of each RNA foci (substracting the background) per condition using a customized macro. Cells treated with doxorubicin had more abundance of centromeric RNA, occupied more area in the nucleus and had increased intensity of cenRNA as compared to untreated (**figure 33 A**). No enrichment in centromere transcript was observed upon treating cells with nutlin 3a (**figure 33 A-D**).

Altogether, our data suggest that centromeric transcriptions at SADS might be differently regulated based on the context of senescence. This may be attributed to different pattern of histone modifications due to differential effect of doxorubicin and nutlin 3a (from the preliminary data of nutlin 3a **figure 32 C**) and needs to be investigated further.

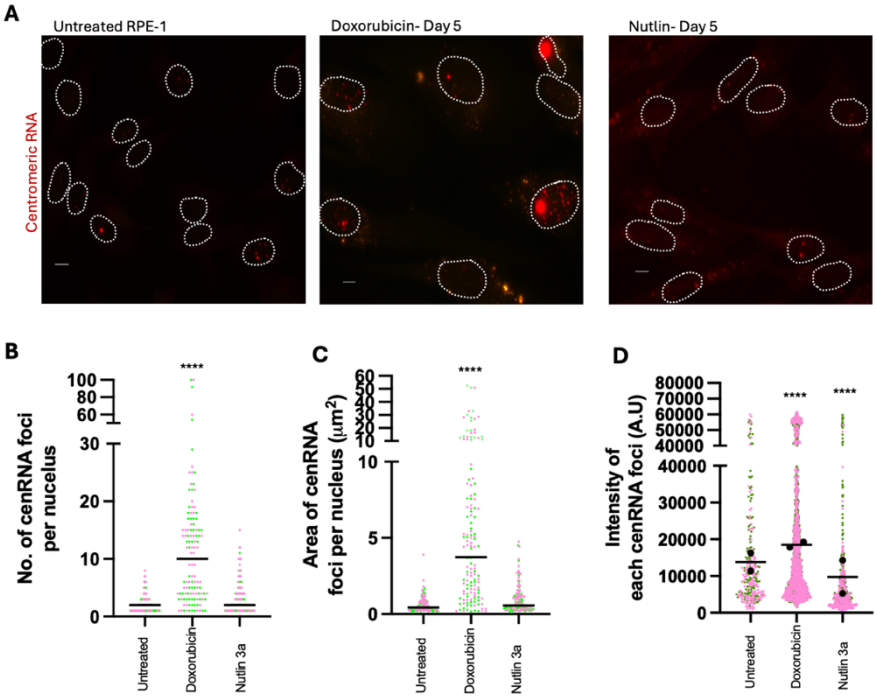


Figure 33: Centromeric DNA undergoes differential transcription based on the context of senescence.

A. *Representative projected fluorescence images of centromeric RNA-FISH signal of RPE-1 cells treated with and without doxorubicin or nutlin 3a. Scale bar 10 μm.*

*B-D. Quantification of centromeric RNA foci detected per nucleus (B); total area of cenRNA foci per nucleus (C) and the intensity of each cenRNA foci (D). More than 50 cells per replicate were analyzed and unpaired t-test was performed ****p<0.0001*

2.3 Effect of histone modifications on α-satellite distension during senescence

In the previous part, I showed that senescence was accompanied by histones modifications at the centromere. In order to address if such epigenetic alterations have a direct or indirect impact on centromeric compaction, I induced epigenetic alterations using specific compounds. To induce global histone acetylation to make the DNA more accessible, I used Trichostatin A (TSA), an inhibitor of histone deacetylase (Tóth et al., 2004). Vice versa, to promote chromatin condensation through histone hypermethylation I treated the cells with Methylstat, an inhibitor of histone demethylase (Biggs et al., 2019).

RPE-1 cells were treated separately with 330 nM TSA and 3 μM Methylstat with simultaneous addition of a double pulse of 100 nM doxorubicin and the cells were analysed after 3 and 5 days of treatment. While addition of TSA together with doxorubicin did not affect centromere distension (**figure 34 B**), treating cells with methylstat reduced the impact of centromere distension (**figure 34 A, C**).

I then tested if the observed reduction in SADS was not due to a lack of DNA damage following doxorubicin treatment due to an indirect effect of methylstat such as cell cycle blockage. To this end, I stained for γH2AX, a marker of DNA double strand break (**figure 34 D**) and found only a minor decrease of DNA damage signal in doxorubicin treated cells, likely not sufficient to explain the reduced SADS formation.

Taken together, these results show that inducing chromatin condensation through epigenetic modifications could reduce SADS formation, suggesting that SADS have more decompacted chromatin. As modified histones impact chromatin compaction, it will also be interesting to look into the abundancy of nucleosome in the centromeric DNA of senescent cells together with modifications in the centromeric DNA, such as DNA methylation.

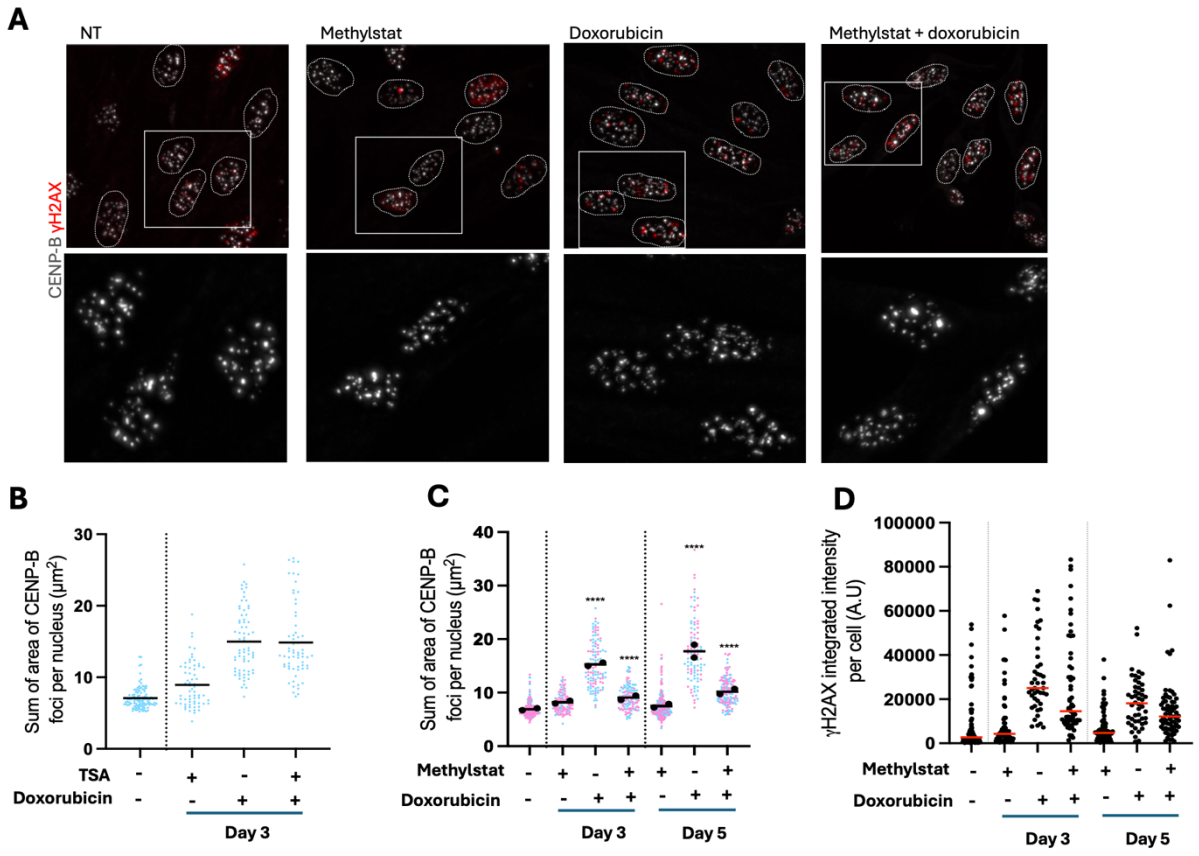


Figure 34: Inducing chromatin compaction impacts centromeric DNA distension during senescence.

- ***A.*** *Representative projected fluorescence images of RPE-1 cells treated with Methylstat and Doxorubicin on day 5.*
- ***B.*** *Quantification of distension of CENP-B signal on day 3 with TSA and doxorubicin treatment (1 replicate).*
- ***C.*** *Quantification of distension of CENP-B signal on day 3 and 5 with methylstat and doxorubicin treatment (2 replicates). More than 40 cells per replicate were analyzed.*
- ***D.*** *Quantification of the intensity of γH2AX on day 3 and 5 upon treatment with methylstat and doxorubicin. Unpaired t-test ****p<0.0001.*

3. Insights on cGAS at centromeres of senescent and cycling cells

3.1 Impact of cGAS on the distension of centromeric DNA during senescence

An additional phenotype of senescent cells is the secretory phenotype, partly mediated by cytosolic DNA sensor and innate immune activator cGAS. As cGAS is also found to be enriched at the centromere (Gentili et al., 2019), I evaluated the effect of cGAS on centromere distension in BJ cells during senescence onset by treatment with doxorubicin. To this end, I knocked out (KO) cGAS in BJ cells using CRISPR-Cas9 system through lentivirus transduction (**figure 35 A**)**,** then the cells were treated with doxorubicin and collected for monitoring the distension of centromeric DNA. Both cGAS WT and deficient cells expressed p53 upon treatment with doxorubicin (**figure 35 B**). While I did not observe an enhanced distension of α-satellite DNA in cGAS KO cells versus control without any treatment, enhanced SADS were visible in cGAS KO after 3 and 5 days of doxorubicin treatment (**figure 35 C-E**)**.** Similar preliminary results were obtained on U-2 OS cells deficient for cGAS (**figure 35 F**).

As cGAS was reported to play a role in DNA compaction (H. Jiang et al., 2019) and to be localized at centromeres, these results suggest that cGAS plays a role in preserving centromere structure, at least during early stages of SADS formation. It is therefore interesting to see if cGAS accumulates at centromeres during SADS formation.

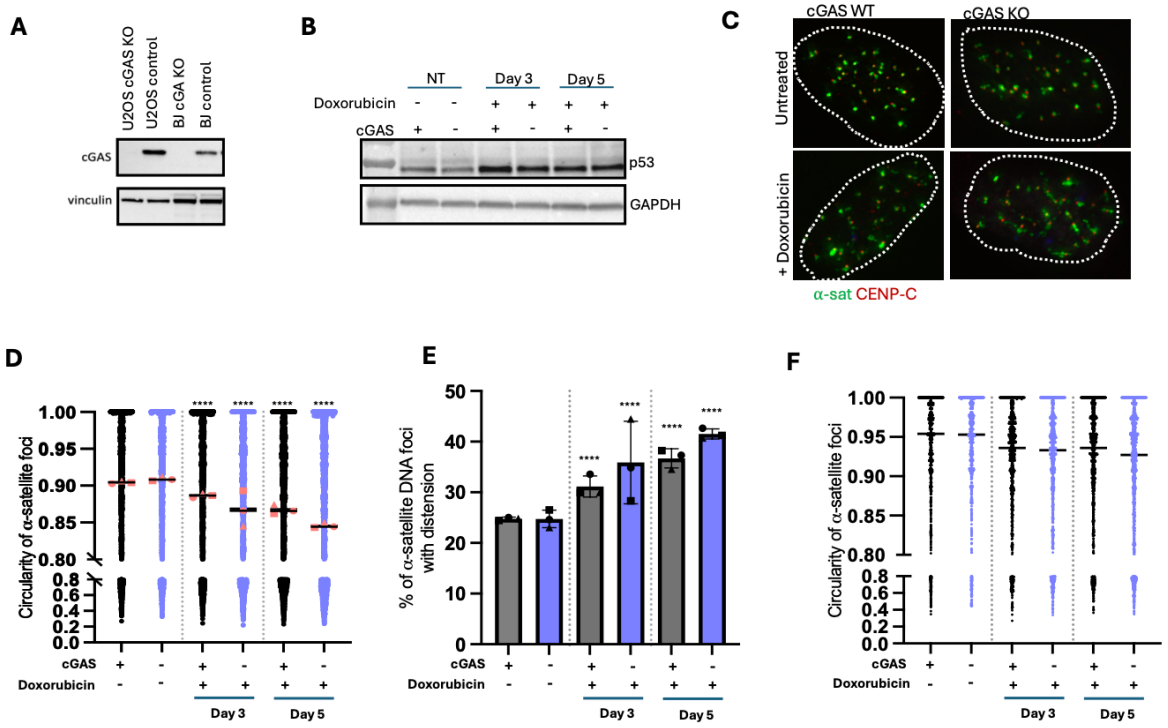


Figure 35: cGAS KO further enhances the doxorubicin-induced distension of α-satellite DNA

- ***A.*** *Western blot showing cGAS of BJ and U-2 OS cells transduced with empty plasmid (control) or cGAS KO.*
- ***B.*** *Western blot showing p53 expression in BJ cells transduced with or without cGAS, treated with doxorubicin for day 3 and 5.*
- ***C.*** *Representative projected fluorescence images of BJ cells with and without cGAS, in untreated and doxorubicin treated condition.*
- ***D.*** *Quantification of the circularity of α-satellite DNA foci in BJ cells on day 3 and 5 after treatment with doxorubicin. The mean of the 3 replicates is marked in red points and at least 30 cells per replicate were analyzed. Unpaired t test: ****p<0.0001.*
- ***E.*** *Percentage of α-sat DNA foci with distension in BJ cells with or without cGAS, with day 3 and 5 of doxorubicin treatment in 3 replicates. Unpaired t test: ****p<0.0001.*
- ***F.*** *Quantification of the circularity of α-satellite DNA foci in U-2 OS cells on day 3 and 5 after treatment with doxorubicin. The mean of 1 replicate, n>30 cells.*

3.2 Assess cGAS enrichment at the centromere during senescence

cGAS contributes to mediate centromere decompaction when cells undergo senescence. To understand if this happens through spatial mechanisms and loss of physical interaction of cGAS with centromeres, I investigated the enrichment of full length cGAS in the nucleus of untreated and senescent U-2 OS cells.
U-2 OS cells were overexpressed with cGAS (FL)-GFP tagged with nuclear localizing signal through lentiviral transduction and then treated with doxorubicin for 3 and 5 days. Ectopic cGAS signal colocalized with many CENP-B foci, of both untreated and doxorubicin treated cells (**figure 36 A**). However, the enrichment of cGAS at centromeres decreased in cells treated with doxorubicin, as evident from the reduced cGAS signal intensity at CENP-B foci (**figure 36 B**). The total intensity of ectopic cGAS in the nucleus is also slightly reduced when cells were treated with doxorubicin (**figure 36 C**).

Overall, I observed that cGAS enrichment at centromeres is reduced when cells undergo through senescence, at least in the case of DNA damage-induced senescence. Since loss of cGAS leads to enhanced distension of centromeric DNA (**figure 35**), it will be therefore interesting to investigate which factors hold cGAS to centromeres in proliferating cells and leads to its loss during senescence.

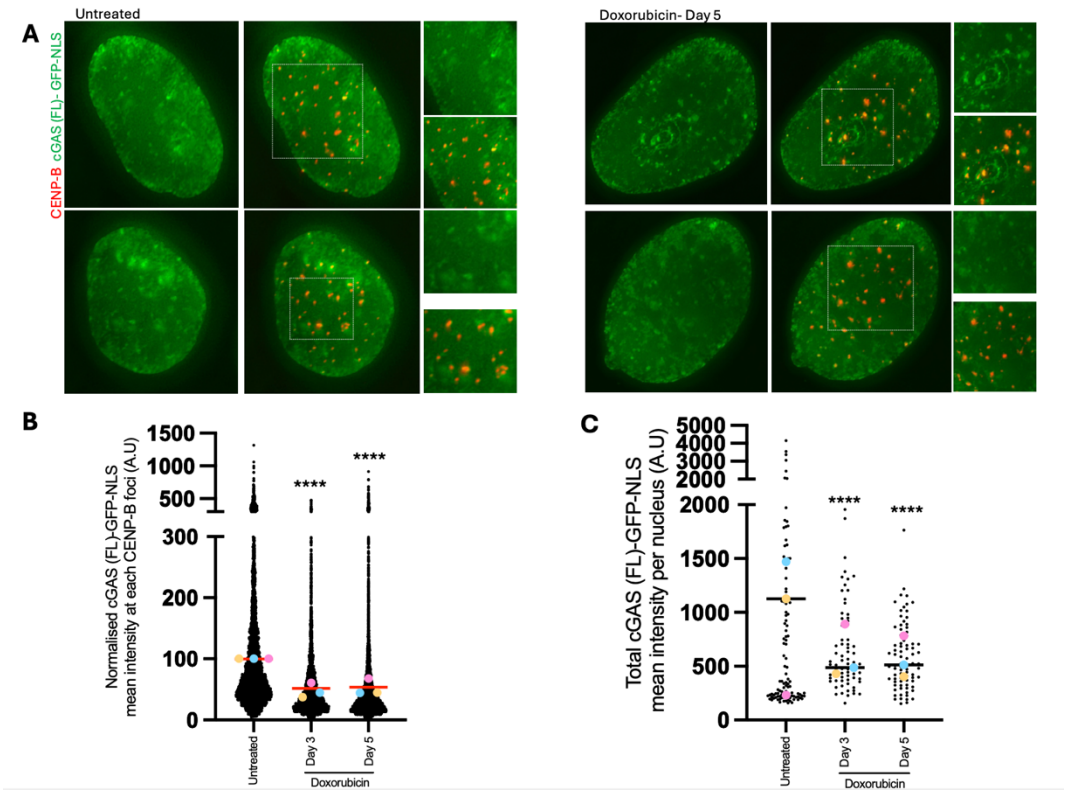


Figure 36: cGAS enrichment is reduced at centromeres when U-2 OS cells undergo doxorubicin-induced senescence

- ***A.*** *Representative projected fluorescence images of U-2 OS cells overexpressing ectopic cGAS (FL)-GFP- NLS (green) and enrichment at the centromeres marked with CENP-B foci signal (red) in untreated (left panel) and doxorubicin day 5 (right panel).*
- ***B.*** *Quantification of the intensity of cGAS (FL)-GFP-NLS signal intensity enriched at CENP-B foci of untreated, doxorubicin day 3 and 5 treated cells. The mean of the three replicates is shown in colored dots. Unpaired t test ****p<0.0001*
- ***C.*** *Quantification of the intensity of total cGAS (FL)-GFP-NLS signal intensity at nucleus. The mean of the three replicates is shown in colored dots.*

3.3 Factors recruiting cGAS to centromere

Previous work found that cGAS is enriched at the centromeres (Gentili et al., 2019), which raise the question on how its recruited to this region. To address this question, I investigated the role of centromeric proteins to recruit cGAS. To test if the recruitment of cGAS was mediated by centromeric proteins I took advance of the bacterial LacO/I system that was integrated into the human genome in a single locus of U-2 OS cells (Janicki et al., 2004; Logsdon et al., 2015) (**figure 37 A**)**.** This system is based on the lactose operon where, in absence of lactose, the lac operator in the DNA is bound and silenced by the lac repressor encoded by LacI (Lennarz & Lane, 2004). Full length cGAS tagged with green fluorescent protein and nuclear localizing signal (cGAS (FL)-GFP-NLS) was ectopically overexpressed via lentiviral transduction in U-2 OS cells containing a LacO array, the DNA-binding sites for LacI. Cells were then transfected with lac repressor HA-LacI fused with either CENP-A, CENP-B or histone 3.1 as control. These LacI constructs that strongly localize to the LacO will act like a bait to the prey cGAS (FL)-GFP-NLS. The transfected U-2 OS cells were then cultured for 48 hours before collecting cells for the visualization of enrichment of cGAS.

As expected, LacI-H3.1 was recruited to the LacO array whereas both LacI-CENP-A and LacI-CENP-B were recruited to both the LacO array but also at the endogenous centromeres (**figure 37 B**). The enrichment of cGAS (FL)-GFP-NLS at the LacO site and at centromeres was then categorized as follow: cells with no colocalization at centromeres or LacO site, cells with only few centromeres colocalization, and cells where cGAS (FL)-GFP-NLS colocalization at most centromeres or the LacO site. As expected for the negative control, cells transfected with H3.1 did not have enrichment of cGAS neither at the LacO site nor a lot at the centromeres. On the contrary, cells transfected with CENP-B had most enrichment at the centromeres, but not at the Lac O site (**figure 37 B, C**). Similar pattern was observed for CENP-A, although at a lesser extent.

The fact that cGAS is not recruited at the LacO via either CENP-A or CENP-B indicates that its recruitment to the centromere is not directly mediated by CENP-A or CENP-B. However, the fact that cGAS is enriched at centromeres when CENP-A or CENP-B are also enriched strongly suggest that its binding is mediated by changes in centromere architecture/epigenetics following CENP-A or CENP-B overexpression.

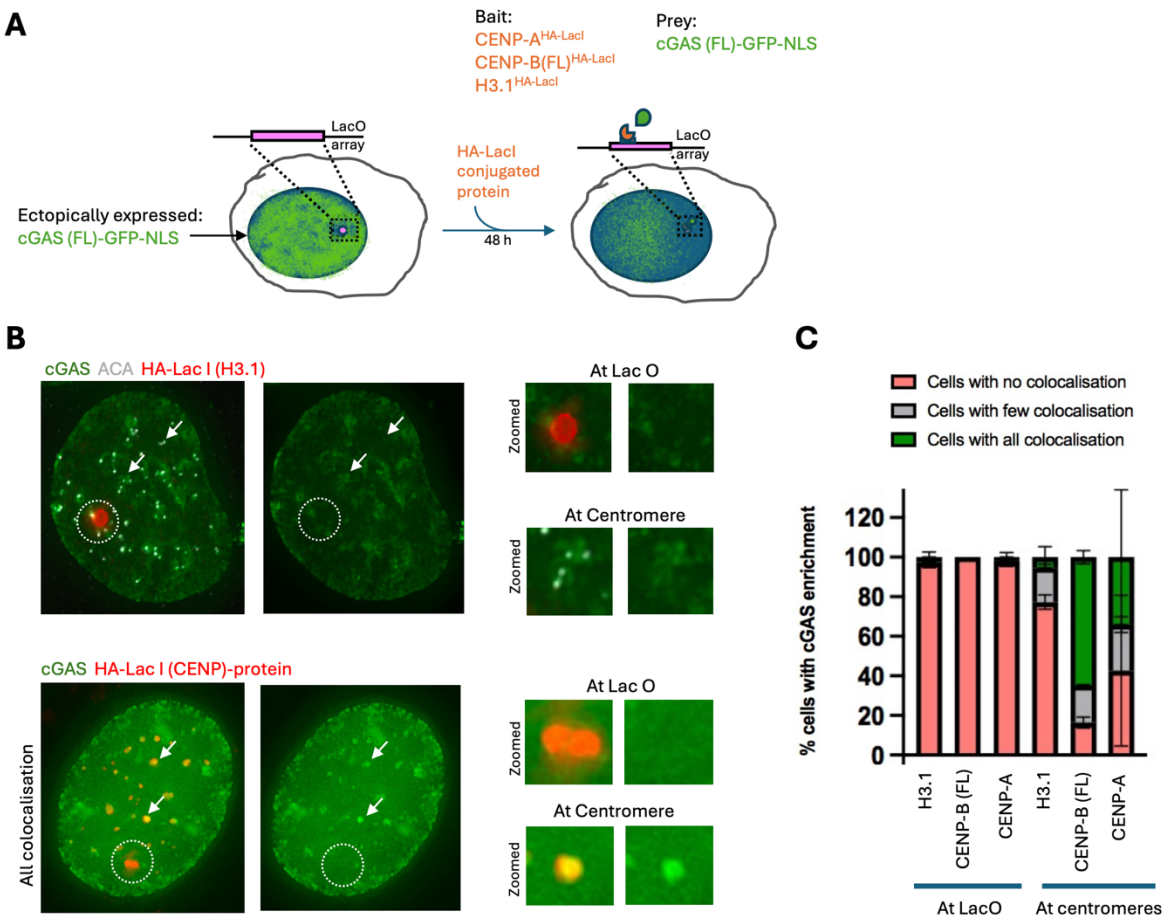


Figure 37: cGAS preferentially enriches at the centromere following CENP-B or CENP-A enrichment to centromeric DNA through the LacO-LacI system

- ***A.*** *Schematic of the LacO-LacI interaction in U-2 OS cells to investigate recruitment of cGAS at centromere.*
- ***B.*** *B: Representative projected fluorescence images of ectopically overexpressed cGAS (FL)-GFP-NLS in U-2 OS cells at the LacO array and at centromeres in cells transfected with HA-LacI conjugated centromeric protein. Inside the dotted circle is the Lac O position. The centromeric DNA is marked in arrows.*
- ***C.*** *Quantification of the percentage of U-2 OS cells with enrichment of cGAS at the LacO site and centromeres after transfection with HA LacI conjugated H3.1, CENP-A and CENP-B with standard error of mean. N= 3 independent replicates with n> 20 cells per replicate.*

Discussion and Perspectives

I have established distension of α-satellite DNA in normal immortalized human BJ and RPE-1 cells through DNA damage (using doxorubicin), chromosome mis segregation (MPS1i) and p53 stabilization (nutlin 3a) and in later sections in cancerous DLD-1 and U-2 OS cell lines through DNA damage. This reflects the universality of the phenomenon of distension of centromeric DNA compaction and its architecture as cells proceed to senescence in different human cell types and senescence conditions. I have investigated various factors that have influence in SADS formation as cells go to senescence.

1. The role of DNA damage response and p53 on α-satellite distension

As cells encounter genotoxic stress it leads to transient activation of p53, that can lead to senescence (Sheekey & Narita, 2023). Here I show that treating cells with Doxorubicin, MPS1i and Nutlin 3a led to induction of p53 and SADS (**figure 24 B and 25 B**). In fact, activating p53 through nutlin 3a was sufficient to form SADS, suggesting that p53 has the ability to induce re-arrangement in the genome organization either directly or indirectly. Indeed, it was shown that activating p53 could induce 3D conformational changes in the chromatin arrangement, by bringing p53-bound distal enhancer-promoter, forming DNA loops to enable p53 transcriptional response and gain of compartmental activity mediated by cohesin (Serra et al., 2024). Through such loop formation, the interaction of p53 bound enhancer was observed with many distal promoters including that of chromatin remodelers like BRD7 and CHDL1, leading to their transcriptional activation. Additionally, p53 has intrinsically disordered regions (IDR) that can potentially form liquid-liquid condensates that may possibly act to remodel chromatin structure. Whether p53 through its IDR can cause changes in genome architecture remains uninvestigated (Serra et al., 2024).

Interestingly, p53 was found to be dispensable in formation of SADS when cells face DNA damage by doxorubicin (**figure 26 B left** and **C**). DNA damage leads to different response factors, mainly the PI3-kinase related proteins ATM/ATR that act upstream of p53, by phosphorylating it either directly or through CHK1/CHK2. Indeed, ATM/ATR has been known to phosphorylate more than 700 proteins under ionizing radiation (Matsuoka et al., 2007) representing an extensive network involved in DDR and repair. Under conditions of DNA damage, induction of SADS occurs independently of the presence of ATM/ATR in early time points (as seen in day 3 of **figure 27 C**). However, as cells go further into senescence program and/or expose to more DNA damage, a minor effect of ATM seem to be required for maintenance of SADS. Therefore, DNA damage per se is sufficient to cause SADS, independently of p53 and with minor requirement of ATM.

Inhibiting ATM's activity in response to ds DNA break has more effect on reducing SADS compared to ATR (**figure 27 C**). Doxorubicin mainly targets topoisomerase II leading to double strand (ds) breaks formation, this could explain why ATR role was not important for SADS formation as ATR is activated in response to single stranded DNA whereas ATM by dsDNA breaks. In fact, previous reports have highlighted ATM's role and found that ATM is the main kinase for phosphorylation of γH2AX following DNA break compared to other kinase like ATR, which may explain the importance of ATM in DDR and hence SADS formation compared to ATR (Arnould et al., 2021b) (Caron et al., 2015).

It is seen that during DSB, chromatin dynamics occur in the vicinity of DSB, upstream of p53, partly mediated by DDR (Arnould et al., 2021). During DNA damage response, the topologically associating domains (TADs ~100 kb to few megabase) on both sides of the DSB undergo loop extrusion mediated by cohesin complex to enable phosphorylation of H2AX histones by DDR kinase. They found that ATM is involved in the recruitment of SCC1, a subunit of cohesin, at the site of DSBs enabling loop extrusion within a damaged TAD. They speculate that ATM mediated phosphorylation of cohesin complex can impact the properties of cohesin protein, thereby affecting chromatin dynamics following DNA damage. However, such chromatin dynamics may have the possibility to lead to secondary effect that can arise in the form of SADS.

Furthermore, it is found that ATM also phosphorylate chromatin remodeler like MOF, histone acetyltransferase for H4 lysine 16 acetylation after DNA damage, which is known to facilitate chromatin decompaction (Gupta et al., 2014) (Sharma et al., 2010). Additionally, in senescent murine cells, the role of histone chaperon FACT (facilitates chromatin transcription complex), has been shown to induce CENP-A delocalization (Hédouin et al., 2017). Therefore, it becomes imperative to investigate the involvement of chromatin remodelers in SADS formation, in cells undergoing senescence following DNA damage.

Despite inhibiting ATM and also ATR, cells still accumulate significant amount of SADS. This may be possible due to activation of alternate kinases upstream of p53, like the DNA-dependent protein kinase (DNA-PK) (Trakarnphornsombat & Kimura, 2023). Indeed, upon doxorubicin treatment, cells with inhibited ATM and ATR still accumulate γH2AX (**figure 27 D**)**,** possibly due to activation of DNA-PK. It was earlier shown that DNA-PK immediately binds to DSB within minutes and phosphorylate H2AX, whereas the role of ATM remains dispensable for such immediate early phosphorylation (Trakarnphornsombat & Kimura, 2023).In this scenario, it is important to investigate the role of DNA-PK in chromatin dynamics and SADS formation.

In case of DNA damage, while cells accumulate more SADS independently of p53, ATM or ATR, their activity is important for senescence progression (as measured by β-

galactosidase) (**figure 26 D, 27 E**). Despite p53 stabilization, the inhibition of ATM or ATR led to reduced SADS formation, indicating that probably the post translational modification of p53 mediated by ATM/ATR is necessary for senescence progression (**figure 27 E**). A full transcriptional activation of p53 occurs by concurrence of two phenomenon: p53 stabilization as well as stress-induced post-translation modifications of p53. Indeed, p53 is implied in senescence, not just by its accumulation, but also by the numerous PTMs influencing its binding with DNA and other interacting partners. For example, in response to DNA damage, p53 undergoes phosphorylation at its N-terminus, acetylation at its C-terminus, partially localize to senescence associated PML bodies and form foci called DNA SCARS (DNA segments with chromatin alterations reinforcing senescence). These PML bodies fuse together in senescent cells to form PML-DNA-SCARS (Rodier et al., 2011) and sequester p53 through its interaction with FOXO4, a transcription factor linked to apoptosis. If this interaction is inhibited, p53 is excluded out of the nuclei leading to apoptosis (Baar et al., 2017) (Parry & Narita, 2016).

Overall, p53 stabilization and accumulation can lead to SADS, yet under conditions of DNA damage, p53 and the DDR protein (ATM/ATR) is dispensable in SADS formation (summarized in **figure 38**). p53 stabilization and DNA damage can induce expression of β-gal. However, under conditions when p53 is deficient or possibly p53 modifications mediated by ATM/ATR is missing, there is decoupling of SADS from other senescence hallmarks like β-gal. In the later section of the discussion, I will discuss how in p53 proficient cells, SADS is coupled to senescence hallmarks like β-gal.

It must be noted that in response to cellular stress, other than the ATM/ATR-p53-p21 axis, cells can also undergo cell cycle arrest through activation of CDK inhibitor $p16^{INK4A}$ (**figure 38 A**), leading to phosphorylation of Rb and prevent cell cycle progression. Indeed, I see that the amount of SADS and β-gal staining in p53sh RPE-1 cells as well as ATM/ATR inhibited cells treated with doxorubicin are higher compared to wild type p53 untreated cells. This hints of the possibility of the activation of the alternate p16-pRb pathway that can influence distension of α-satellite DNA as well as senescence progression. It was previously shown that senescent cells resume proliferation in absence of p53 as well as p16 (Coppé et al., 2008) (Sheekey & Narita, 2023). However, in my work we found that SADS still occur independent of p53 in p16 low BJ cells upon DNA damage or chromosome missegregation. This shows similar behavior to induction of SASP or SAHF where even though inactivation of p53 in low p16 BJ cells resumed proliferation but the cells still have SASP (Coppé et al., 2008) and SAHF (Di Micco et al., 2011). Therefore, in absence of p53 and p16, senescent cells may resume cell proliferation while maintaining senescence hallmarks like SASP, SAHF and possibly SADS as well (Salama et al., 2014). Hence investigation of the effect of p16 on SADS under conditions of DNA damage in RPE-1 becomes important to understand the factors influencing dynamics of centromere.

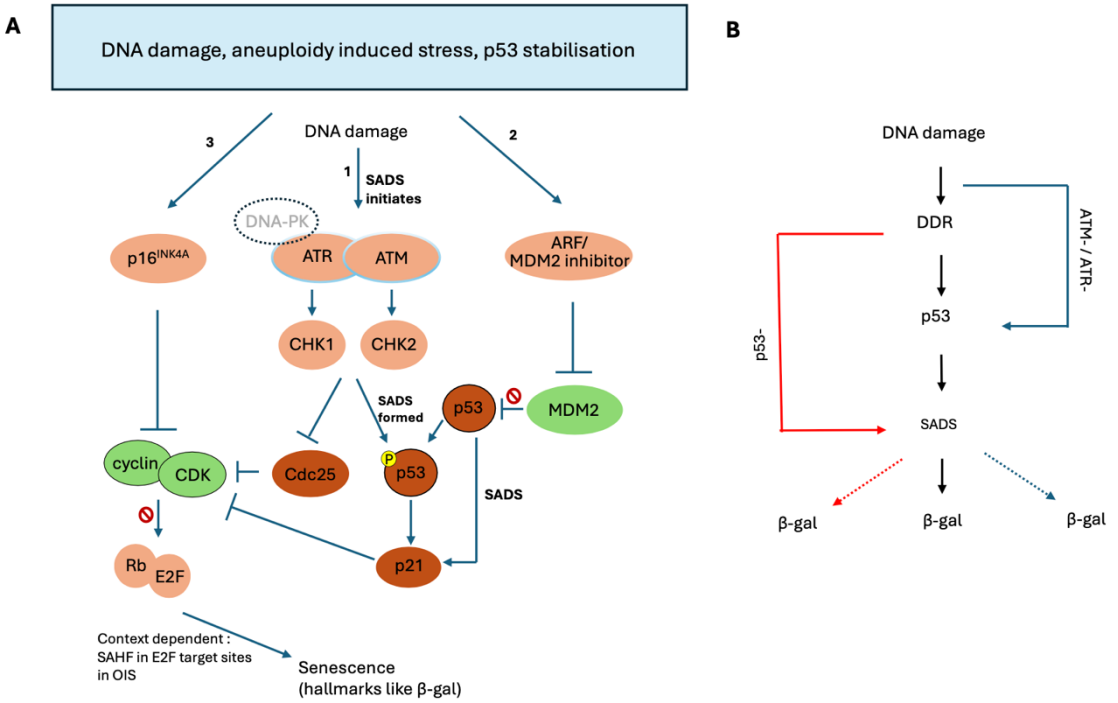


Figure 38: Mechanism of senescence progression and distension of α-satellite DNA

A) 1: Aneuploidy induced stress, telomere attrition and oncogene activation have been associated with DNA damage and activating the DNA damage response (DDR). Initiation of the distension of α-satellite DNA (SADS) following DNA damage occurs independently of the activation of ATR/ATM kinase. ATM/ATR then phosphorylate p53 directly or through CHK1/CHK2. ATM/ATR activity is required for maintenance of SADS as cells progress to senescence. Other kinases like DNA-PK following DNA damage may also get activated. CHK1/2 mediated phosphorylation of Cdc25 phosphatase cause its deactivation and prevent dephosphorylation of cyclin-CDK which in turn lead to cell cycle arrest. Phosphorylation of p53 by CHK1/2 or directly through ATM/ATR leads to its transcriptional activation and upregulation of its target p21, an endogenous CDK inhibitor thereby inhibiting cell cycle progression. Distension of α-satellite DNA can be achieved irrespective of the presence of p53 or its modifications by ATM/ATR but the role of DNA-PK is yet to be investigated.

2: Tumor suppressor ARF which is encoded from the alternate reading frame of p16, inhibits MDM2 from interacting with p53. MDM2 can also be degraded by chemical inhibitors, resulting in stabilization of p53 further leading to senescence.

3: Another barrier to cell cycle other than p53-p21 axis is through the CDK inhibitor p16. Inhibiting CDKs leads to inhibition of phosphorylation of RB protein pockets and thereby letting TF E2F sequestered. p16-Rb signal partly mediates formation of senescent hallmark SAHF at E2F target promoters but the role of p16-Rb signaling in SADS remains to be investigated (Adapted and modified from Lanigan et al., 2011).

B) Distension of α-satellite DNA is coupled to the appearance of late senescence hallmarks like accumulation of β-galactosidase in presence of a proper DDR and WT p53. Following DNA damage, cells initiate SADS which is also impacted by the DDR. DDR activates the p53/p21 signaling axis and later cause increase in β-galactosidase. SADS do not impact p53/p21 expression but boosts β-galactosidase accumulation. Under conditions where the p53 is missing, or when ATM/ATR is inhibited, α-satellite DNA distension is decoupled from senescence progression (scored by β-galactosidase).

2. The role of centromeric proteins during senescence

Regulated protein profile of a cell will ensure that the protein turnover is rightly maintained with a balance of production versus degradation, correct folding, assembly and deposition at the right location in appropriate concentration and bear the necessary post translational modifications (Llewellyn et al., 2023), also called as proteostasis. Metabolically active senescent cells exhibit altered proteostasis, with unique transcriptional and translational demands, for example increase in its inflammatory secretome (Payea et al., 2022) or reduction of proteins related to cell-cycle progression. As cells go to senescence with doxorubicin and MPS1i, we see that that the overall production of centromeric proteins decline (**figure 28 A and B**). The turnover and assembly of centromeric proteins occur in a cell-cycle dependent manner. For example, CENP-A is synthesized in G2 phase of the cell cycle but is only deposited at the centromere during mitosis exit and early G1 (Jansen et al., 2007). As senescent cells stop cycling, this leads to decrease in the production of centromeric proteins.

Despite this global, cell cycle mediated decline, the concentration of centromeric proteins bound at centromeres remain widely unchanged, except for minor reduction in CENP-B (**figure 28 C and D**). This is similar to what was observed by Swanson et al., (2013) that were showing stable CENP-A and -B association with centromeres under SADS condition. These stability of CENP-A, CENP-B and CENP-C at centromeres under senescence condition is not that surprising, as compared to other canonical nucleosomes, CENP-A nucleosomes are very stable and long-lived (Bodor et al., 2013) (Smoak et al., 2016) and this stability is mediated by other centromeric proteins like CENP-C and CENP-N (Allu et al., 2019). Moreover, CENP-B bound at CENP-B box helps to retain the previously assembled CENP-A on the centromeric DNA through specific interactions (Fujita et al., 2015) and also stabilize CENP-C (Fachinetti et al., 2015).

However, other studies on aged primary human fibroblast found that the level of centromeric protein CENP-A decline in a p53 dependent manner and CENP-B level increases as cells undergo senescence (Maehara et al., 2010b). Sikder et al., (*Biorixv* 2023) found similar with no changes in CENP-B in aged human fibroblast but increased CENP-B in doxorubicin treated human fibroblast. Additionally, in senescent mouse embryonic fibroblast, nucleosomal CENP-A has been seen to be mislocalized from the centromere (Hédouin et al., 2017).
This disparity in the abundance of centromeric proteins may be linked to specific cell type or senescence induction. It is also possible that the cellular outcome from the abundancy of a given protein may not necessarily equate with the outcomes due to its loss or gain of function. For example, certain protein might be still present but lack functionality due to absence/changes of post-translational modifications. Indeed, it is known that certain centromeric proteins undergo PTMs that might confer stability to centromeres and/or centromeric nucleosome. For example, maintenance of the levels

of SUMOylation (Small Ubiquitin Modifier) of centromeric proteins including CENP-B and CENP-C leads to stabilization of CENP-A nucleosome (Mitra et al., 2020) (Keiten-Schmitz et al., 2020). Further, many PTMs in CENP-A can also influence conformation of centromeric chromatin (Bailey et al., 2013) (Srivastava et al., 2018). Therefore, studying PTMs in centromeric proteins during senescence can be a new interesting route of investigation.

As mentioned in the introduction, CENP-A, CENP-B and CENP-C are involved in higher order structure of centromeres (Andronov et al., 2019) (Chardon et al., 2022) (Melters et al., 2019). Via acute depletion of each of these proteins, I revealed that they are involved in centromeric DNA compaction (**figure 29 C and D**), especially CENP-B depletion, mimicking what is observed under condition of SADS formation. On this regard, overexpressing ectopic full-length CENP-B in doxorubicin-induced senescent BJ cells is able to partially overcome the spatial distension of α-satellite DNA compared to cells with WT CENP-B, but only at early time points of senescence onset (**figure 30 C**) (summarized in **figure 39**). How CENP-B promote centromere compaction in normal proliferating cells have been previously investigated and could explain my own observation on senescent cells. CENP-B has been seen to mediate epigenetic modifications in centromeric DNA by enhancing H3K9me3 (Okada et al., 2007) and H3K36 methylation (Otake et al., 2020). It was identified that the dimerization domain of CENP-B is involved in centromere compaction via DNA loop formation (Yoda et al., 1998) (Chardon et al., 2022).

We have investigated in senescent cells, the involvement of the different CENP-B domains in mediating SADS. We found that, in addition to dimerization domain, the acidic 2 domain of CENP-B impacts the percentage of centromeres with SADS features, even more than CENP-B KO (**figure 31 E**). This highlight a crucial role of the acidic 2 domain of CENP-B in regulating the centromeric DNA architecture during senescence. It might be possible that this region acts as a site of recruitment for various chromatin modifiers that can regulate SADS formation. Indeed, it was previously shown that the acidic region of CENP-B has been implicated in promoting heterochromatin by recruitment of HP1 and SUV39h1 creating epigenetic chromatin states permissive for either heterochromatin assembly in the alphoid DNA of integrated artificial chromosomes or CENP-A deposition in HeLa cells (Otake et al., 2020). As a mutually exclusive function, the same team highlighted that CENP-B acidic domains have also been known to recruit the chromatin remodeler H3K36 methylase ASHL1 that promote open chromatin to further facilitate CENP-A deposition. How CENP-B can promote these two opposite chromatin states is not known and certainly, this can be an interesting area of investigation during senescence onset.

Acidic domains, like the one of CENP-B, are known to have intrinsically disordered regions (IDR). These regions do not fold into a stabilized 3D structure and are generally

known to have potential biological role such as promoting liquid-liquid phase separation. It has been shown that CENP-B promotes clustering of several centromeres in a cell (Chardon et al., 2022). Such clustering may be promoted by phase separation that can impact biological processes like DNA replication or repair. In addition, centromeres have been already associated to phase separation: regions of the inner centromere that comprise of α-satellite DNA, phosphorylated histone H3, phosphorylated HP1α, Aurora B as well as Borealin, a component of the chromosomal passenger complex (CPC) (Trivedi et al., 2019) (Ong & Torres, 2020). Disruption of this phase separation has been linked with increased chromosome missegregation. These observations highlight the importance to investigate whether unfolding of centromeric DNA during senescence disrupts any biomolecular condensates and alters biological processes that rely on phase separation.

The observed delay in SADS via CENP-B OE is also associated with the delay in the appearance of β-galactosidase accumulation (**figure 30 F**, summarized in **figure 39**). This is quite remarkable as DNA damage was the source of senescence induction and that these cells have intact WT p53 Therefore, this data indicates that SADS formation may participate to the appearance of late senescence hallmarks like β-gal, possibly providing some sort of feedback to the whole cell. This should be independent from a direct connection of SADS to p53 as when cells face DNA damage p53 was shown to be dispensable for their formation (**figure 30 D**). How do SADS influence late senescence markers like β-galactosidase is still unknown and is a matter to be investigated further. I am also currently looking into whether SADS can influence other senescent markers like SASP during senescence induced by DNA damage and non-DNA damage-based stress. As SASP is meant for immune clearance of senescent cells and misregulated chronic SASP may cause tumorigenesis, therefore factors influencing SASP becomes important.

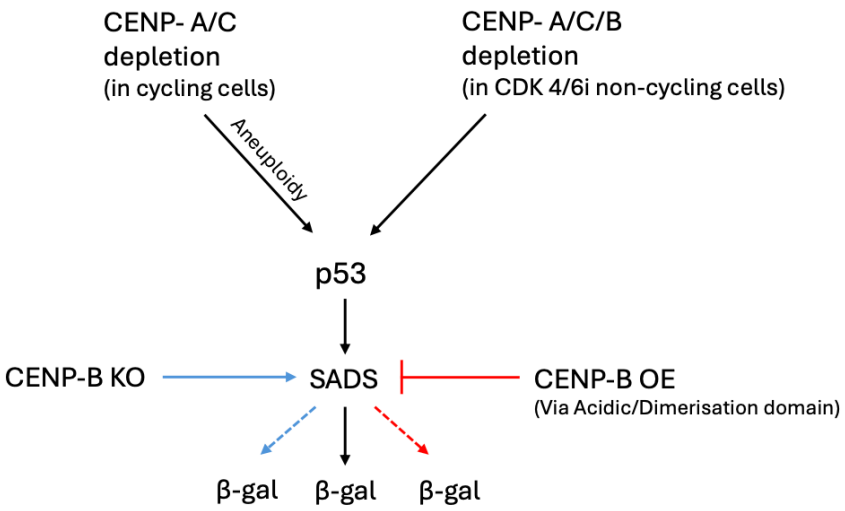


Figure 39: Possible pathways of SADS formation involving centromeric dysfunction.
Depletion of CENP-A/C in cycling cells cause aneuploidy and activate the p53 mediated cell cycle arrest, senescence and hence SADS. Inhibition of CDK 4/6 through palbociclib cause cell cycle arrest. Depletion of CENP-A/C with or without CENP-B KO in non-cycling cells promotes SADS formation. However, with CENP-B KO there is SADS, but cells do not go to senescence. Overexpressing CENP-B can partially delay SADS as well a β-galactosidase expression.

3. cGAS at centromeres during senescence

The cGAS-STING pathway is an important signaling cascade to induce the innate immune response and produce SASP in senescent cells. It is seen that in STING deficient cells such as U-2 OS and human embryonic kidney HEK 293T, the mere presence of cGAS is unable to activate IFN-1 (H. Chen et al., 2020). It must be noted that like most SASP components, IFN-β is not universally expressed in senescence (Frisch & MacFawn, 2020) highlighting the context dependent effects of cGAS in senescence progression. Despite many studies about cGAS-STING signaling in senescence and cancer through SASP, its direct role in senescence progression is not clearly established.

Here, based on the finding of cGAS enrichment at centromeric satellites (Gentili et al., (2019a)) and its role in senescence response I have investigated which role (if any) cGAS plays on SADS formation/regulation. I found that cGAS enrichment is depleted at the distended centromeres as cells progress to senescence with doxorubicin treatment (**figure 36 A, B**) and that cGAS knockout led to increase SADS formation, but only under conditions of DNA damage (**figure 35 D, E, F**). Previous studies reveal that cGAS self-oligomerize and enable compaction of dsDNA to form higher ordered structure (H. Jiang et al., 2019). This finding, along with my results, suggests that cGAS is an important factor for keeping centromeric compaction. However, the fact that removal of CGAS per se does not promote SADS in cycling conditions implies that cGAS loss from centromeres might be either a cause or a consequence of centromere decompaction during senescence.

This can be explained by the epigenetic changes that might occur at centromeres during senescence that in turn may further dictate cGAS affinity for centromere and functionality. Indeed it is seen that cGAS localization does not preferentially localize at open chromatin marks such as H3K27Ac (Gentili et al., 2019). Moreover, reduced H3K9me2/3 as well as reduced DNA methylation led to cGAS untethering and release from chromatin leading to cGAS activation (Fang et al., 2023). Additionally, post translational modifications of cGAS may also regulate the function of cGAS (Song et al., 2022). For example, methylation of cGAS by direct binding of SUV39H1 to cGAS blocks the DNA binding and dimerization capacity of cGAS leading to reduced cGAS activity (Fang et al., 2023).

In this PhD work I described that SADS formation induce differential epigenetic modifications of histones at the centromere in a DNA damage-dependent and -independent manner as well as dependent on cell-types (**figure 32 A, B, C**) Doxorubicin caused increased open chromatin marks at centromeres as well as increased closed chromatin marks in DLD-1 cells and modest increase in constitutive heterochromatin in RPE-1 cells. More “open” chromatin dictates activities such as transcription, whereas more repressed state will have reduced transcription. Indeed, doxorubicin treated cells had high transcription of centromeres (**figure 33 B, C, D**)**.** Michalski et al., (2020) show that regions of high transcription that are free of nucleosomes cannot maintain cGAS

leading to its dissociation. This is because cGAS is known to interact with the acidic patch of histones H2A-H2B and not directly on the DNA, hence loss of histone leads to loss of cGAS (De Oliveira Mann & Hopfner, 2021). This is in concurrence to the cGAS loss from centromeres in doxorubicin treated cells (**figure 36 B**). To this end, we are currently investigating the nucleosome positioning at centromeres when cells undergo senescence through assessing chromatin accessibility followed by Fiber-seq, a single molecule long read sequencing method enabling nucleotide-resolution readout of chromatin fibers.
On the other hand, p53 stabilization via nutlin 3a caused opposite epigenetic marks at centromeres compared to DNA damage-induced senescence like reduced open chromatin marks and constitutive heterochromatin with an increase of facultative heterochromatin (**figure 32**). Also, under this condition I observed no change in the transcription status of centromeres (**figure 33 B, C, D**). This hints that SADS formation is not directly caused by changes in the epigenetic landscape, but rather the context of senescence drives different chromatin changes associated to SADS. It will be important to investigate cGAS localization and nucleosomes occupancy, and how they affect centromere compaction following nutlin 3a treatment.

Swanson et al., observed that cycling and senescent cells had similar levels of heterochromatin marks (H3K9me3 and H3K27me3) despite distension of satellites in senescent cells. They proposed that SADS is a change in higher order chromatin involving chromatin loops rather than local changes in DNA wrapping due to histone modification. In this view, the role of factors that are known to regulate higher order chromatin structure like cGAS and centromeric proteins like CENP-B becomes important in this context. Upon investigating what recruits cGAS to centromeres in interphase cells, I found that cGAS is enriched at centromeres once bound by overexpressed CENP-B (**figure 37 B, C**). As CENP-B mediates centromere compaction (Chardon et al., 2022), it may be possible that CENP-B promotes centromeric DNA looping and compaction facilitating the binding of cGAS to centromeres. On similar note Andreeva et al., (2017), found that DNA bending protein like TFAM and HMGB pre-structure the DNA to be nucleated by cGAS (**figure 22 B**). Therefore, it becomes important to address which domain of CENP-B or cGAS is responsible for enrichment of cGAS at the centromeres during interphase through co-IP as this will hint us towards possible occurrence of modifications in different domains of CENP-B or cGAS as cells go to senescence. It is important to highlight that my data strongly argues against a direct recruitment of cGAS via CENP-B, despite it was found through mass spectrometry that cGAS interacts with CENP-B (Lum et al., 2018).

cGAS interacts with centromeres through its disordered N-terminal domain (Gentili et al., 2019), the domain which is also responsible for forming phase-separated condensates of cGAS-dsDNA (Zhou et al., 2021). As centromeres also undergo compaction mediated by CENP-B, which also has intrinsically disordered regions, cGAS

enrichment on centromeres may possibly enhance phase separation of centromeres or, alternatively, be recruited under this condition. Since compact DNA structure restricts and controls activities such as transcription, replication and recombination, it may be possible that compact centromeres (phase-separated or not) carry out such regulated activities. This may be fundamental in maintaining genome stability, especially for the regions in the genome comprising of repetitive sequences.

The untethering of cGAS from centromeres, and overall from chromatin as evident from reduction in cGAS intensity in the whole nucleus during senescence might favor cGAS catalytic activation. In general, in senescent cells induced by DNA damage, cGAS recognizes the chromatin fragments released in the cytosol and activates the innate immune pathway. SASP factors produced during senescence can have context-dependent roles in either senescence progression or tumor progression. To this end, we are performing a transcriptomic profiling for senescent cells with or without compact centromeres. Centromeric compaction will be modulated by overexpressing CENP-B and senescence will be induced by doxorubicin. This will enable to understand if SADS can influence the secretome and hence the phenotype of the senescent cells which in an *in vivo* context will determine the tissue microenvironment, further impacting the behavior of neighboring cells.

4. Summary:

In summary, in my PhD work I have investigated the factors that influence SADS formation as cells go to senescence including the role of DDR proteins, p53, centromeric proteins as well as innate immune activator cGAS. I have also investigated the epigenetic changes associated with SADS at centromere like histone modifications and transcriptional changes associated with SADS during senescence (**figure 40**). Specifically: 1-2: p53 activation or DNA damage is sufficient to activate SADS formation. 3-4: Depleting centromeric proteins and cGAS are correlated with SADS formation. 5: Based on the context of senescence, SADS can have outcomes like altered epigenetics (like histone modifications) and transcription at the centromere. Other processes like histone occupancy and DNA methylation remains to be investigated. These processes may also in turn influence SADS formation. 6: SADS is coupled to β-gal in a p53 dependent manner. 7: SADS may also be linked to other senescence phenotypes like SASP, which is currently under investigation by our lab.

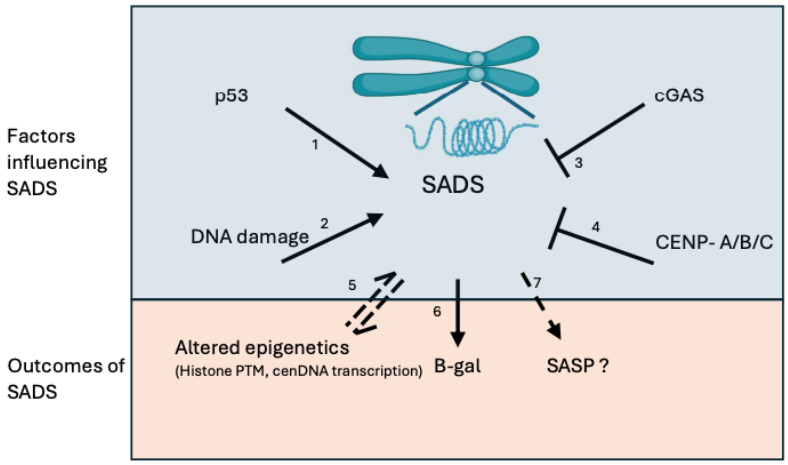


Figure 40: Factors regulating SADS formation and probable outcomes of SADS.

Materials and Methods

Cell culture:

Cells were cultivated in 37 °C and 5% CO_2 in a humidified chamber. Cells containing doxycycline inducible construct were cultured in 10% tetracycline free Fetal Bovine Serum (FBS) (Biowest, S140T), otherwise cultured in regular FBS (BioSera, FB-1001). Immortalised hTERT human foreskin fibroblast BJ cells were cultured in Dulbecco modified minimum essential media (DMEM) GlutaMAX medium (Gibco 61965-059), 20% Medium 199 (Sigma Aldrich M4530) supplemented with 10% FBS. hTERT retinal pigment epithelial cells (RPE-1) were cultured in DMEM: F12 GlutaMAX (Gibco, 31331-028) and 0.123% sodium bicarbonate. DLD-1 and U-2 OS cells were cultured in DMEM supplemented with 10% FBS.

Lentiviral particles production:

To generate lentiviral particles, 293-T cells were co-transfected overnight with 6 µg of the constructs in specific plasmid backbone, 4 µg of psPAX2 (gag, pol, rev) and 2 µg pMD2.G (VSV-G env) (12 µg total DNA), using a 1:3 ratio of DNA:FuGENE®HD (Promega, E2311) in 5 mL of OptiMEM.(Gibco, #31985062) After 12 -16h the transfection media was replaced by 6 mL fresh culture media. The supernatant containing lentiviral particles was collected after 48 hours, filtered through a Minisart® 0.45 µm polyethersulfone syringe filter (Sartorius, 16537-K). An appropriate volume of lentiviral supernatant was added to the target cells in culture in the presence of 8 µg/mL polybrene (Santa Cruz, sc-134220) for 48 hours. Then the lentiviral supernatant was removed, and the cells were selected with construct-specific antibiotic selection for up to 2 weeks. When a fluorescent marker was used, the cells were allowed to grow for 2-4 days, with frequent media changes and then the construct was expressed by adding doxycycline before sorting the fluorescent positive cells on a SONY SH800 cell sorter.

Stable cell line generation:

For knock-out experiments through CRISPR/Cas9, BJ cells (human diploid fibroblast cell line) and U-2 OS cells (human osteosarcoma cell line) were transduced with pLentiCRISPR-CMV containing guide RNA for targeting against human cGAS (guide RNA sequence GGGGATCCCGACTTCCTGGC) and negative control (described in Gentili et al., 2015). The cells were selected by adding 2 µg/mL Puromycin 2 days after the transduction. The knock-out efficiency was measured by western blot after 2 weeks. The plasmids were obtained from Nicolas Manel (Institut Curie).

For overexpressing EGFP tagged human NLS-cGAS (FL), U-2 OS cells were lentiviral transduced with pTRIP-SFFV plasmid cloned with EGFP-NLS-FLAG-cGAS as described in

(Gentili et al., 2019). The transduced cells were cultured and after 8-10 days, EGFP positive cells were sorted. The plasmid is obtained from Nicolas Manel.

For overexpressing CENP-B (FL) mcherry, BJ cells were transduced with doxycycline inducible lentiviral plasmids. The plasmid was generated by cloning CENP-B (FL) tagged with mCherry into a pInducer-20 backbone through Restriction cloning that required insertion of BsmBI and BstBI sites through PCR amplification. After selection for upto 2 weeks with 400 μg/mL neomycin, the construct expression was induced overnight by adding 0.1 μg/mL of doxycycline before sorting mCherry positive cells.

FRT/Flp-in system was used to generate DLD-1 cell line (Fachinetti et al. 2013) integrating cDNA of carboxy-terminal tagged mCherry of CENP-B Full Length (FL) or CENP-BΔC-Ter or CENP-BΔC-TerΔAcidic2 domains into a pcDNA5/FRT plasmid harboring a promoter and start codon lacking hygromycin resistance cassette and co-transfected with a pOG44 plasmid (Flp-recombinase) as described in S.Hoffman *et al*. (2020). Single clones were isolated after selection with 0.3 mg/mL hygromycin (Invitrogen), and integration was verified by immunofluorescence microscopy.

Transient transfection:

LacI constructs: mCherry-LacI-CENP-A, mCherry-LacI-CENP-B (FL) were generated according to (Hoffmann et al., 2020) and Plasmids for HA-LacI-H.3.3 were received from Ben Black (UPenn). U-2 OS cells containing Lac O array was transiently transfected with these constructs using a non-liposomal transfection reagent (FuGENE®HD) in the ratio of 1:5.6 (μg of DNA : μL of FuGENE) in a total volume of 150 μL OptiMEM.The transfected cells were cultured for 48 hours in 2 mL culture before taking them for immunofluorescence and visualization under the microscope.

Western blot:

Whole cell lysates were prepared by lysing the cells using 4% SDS, 20% Glycerol, 100mM Tris-HCl, pH 7.6 buffer and the protein concentration was determined by BCA reagent (Thermo scientific, 23227). Proteins were separated gradually by 4-20% Polyacrylamide Gel Electrophoresis (BIO-RAD, Criterion TGX Precast Gels, #5671094), transferred to nitrocellulose membranes (BIO-RAD, Trans-Blot Turbo Transfer Pack, #1704159) using Trans-Blot Turbo Transfer System (BIO-RAD).
Primary antibodies diluted (1:000) in 5% milk/1X TBS-Tween 20, were incubated overnight: mouse anti-p53 (Monoclonal, Cell Signaling 48818S), anti-CENP-A (Monoclonal, ADI-KAM-CC06-E), anti-CENP-B (Polyclonal, abcam, ab25734 or Monoclonal, clone EPR24047-64, Abcam, ab259855), and anti-CENP-C (gift from Aaron Straight), anti-Lamin B1 (Proteintech/euromedex, 12987-1-AP), rabbit-GAPDH (dilution 1:5000, Monoclonal, clone 14C10, Cell Signaling Technology, 2118), anti-rabbit cGAS

(Monoclonal, Cell Signaling Technologies, 15102). Samples were then washed thrice with 1X TBS-T before 1h secondary antibodies incubation. Secondary antibodies were either donkey anti-human-HRP (Polyclonal, Jackson ImmunoResearch, 709-035-149) or donkey anti-rabbit-HRP (GE Healthcare, Na934vs), and chemiluminescence was determined using ECL detection system (BIO-RAD, Clarity Western ECL Substrate, #170-5062). The membranes were stripped and re-probed with human-ACA Centromere Protein (4 µg/mL, Antibodies-Online GmbH, 15-235-0001), anti-γ-H2AX (1:1000, clone 20E3, Cell Signaling Technology, #9718). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control.

BrdU staining and cell cycle analysis:

For cell cycle and Brdu analysis, cells treated with doxorubicin, MPS1i and nutlin 3a for 3, 5 or 7 days were incubated with 30 µM Brdu for 24 hours or 30 minutes before collection and later fixing them by dropwise addition of 3 mL of 75% ethanol followed by incubation in ice for atleast 1 hour or overnight storage at 4 °C. The cells are then pelleted and washed once with 1% Bovine serum albumin (BSA) diluted in PBS and then denatured by suspending them in 1 mL 2N HCl for 30 minutes at RT. Next, the cells are pelleted, and the acid is neutralized by addition of 0.1M borax for 2 minutes and washed once with 1% BSA/ PBS. The cells were suspended in 0.5% Tween 20, 1% BSA diluted in PBS before adding 100 µL primary antibody anti-BrdU (BD, 555627) 5 ng/µL (diluted 1:100 in 0.5% Tween 20, 1% BSA diluted in PBS) for 1 hour at room temperature.

The cells are then pelleted and washed with 1% BSA/PBS and incubated with 50 µL of anti-mouse FITC secondary antibody (Invitrogen, 62-6511) diluted in 1:200 in 1% BSA/PBS for 30 minutes.

The cells are then washed with 1% BSA/PBS 1X and suspended in 200 µL 1x PBS containing 2 µg/mL of propidium iodide (Invitrogen, P3566) and 150 µg/mL RNAse A (Invitrogen PureLinkTM, 12091039) at 4 °C overnight. The cells are passed through the LSR II flow cytometer (BD) for obtaining the PI and BrdU intensities.

Immunofluorescence (IF):

Cells treated with doxorubicin (100 nM diluted in DMSO), MPS1i (500 nM diluted in DMSO) nutlin 3a (10 µM diluted in DMSO) were cultured up to 70-80% confluency on 1.5H glass coverslips (Marienfeld SuperiorTM, 0117520). Cells were fixed with 4% Formaldehyde in 0.1% Triton X-100 in 1X PBS for 10 minutes, then washed thrice with 0.1% Triton X-100 in 1X PBS and blocked in Triton Blocking Buffer (0,2 M glycine, 2,5% FBS and 0,1% Triton X-100) for 30 minutes at room temperature or overnight at 4 °C. The Primary antibodies diluted (1:1000) in blocking buffer were incubated for 1 hour at room temperature in Triton Blocking Buffer : mouse anti-CENP-A (Monoclonal ADI-KAM-CC06-E) rabbit anti-CENP-B (Polyclonal, Abcam, ab25734 or clone EPR24047-64, Abcam,

ab259855), guinea pig-anti-CENP-C (MBL, PD030), mouse-anti-p21 (dilution 1:200, clone EA10, calbiochem, OP64) or human-anti-ACA (Antibodies-Online GmbH, 15-235-0001), rabbit-anti-mCherry (Polyclonal, A-P-R#13, Protlab Institut Curie), rabbit-anti-H3K4me3 (Polyclonal, Abcam, ab8580), rabbit-anti-H3K9me3 (Polyclonal, Abcam, ab8898), rabbit-anti-H4K5/8/12/16ac (Polyclonal, Abcam, ab177790), rabbit-anti-H3K27me3 (Monoclonal, clone C36B11, Cell Signaling Technology, 9733), mouse-anti-H3K36me3 (1:500, Monoclonal, clone 0333, Active Motif, AB 2614986), anti-rabbit cGAS (Monoclonal, Cell Signaling Technologies, 15102).
Cells were washed three times with 0,1% Triton X-100 in 1X PBS before incubation with species-specific secondary antibodies for 45 min at room temperature in Triton Blocking Buffer. Alexa Fluor® 488, Cy3 and Alexa Fluor® 647 conjugated secondary antibodies were used at 1:500, all from Jackson ImmunoResearch Laboratories and with GFP-booster (ChromoTek gba-488). Cells were washed thrice with 0,1% Triton X-100 in 1X PBS before DAPI staining (4',6-diamidino-2-phenylindole ,1 µg/mL in 1X PBS), washed and subsequent mounting with ProLong Gold Antifade Mountant (Invitrogen, P36934).

IF-FISH of centromeric proteins-α-satellite DNA:

Coverslips were washed three times with PBS and permeabilized with 1mL of PBS- 0.1% Triton X-100 (v/v) (Euromedex) for 10 minutes at RT. Washed again three times with PBS and incubated with blocking buffer (0.2 M glycine, 2.5% FBS (v/v), 0.1% triton X-100 (v/v) in PBS) overnight at 4°C or for 30 minutes at room temperature. For immunofluorescence, the cells were then incubated in the mentioned primary antibodies (diluted in blocking buffer) for 1 hour at room temperature followed by secondary antibodies (Alexa488 or Cy3, at 1:500 Jackson ImmunoResearch), diluted in blocking buffer for 45 minutes at room temperature and washed thrice with PBS-0.1% Triton. Following IF, prior to FISH, cells were treated for 30 minutes at 37 °C with 1 µg/mL of RNase A (Fisher Scientific 12091039) diluted in water, washed thrice with PBS and then treated with ethanol:acetic acid (3:1) for 15 min at room temperature. The coverslips were quickly rinsed in 80% ethanol and air-dried. Then peptide nucleic acid α-satellite probes mix conjugated to biotin (listed in **Table S1** as described (Giunta et al., 2021b)) (diluted to 1:100 from 100 mM stock in hybridization buffer having 10% dextran, 50% formamide in 2X SSC and 1% tween 20) was applied on the samples and denatured at 75°C for 3 minutes followed by overnight incubation at 37°C in Leica Biosystems ThermoBrite. The slides are then washed in 0.4X SSC at 72°C for 2 minutes followed by 30 seconds wash in 0.05 % Tween 20 (v/v) diluted in 2x SSC. After wash, the coverslips were incubated with streptavidin conjugated with Alexa647 or 594 (used at 1:1000 in PBS from 1 mg/mL stock, Fisher Scientific S21374) for at least an hour at room temperature and then washed with PBS three times. The coverslips are then counter-stained with 1µg/mL DAPI, washed with PBS before mounting on slides with prolong gold anti-fade reagent (Invitrogen P36934). The α-satellite probe mix consists of 6 oligonucleotides of

approximately 50 bp. Oligos were designed to match the consensus sequence of 21 Higher Order Repeats families (HORs), distributed across all human centromeres (listed in **Table S1**). Images of ~3.5 to 4.0 μm stack (with step size of z= 0.2 μm) were acquired on an inverted widefield Deltavision Core Microscope Applied Precision at 100X Olympus UPlanSApo 100 oil immersion objective with numerical aperture 1.4 and Xenon lamp of 250 W along with Photometric CoolSNAP HQ2 Camera. Images were also acquired on inverted widefield Thunder microscope at 100X oil immersion objective with a numerical aperture 1.4 and LED lamp with Leica K8 camera.

RNA-FISH of centromeric DNA:

Cells cultured upto 70-80% confluency on 1.5H glass coverslips (Marienfeld SuperiorTM, 0117520) were treated with doxorubicin and nutlin 3a and collected at day 5. The coverslips were washed once with 1X PBS and fixed in 4% formaldehyde (Sigma Aldrich, 252549) prepared in 1X PBS, for 10 minutes at room temperature (RT). The cells were rinsed twice in 1X PBS for 10 minutes and permeabilized in 1 mL of 70% ethanol in 4 °C for at least one hour or optionally for few days. Next, cells were rinsed for 10 mins in 1X PBS and 5 minutes in Wash solution (10% deionized formamide, 2X SSC dissolved in nuclease free water). Then oligonucleotide probe set with sequences complementary to pan-chromosomal consensus α-satellite sequence conjugated to fluorophore Quasar 570 (listed as ASAT oligos in Bury et al., 2020) were used. The probe set, diluted in hybridization buffer (10% dextran sulfate, 10% deionized formamide, 2X SSC prepared in nuclease free water), was applied on the samples (diluted to 100 nM in Tris EDTA solution from a stock of 25 μM). The coverslip is sealed well with a glue and incubated in dark overnight at 37 °C in hybridization oven (Stuart, SI30H). Next, the coverslip is rinsed twice for 30 minutes each with pre-incubated wash solution at 37 °C and rinsed quickly with 2X SSC followed by 5 minutes rinse with 1X PBS. The coverslips were then counter stained with 1μg/mL DAPI.

Immunofluorescence image analysis:

Images were processed and analyzed with ImageJ Fiji. A Fiji macro was created to detect and compute the intensity of α-satellite DNA together with centromeric proteins. A nuclei mask is run using a preselected auto thresholding method on the DAPI channel (through Huang method) (it requires MorphoLibJ plugin for advanced watershedding). Then it searches for α-satellite signal (“centromere channel” on the macro) on the projected images based on the local maxima of the fluorescence intensity to set the region of interest (ROI) by a circle of user-defined radius. Within that ROI, based on different channels, the mean and integrated fluorescence intensity together with corrected mean fluorescence of α-satellite and centromeric proteins are computed. The corrected mean fluorescence is measured by subtracting the value of the pixel with the lowest intensity in the ROI circle from the mean intensity of the circle. This macro was used to measure

the intensity of centromeric proteins (CENP-A/ CENP-B/ CENP-C) as cells underwent senescence. This macro was written by Solène Hervé (Giunta et al., 2021)

Computation of area, circularity and signal intensity:

Images were processed and analyzed with ImageJ Fiji. A Fiji macro was created to detect and compute shape descriptors like circularity and area of α-satellite DNA together with measuring the intensity of centromeric proteins. This macro first creates a z-projection based on maximum intensity. Before processing the α-satellite signals, the macro allows specifying the parameters for analysis, like the minimum area of nuclei or α- satellite structures. A nuclei mask is run using a preselected auto thresholding method on the DAPI channel (through Huang2 method). Additionally, this macro requires PTBIOP plugin. Then the macro searches for α-satellite signal ("structure channel" on the macro) on the projected images based on the local maxima of the fluorescence intensity. Segmentation of the structure within region of interest (ROI) is done based on the choice of method of auto local threshold (through Bernsen method). Within that ROI the macro computes the area and circularity as well as the mean and integrated fluorescence intensity of all the channels. To remove the background inside the ROI circle, corrected mean fluorescence is measured by substracting the value of the pixel with the lowest intensity in the ROI circle from the mean intensity of the circle. Along with computing the shape of centromeric DNA, this macro was used to measure the intensity of epigenetic histone marks and the intensity of cGAS at centromere of cells. This macro was built by Anne-Sophie Mace (Institut Curie, Paris).

Senescence-Associated β-galactosidase assay:

Cells were fixed and stained for β-Galactosidase staining assay by following the Senescence β-Galactosidase Staining Kit protocol (Cell Signaling Technology, #9860). Staining solution was equilibrated at pH 6.0 and added to the fixed cells, then sealed with parafilm and covered from light for an overnight incubation at 37°C and the images were acquired using ECLIPSE T*i*-U (Nikon), an inverted brightfield microscope.

Bibliography:

Afifi, M. M., Crncec, A., Cornwell, J. A., Cataisson, C., Paul, D., Ghorab, L. M., Hernandez, M. O., Wong, M., Kedei, N., & Cappell, S. D. (2023). Irreversible cell cycle exit associated with senescence is mediated by constitutive MYC degradation. *Cell Reports*, *42*(9), 113079. https://doi.org/10.1016/j.celrep.2023.113079

Ali-Ahmad, A., Bilokapić, S., Schäfer, I. B., Halić, M., & Sekulić, N. (2019). CENP -C unwraps the human CENP -A nucleosome through the H2A C-terminal tail. *EMBO Reports*, *20*(10), e48913. https://doi.org/10.15252/embr.201948913

Alimonti, A., Nardella, C., Chen, Z., Clohessy, J. G., Carracedo, A., Trotman, L. C., Cheng, K., Varmeh, S., Kozma, S. C., Thomas, G., Rosivatz, E., Woscholski, R., Cognetti, F., Scher, H. I., & Pandolfi, P. P. (2010). A novel type of cellular senescence that can be enhanced in mouse models and human tumor xenografts to suppress prostate tumorigenesis. *Journal of Clinical Investigation*, *120*(3), 681–693. https://doi.org/10.1172/JCI40535

Allu, P. K., Dawicki-McKenna, J. M., Van Eeuwen, T., Slavin, M., Braitbard, M., Xu, C., Kalisman, N., Murakami, K., & Black, B. E. (2019). Structure of the Human Core Centromeric Nucleosome Complex. *Current Biology*, *29*(16), 2625-2639.e5. https://doi.org/10.1016/j.cub.2019.06.062

Altemose, N., Logsdon, G. A., Bzikadze, A. V., Sidhwani, P., Langley, S. A., Caldas, G. V., Hoyt, S. J., Uralsky, L., Ryabov, F. D., Shew, C. J., Sauria, M. E. G., Borchers, M., Gershman, A., Mikheenko, A., Shepelev, V. A., Dvorkina, T., Kunyavskaya, O., Vollger, M. R., Rhie, A., … Miga, K. H. (2022). Complete genomic and epigenetic maps of human centromeres. *Science*, *376*(6588), eabl4178. https://doi.org/10.1126/science.abl4178

Altemose, N., Maslan, A., Smith, O. K., Sundararajan, K., Brown, R. R., Mishra, R., Detweiler, A. M., Neff, N., Miga, K. H., Straight, A. F., & Streets, A. (2022). DiMeLo-seq: A long-read, single-molecule method for mapping protein–DNA interactions genome wide. *Nature Methods*, *19*(6), 711–723. https://doi.org/10.1038/s41592-022-01475-6

Andreeva, L., Hiller, B., Kostrewa, D., Lässig, C., De Oliveira Mann, C. C., Jan Drexler, D., Maiser, A., Gaidt, M., Leonhardt, H., Hornung, V., & Hopfner, K.-P. (2017). cGAS senses long and HMGB/TFAM-bound U-turn DNA by forming protein–DNA ladders. *Nature*, *549*(7672), 394–398. https://doi.org/10.1038/nature23890

Andresen, K., Jimenez-Useche, I., Howell, S. C., Yuan, C., & Qiu, X. (2013). Solution Scattering and FRET Studies on Nucleosomes Reveal DNA Unwrapping Effects of H3 and

H4 Tail Removal. *PLoS ONE*, *8*(11), e78587. https://doi.org/10.1371/journal.pone.0078587

Andronov, L., Ouararhni, K., Stoll, I., Klaholz, B. P., & Hamiche, A. (2019). CENP-A nucleosome clusters form rosette-like structures around HJURP during G1. *Nature Communications*, *10*(1), 4436. https://doi.org/10.1038/s41467-019-12383-3

Angelov, D., Molla, A., Perche, P.-Y., Hans, F., Côté, J., Khochbin, S., Bouvet, P., & Dimitrov, S. (2003). The Histone Variant MacroH2A Interferes with Transcription Factor Binding and SWI/SNF Nucleosome Remodeling. *Molecular Cell*, *11*(4), 1033–1041. https://doi.org/10.1016/S1097-2765(03)00100-X

Arnould, C., Rocher, V., Finoux, A.-L., Clouaire, T., Li, K., Zhou, F., Caron, P., Mangeot, Philippe. E., Ricci, E. P., Mourad, R., Haber, J. E., Noordermeer, D., & Legube, G. (2021). Loop extrusion as a mechanism for formation of DNA damage repair foci. *Nature*, *590*(7847), 660–665. https://doi.org/10.1038/s41586-021-03193-z

Asbury, C. (2017). Anaphase A: Disassembling Microtubules Move Chromosomes toward Spindle Poles. *Biology*, *6*(1), 15. https://doi.org/10.3390/biology6010015

Astle, M. V., Hannan, K. M., Ng, P. Y., Lee, R. S., George, A. J., Hsu, A. K., Haupt, Y., Hannan, R. D., & Pearson, R. B. (2012). AKT induces senescence in human cells via mTORC1 and p53 in the absence of DNA damage: Implications for targeting mTOR during malignancy. *Oncogene*, *31*(15), 1949–1962. https://doi.org/10.1038/onc.2011.394

Baar, M. P., Brandt, R. M. C., Putavet, D. A., Klein, J. D. D., Derks, K. W. J., Bourgeois, B. R. M., Stryeck, S., Rijksen, Y., Van Willigenburg, H., Feijtel, D. A., Van Der Pluijm, I., Essers, J., Van Cappellen, W. A., Van IJcken, W. F., Houtsmuller, A. B., Pothof, J., De Bruin, R. W. F., Madl, T., Hoeijmakers, J. H. J., … De Keizer, P. L. J. (2017). Targeted Apoptosis of Senescent Cells Restores Tissue Homeostasis in Response to Chemotoxicity and Aging. *Cell*, *169*(1), 132-147.e16. https://doi.org/10.1016/j.cell.2017.02.031

Bai, J., & Liu, F. (2022). Nuclear cGAS: Sequestration and beyond. *Protein & Cell*, *13*(2), 90–101. https://doi.org/10.1007/s13238-021-00869-0

Bailey, A. O., Panchenko, T., Sathyan, K. M., Petkowski, J. J., Pai, P.-J., Bai, D. L., Russell, D. H., Macara, I. G., Shabanowitz, J., Hunt, D. F., Black, B. E., & Foltz, D. R. (2013). Posttranslational modification of CENP-A influences the conformation of centromeric chromatin. *Proceedings of the National Academy of Sciences*, *110*(29), 11827–11832. https://doi.org/10.1073/pnas.1300325110

Baker, D. J., Childs, B. G., Durik, M., Wijers, M. E., Sieben, C. J., Zhong, J., A. Saltness, R., Jeganathan, K. B., Verzosa, G. C., Pezeshki, A., Khazaie, K., Miller, J. D., & Van Deursen, J. M. (2016). Naturally occurring p16Ink4a-positive cells shorten healthy lifespan. *Nature*, *530*(7589), 184–189. https://doi.org/10.1038/nature16932

Banerjee, D., Langberg, K., Abbas, S., Odermatt, E., Yerramothu, P., Volaric, M., Reidenbach, M. A., Krentz, K. J., Rubinstein, C. D., Brautigan, D. L., Abbas, T., Gelfand, B. D., Ambati, J., & Kerur, N. (2021). A non-canonical, interferon-independent signaling activity of cGAMP triggers DNA damage response signaling. *Nature Communications*, *12*(1), 6207. https://doi.org/10.1038/s41467-021-26240-9

Bazzar, W., Bocci, M., Hejll, E., Högqvist Tabor, V., Hydbring, P., Grandien, A., Alzrigat, M., & Larsson, L.-G. (2021). Pharmacological inactivation of CDK2 inhibits MYC/BCL-XL-driven leukemia in vivo through induction of cellular senescence. *Cell Cycle*, *20*(1), 23–38. https://doi.org/10.1080/15384101.2020.1855740

Biggs, R., Liu, P. Z., Stephens, A. D., & Marko, J. F. (2019). Effects of altering histone posttranslational modifications on mitotic chromosome structure and mechanics. *Molecular Biology of the Cell*, *30*(7), 820–827. https://doi.org/10.1091/mbc.E18-09-0592

Black, B. E., Foltz, D. R., Chakravarthy, S., Luger, K., Woods, V. L., & Cleveland, D. W. (2004). Structural determinants for generating centromeric chromatin. *Nature*, *430*(6999), 578–582. https://doi.org/10.1038/nature02766

Blais, A., & Dynlacht, B. D. (2007). E2F-associated chromatin modifiers and cell cycle control. *Current Opinion in Cell Biology*, *19*(6), 658–662. https://doi.org/10.1016/j.ceb.2007.10.003

Blower, M. D., & Karpen, G. H. (2001). The role of Drosophila CID in kinetochore formation, cell-cycle progression and heterochromatin interactions. *Nature Cell Biology*, *3*(8), 730–739. https://doi.org/10.1038/35087045

Bodor, D. L., Mata, J. F., Sergeev, M., David, A. F., Salimian, K. J., Panchenko, T., Cleveland, D. W., Black, B. E., Shah, J. V., & Jansen, L. E. (2014). The quantitative architecture of centromeric chromatin. *eLife*, *3*, e02137. https://doi.org/10.7554/eLife.02137

Bodor, D. L., Valente, L. P., Mata, J. F., Black, B. E., & Jansen, L. E. T. (2013). Assembly in G1 phase and long-term stability are unique intrinsic features of CENP-A nucleosomes. *Molecular Biology of the Cell*, *24*(7), 923–932. https://doi.org/10.1091/mbc.e13-01-0034

Bowman, G. D., & Poirier, M. G. (2015). Post-Translational Modifications of Histones That Influence Nucleosome Dynamics. *Chemical Reviews*, *115*(6), 2274–2295. https://doi.org/10.1021/cr500350x

Brouhard, G. J., & Hunt, A. J. (2005). Microtubule movements on the arms of mitotic chromosomes: Polar ejection forces quantified *in vitro*. *Proceedings of the National Academy of Sciences*, *102*(39), 13903–13908. https://doi.org/10.1073/pnas.0506017102

Bunning, A. R., & Gupta Jr., M. L. (2023). The importance of microtubule-dependent tension in accurate chromosome segregation. *Frontiers in Cell and Developmental Biology*, *11*, 1096333. https://doi.org/10.3389/fcell.2023.1096333

Bury, L., Moodie, B., Ly, J., McKay, L. S., Miga, K. H., & Cheeseman, I. M. (2020). Alpha-satellite RNA transcripts are repressed by centromere–nucleolus associations. *eLife*, *9*, e59770. https://doi.org/10.7554/eLife.59770

Cai, Y., Zhang, Y., Loh, Y. P., Tng, J. Q., Lim, M. C., Cao, Z., Raju, A., Lieberman Aiden, E., Li, S., Manikandan, L., Tergaonkar, V., Tucker-Kellogg, G., & Fullwood, M. J. (2021). H3K27me3-rich genomic regions can function as silencers to repress gene expression via chromatin interactions. *Nature Communications*, *12*(1), 719. https://doi.org/10.1038/s41467-021-20940-y

Capparelli, C., Chiavarina, B., Whitaker-Menezes, D., Pestell, T. G., Pestell, R. G., Hulit, J., Andò, S., Howell, A., Martinez-Outschoorn, U. E., Sotgia, F., & Lisanti, M. P. (2012). CDK inhibitors (p16/p19/p21) induce senescence and autophagy in cancer-associated fibroblasts, "fueling" tumor growth via paracrine interactions, without an increase in neo-angiogenesis. *Cell Cycle*, *11*(19), 3599–3610. https://doi.org/10.4161/cc.21884

Caron, P., Choudjaye, J., Clouaire, T., Bugler, B., Daburon, V., Aguirrebengoa, M., Mangeat, T., Iacovoni, J. S., Álvarez-Quilón, A., Cortés-Ledesma, F., & Legube, G. (2015). Non-redundant Functions of ATM and DNA-PKcs in Response to DNA Double-Strand Breaks. *Cell Reports*, *13*(8), 1598–1609. https://doi.org/10.1016/j.celrep.2015.10.024

Carroll, C. W., Silva, M. C. C., Godek, K. M., Jansen, L. E. T., & Straight, A. F. (2009). Centromere assembly requires the direct recognition of CENP-A nucleosomes by CENP-N. *Nature Cell Biology*, *11*(7), 896–902. https://doi.org/10.1038/ncb1899

Cavalieri, V. (2021). The Expanding Constellation of Histone Post-Translational Modifications in the Epigenetic Landscape. *Genes*, *12*(10), 1596. https://doi.org/10.3390/genes12101596

Chandra, T., Kirschner, K., Thuret, J.-Y., Pope, B. D., Ryba, T., Newman, S., Ahmed, K., Samarajiwa, S. A., Salama, R., Carroll, T., Stark, R., Janky, R., Narita, M., Xue, L., Chicas, A., Nũnez, S., Janknecht, R., Hayashi-Takanaka, Y., Wilson, M. D., … Narita, M. (2012). Independence of Repressive Histone Marks and Chromatin Compaction during Senescent Heterochromatic Layer Formation. *Molecular Cell*, *47*(2), 203–214. https://doi.org/10.1016/j.molcel.2012.06.010

Chandra, T., & Narita, M. (2013). High-order chromatin structure and the epigenome in SAHFs. *Nucleus*, *4*(1), 23–28. https://doi.org/10.4161/nucl.23189

Chantalat, S., Depaux, A., Héry, P., Barral, S., Thuret, J.-Y., Dimitrov, S., & Gérard, M. (2011). Histone H3 trimethylation at lysine 36 is associated with constitutive and facultative heterochromatin. *Genome Research*, *21*(9), 1426–1437. https://doi.org/10.1101/gr.118091.110

Chardon, F., Japaridze, A., Witt, H., Velikovsky, L., Chakraborty, C., Wilhelm, T., Dumont, M., Yang, W., Kikuti, C., Gangnard, S., Mace, A.-S., Wuite, G., Dekker, C., & Fachinetti, D. (2022). CENP-B-mediated DNA loops regulate activity and stability of human centromeres. *Molecular Cell*, *82*(9), 1751-1767.e8. https://doi.org/10.1016/j.molcel.2022.02.032

Charles Molnar and Jane Gair. (2015). *Concepts of Biology* (1st Canadian Edition). British Columbia Ministry of Advanced Education. https://pressbooks.bccampus.ca/conceptsofbiologymolnarcamosun/front-matter/about-the-book/

Chen, H., Chen, H., Zhang, J., Wang, Y., Simoneau, A., Yang, H., Levine, A. S., Zou, L., Chen, Z., & Lan, L. (2020). cGAS suppresses genomic instability as a decelerator of replication forks. *Science Advances*, *6*(42), eabb8941. https://doi.org/10.1126/sciadv.abb8941

Chen, R., Kang, R., Fan, X.-G., & Tang, D. (2014). Release and activity of histone in diseases. *Cell Death & Disease*, *5*(8), e1370–e1370. https://doi.org/10.1038/cddis.2014.337

Chen, Y., Zhang, Q., Teng, Z., & Liu, H. (2021). Centromeric transcription maintains centromeric cohesion in human cells. *Journal of Cell Biology*, *220*(7), e202008146. https://doi.org/10.1083/jcb.202008146

Cheng, N., Kim, K.-H., & Lau, L. F. (2022). Senescent hepatic stellate cells promote liver regeneration through IL-6 and ligands of CXCR2. *JCI Insight*, *7*(14), e158207. https://doi.org/10.1172/jci.insight.158207

Cho, M.-G., Kumar, R. J., Lin, C.-C., Boyer, J. A., Shahir, J. A., Fagan-Solis, K., Simpson, D. A., Fan, C., Foster, C. E., Goddard, A. M., Lerner, L. M., Ellington, S. W., Wang, Q., Wang, Y., Ho, A. Y., Liu, P., Perou, C. M., Zhang, Q., McGinty, R. K., … Gupta, G. P. (2024). MRE11 liberates cGAS from nucleosome sequestration during tumorigenesis. *Nature*, *625*(7995), 585–592. https://doi.org/10.1038/s41586-023-06889-6

Clapier, C. R., Iwasa, J., Cairns, B. R., & Peterson, C. L. (2017). Mechanisms of action and regulation of ATP-dependent chromatin-remodelling complexes. *Nature Reviews Molecular Cell Biology*, *18*(7), 407–422. https://doi.org/10.1038/nrm.2017.26

Cleveland, D. W., Mao, Y., & Sullivan, K. F. (2003). Centromeres and Kinetochores. *Cell*, *112*(4), 407–421. https://doi.org/10.1016/S0092-8674(03)00115-6

Collins, K. A., Castillo, A. R., Tatsutani, S. Y., & Biggins, S. (2005). De Novo Kinetochore Assembly Requires the Centromeric Histone H3 Variant. *Molecular Biology of the Cell*, *16*(12), 5649–5660. https://doi.org/10.1091/mbc.e05-08-0771

Coppé, J.-P., Patil, C. K., Rodier, F., Sun, Y., Muñoz, D. P., Goldstein, J., Nelson, P. S., Desprez, P.-Y., & Campisi, J. (2008). Senescence-Associated Secretory Phenotypes Reveal Cell-Nonautonomous Functions of Oncogenic RAS and the p53 Tumor Suppressor. *PLoS Biology*, *6*(12), e301. https://doi.org/10.1371/journal.pbio.0060301

Corneo, G., Ginelli, E., & Polli, E. (1968). Isolation of the complementary strands of a human satellite DNA. *Journal of Molecular Biology*, *33*(1), 331–335. https://doi.org/10.1016/0022-2836(68)90301-X

Corneo, G., Ginelli, E., & Polli, E. (1970). Repeated sequences in human DNA. *Journal of Molecular Biology*, *48*(2), 319–327. https://doi.org/10.1016/0022-2836(70)90163-4

Corneo, G., Ginelli, E., & Polli, E. (1971). Renaturation properties and localization in heterochromatin of human satellite DNA's. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis*, *247*(4), 528–534. https://doi.org/10.1016/0005-2787(71)90689-7

Courtois-Cox, S., Jones, S. L., & Cichowski, K. (2008). Many roads lead to oncogene-induced senescence. *Oncogene*, *27*(20), 2801–2809. https://doi.org/10.1038/sj.onc.1210950

Crofts, S. J. C., Latorre-Crespo, E., & Chandra, T. (2023). DNA methylation rates scale with maximum lifespan across mammals. *Nature Aging*, *4*(1), 27–32. https://doi.org/10.1038/s43587-023-00535-6

Crouch, J., Shvedova, M., Thanapaul, R. J. R. S., Botchkarev, V., & Roh, D. (2022). Epigenetic Regulation of Cellular Senescence. *Cells*, *11*(4), 672. https://doi.org/10.3390/cells11040672

Crozier, L., Foy, R., Adib, R., Kar, A., Holt, J. A., Pareri, A. U., Valverde, J. M., Rivera, R., Weston, W. A., Wilson, R., Regnault, C., Whitfield, P., Badonyi, M., Bennett, L. G., Vernon, E. G., Gamble, A., Marsh, J. A., Staples, C. J., Saurin, A. T., … Ly, T. (2023). CDK4/6 inhibitor-mediated cell overgrowth triggers osmotic and replication stress to promote senescence. *Molecular Cell*, *83*(22), 4062-4077.e5. https://doi.org/10.1016/j.molcel.2023.10.016

Dai, Z., Ramesh, V., & Locasale, J. W. (2020). The evolving metabolic landscape of chromatin biology and epigenetics. *Nature Reviews Genetics*, *21*(12), 737–753. https://doi.org/10.1038/s41576-020-0270-8

Daley, J. M., & Sung, P. (2014). 53BP1, BRCA1, and the Choice between Recombination and End Joining at DNA Double-Strand Breaks. *Molecular and Cellular Biology*, *34*(8), 1380–1388. https://doi.org/10.1128/MCB.01639-13

Dambacher, S., Deng, W., Hahn, M., Sadic, D., Fröhlich, J., Nuber, A., Hoischen, C., Diekmann, S., Leonhardt, H., & Schotta, G. (2012). CENP-C facilitates the recruitment of M18BP1 to centromeric chromatin. *Nucleus*, *3*(1), 101–110. https://doi.org/10.4161/nucl.18955

De Cecco, M., Criscione, S. W., Peckham, E. J., Hillenmeyer, S., Hamm, E. A., Manivannan, J., Peterson, A. L., Kreiling, J. A., Neretti, N., & Sedivy, J. M. (2013). Genomes of replicatively senescent cells undergo global epigenetic changes leading to gene silencing and activation of transposable elements. *Aging Cell*, *12*(2), 247–256. https://doi.org/10.1111/acel.12047

De Magalhães, J. P. (2024). Cellular senescence in normal physiology. *Science*, *384*(6702), 1300–1301. https://doi.org/10.1126/science.adj7050

De Oliveira Mann, C. C., & Hopfner, K. (2021). Nuclear cGAS: Guard or prisoner? *The EMBO Journal*, *40*(16), e108293. https://doi.org/10.15252/embj.2021108293

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre', M., Giovanni Nuciforo, P., Bensimon, A., Maestro, R., Giuseppe Pelicci, P., & d'Adda Di Fagagna, F. (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature*, *444*(7119), 638–642. https://doi.org/10.1038/nature05327

Di Micco, R., Sulli, G., Dobreva, M., Liontos, M., Botrugno, O. A., Gargiulo, G., Dal Zuffo, R., Matti, V., d'Ario, G., Montani, E., Mercurio, C., Hahn, W. C., Gorgoulis, V., Minucci, S., & d'Adda Di Fagagna, F. (2011). Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. *Nature Cell Biology*, *13*(3), 292–302. https://doi.org/10.1038/ncb2170

Dou, Z., Ghosh, K., Vizioli, M. G., Zhu, J., Sen, P., Wangensteen, K. J., Simithy, J., Lan, Y., Lin, Y., Zhou, Z., Capell, B. C., Xu, C., Xu, M., Kieckhaefer, J. E., Jiang, T., Shoshkes-Carmel, M., Tanim, K. M. A. A., Barber, G. N., Seykora, J. T., … Berger, S. L. (2017). Cytoplasmic chromatin triggers inflammation in senescence and cancer. *Nature*, *550*(7676), 402–406. https://doi.org/10.1038/nature24050

Dou, Z., Xu, C., Donahue, G., Shimi, T., Pan, J.-A., Zhu, J., Ivanov, A., Capell, B. C., Drake, A. M., Shah, P. P., Catanzaro, J. M., Daniel Ricketts, M., Lamark, T., Adam, S. A., Marmorstein, R., Zong, W.-X., Johansen, T., Goldman, R. D., Adams, P. D., & Berger, S. L. (2015). Autophagy mediates degradation of nuclear lamina. *Nature*, *527*(7576), 105–109. https://doi.org/10.1038/nature15548

Dunleavy, E. M., Almouzni, G., & Karpen, G. H. (2011). H3.3 is deposited at centromeres in S phase as a placeholder for newly assembled CENP-A in G_1 phase. *Nucleus*, *2*(2), 146–157. https://doi.org/10.4161/nucl.2.2.15211

Dunleavy, E. M., Roche, D., Tagami, H., Lacoste, N., Ray-Gallet, D., Nakamura, Y., Daigo, Y., Nakatani, Y., & Almouzni-Pettinotti, G. (2009). HJURP Is a Cell-Cycle-Dependent Maintenance and Deposition Factor of CENP-A at Centromeres. *Cell*, *137*(3), 485–497. https://doi.org/10.1016/j.cell.2009.02.040

Duzdevich, D., Redding, S., & Greene, E. C. (2014). DNA Dynamics and Single-Molecule Biology. *Chemical Reviews*, *114*(6), 3072–3086. https://doi.org/10.1021/cr4004117

Earnshaw, W. C., Ratrie, H., & Stetten, G. (1989). Visualization of centromere proteins CENP-B and CENP-C on a stable dicentric chromosome in cytological spreads. *Chromosoma*, *98*(1), 1–12. https://doi.org/10.1007/BF00293329

Earnshaw, W. C., & Rothfield, N. (1985). Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma*, *91*(3–4), 313–321. https://doi.org/10.1007/BF00328227

Earnshaw, W., Sullivan, K., Machlin, P., Cooke, C., Kaiser, D., Pollard, T., Rothfield, N., & Cleveland, D. (1987). Molecular cloning of cDNA for CENP-B, the major human

centromere autoantigen. *The Journal of Cell Biology*, *104*(4), 817–829. https://doi.org/10.1083/jcb.104.4.817

Fachinetti, D., Diego Folco, H., Nechemia-Arbely, Y., Valente, L. P., Nguyen, K., Wong, A. J., Zhu, Q., Holland, A. J., Desai, A., Jansen, L. E. T., & Cleveland, D. W. (2013). A two-step mechanism for epigenetic specification of centromere identity and function. *Nature Cell Biology*, *15*(9), 1056–1066. https://doi.org/10.1038/ncb2805

Fachinetti, D., Han, J. S., McMahon, M. A., Ly, P., Abdullah, A., Wong, A. J., & Cleveland, D. W. (2015a). DNA Sequence-Specific Binding of CENP-B Enhances the Fidelity of Human Centromere Function. *Developmental Cell*, *33*(3), 314–327. https://doi.org/10.1016/j.devcel.2015.03.020

Falk, S. J., Guo, L. Y., Sekulic, N., Smoak, E. M., Mani, T., Logsdon, G. A., Gupta, K., Jansen, L. E. T., Van Duyne, G. D., Vinogradov, S. A., Lampson, M. A., & Black, B. E. (2015). CENP-C reshapes and stabilizes CENP-A nucleosomes at the centromere. *Science*, *348*(6235), 699–703. https://doi.org/10.1126/science.1259308

Fang, L., Hao, Y., Yu, H., Gu, X., Peng, Q., Zhuo, H., Li, Y., Liu, Z., Wang, J., Chen, Y., Zhang, J., Tian, H., Gao, Y., Gao, R., Teng, H., Shan, Z., Zhu, J., Li, Z., Liu, Y., … Wang, P. (2023). Methionine restriction promotes cGAS activation and chromatin untethering through demethylation to enhance antitumor immunity. *Cancer Cell*, *41*(6), 1118-1133.e12. https://doi.org/10.1016/j.ccell.2023.05.005

Flemming, W. (1882). *Zellsubstanz, Kern und Zelltheilung /*. F.C.W. Vogel,. https://doi.org/10.5962/bhl.title.168645

Foltz, D. R., Jansen, L. E. T., Bailey, A. O., Yates, J. R., Bassett, E. A., Wood, S., Black, B. E., & Cleveland, D. W. (2009). Centromere-Specific Assembly of CENP-A Nucleosomes Is Mediated by HJURP. *Cell*, *137*(3), 472–484. https://doi.org/10.1016/j.cell.2009.02.039

Foltz, D. R., Jansen, L. E. T., Black, B. E., Bailey, A. O., Yates, J. R., & Cleveland, D. W. (2006). The human CENP-A centromeric nucleosome-associated complex. *Nature Cell Biology*, *8*(5), 458–469. https://doi.org/10.1038/ncb1397

Freund, A., Laberge, R.-M., Demaria, M., & Campisi, J. (2012). Lamin B1 loss is a senescence-associated biomarker. *Molecular Biology of the Cell*, *23*(11), 2066–2075. https://doi.org/10.1091/mbc.e11-10-0884

Frisch, S. M., & MacFawn, I. P. (2020). Type I interferons and related pathways in cell senescence. *Aging Cell*, *19*(10), e13234. https://doi.org/10.1111/acel.13234

From the archive: The cell cycle and Antarctic exploration. (2023). *Nature*, *615*(7953), 594–594. https://doi.org/10.1038/d41586-023-00796-6

Fujita, R., Otake, K., Arimura, Y., Horikoshi, N., Miya, Y., Shiga, T., Osakabe, A., Tachiwana, H., Ohzeki, J., Larionov, V., Masumoto, H., & Kurumizaka, H. (2015). Stable complex formation of CENP-B with the CENP-A nucleosome. *Nucleic Acids Research*, *43*(10), 4909–4922. https://doi.org/10.1093/nar/gkv405

Fujita, Y., Hayashi, T., Kiyomitsu, T., Toyoda, Y., Kokubu, A., Obuse, C., & Yanagida, M. (2007). Priming of Centromere for CENP-A Recruitment by Human hMis18α, hMis18β, and M18BP1. *Developmental Cell*, *12*(1), 17–30. https://doi.org/10.1016/j.devcel.2006.11.002

Fukagawa, T., & Earnshaw, W. C. (2014). The Centromere: Chromatin Foundation for the Kinetochore Machinery. *Developmental Cell*, *30*(5), 496–508. https://doi.org/10.1016/j.devcel.2014.08.016

Funayama, R., Saito, M., Tanobe, H., & Ishikawa, F. (2006). Loss of linker histone H1 in cellular senescence. *The Journal of Cell Biology*, *175*(6), 869–880. https://doi.org/10.1083/jcb.200604005

Furuyama, S., & Biggins, S. (2007). Centromere identity is specified by a single centromeric nucleosome in budding yeast. *Proceedings of the National Academy of Sciences*, *104*(37), 14706–14711. https://doi.org/10.1073/pnas.0706985104

Gamba, R., & Fachinetti, D. (2020). From evolution to function: Two sides of the same CENP-B coin? *Experimental Cell Research*, *390*(2), 111959. https://doi.org/10.1016/j.yexcr.2020.111959

Gauchier, M., Kan, S., Barral, A., Sauzet, S., Agirre, E., Bonnell, E., Saksouk, N., Barth, T. K., Ide, S., Urbach, S., Wellinger, R. J., Luco, R. F., Imhof, A., & Déjardin, J. (2019). SETDB1-dependent heterochromatin stimulates alternative lengthening of telomeres. *Science Advances*, *5*(5), eaav3673. https://doi.org/10.1126/sciadv.aav3673

Geiss, C. P., Keramisanou, D., Sekulic, N., Scheffer, M. P., Black, B. E., & Frangakis, A. S. (2014). CENP-A Arrays Are More Condensed than Canonical Arrays at Low Ionic Strength. *Biophysical Journal*, *106*(4), 875–882. https://doi.org/10.1016/j.bpj.2014.01.005

Gentili, M., Kowal, J., Tkach, M., Satoh, T., Lahaye, X., Conrad, C., Boyron, M., Lombard, B., Durand, S., Kroemer, G., Loew, D., Dalod, M., Théry, C., & Manel, N. (2015). Transmission of innate immune signaling by packaging of cGAMP in viral particles. *Science*, *349*(6253), 1232–1236. https://doi.org/10.1126/science.aab3628

Gentili, M., Lahaye, X., Nadalin, F., Nader, G. P. F., Puig Lombardi, E., Herve, S., De Silva, N. S., Rookhuizen, D. C., Zueva, E., Goudot, C., Maurin, M., Bochnakian, A., Amigorena, S., Piel, M., Fachinetti, D., Londoño-Vallejo, A., & Manel, N. (2019a). The N-Terminal Domain of cGAS Determines Preferential Association with Centromeric DNA and Innate Immune Activation in the Nucleus. *Cell Reports*, *26*(9), 2377-2393.e13. https://doi.org/10.1016/j.celrep.2019.01.105

Ghoneim, M., Fuchs, H. A., & Musselman, C. A. (2021). Histone Tail Conformations: A Fuzzy Affair with DNA. *Trends in Biochemical Sciences*, *46*(7), 564–578. https://doi.org/10.1016/j.tibs.2020.12.012

Giunta, S., Hervé, S., White, R. R., Wilhelm, T., Dumont, M., Scelfo, A., Gamba, R., Wong, C. K., Rancati, G., Smogorzewska, A., Funabiki, H., & Fachinetti, D. (2021a). CENP-A chromatin prevents replication stress at centromeres to avoid structural aneuploidy. *Proceedings of the National Academy of Sciences*, *118*(10), e2015634118. https://doi.org/10.1073/pnas.2015634118

Gómez-Márquez, J. (2021). What is life? *Molecular Biology Reports*, *48*(8), 6223–6230. https://doi.org/10.1007/s11033-021-06594-5

Gonzalez-Meljem, J. M., Apps, J. R., Fraser, H. C., & Martinez-Barbera, J. P. (2018). Paracrine roles of cellular senescence in promoting tumourigenesis. *British Journal of Cancer*, *118*(10), 1283–1288. https://doi.org/10.1038/s41416-018-0066-1

Goto, N., Suke, K., Yonezawa, N., Nishihara, H., Handa, T., Sato, Y., Kujirai, T., Kurumizaka, H., Yamagata, K., & Kimura, H. (2024). ISWI chromatin remodeling complexes recruit NSD2 and H3K36me2 in pericentromeric heterochromatin. *Journal of Cell Biology*, *223*(8), e202310084. https://doi.org/10.1083/jcb.202310084

Grosse, L., Wagner, N., Emelyanov, A., Molina, C., Lacas-Gervais, S., Wagner, K.-D., & Bulavin, D. V. (2020). Defined p16High Senescent Cell Types Are Indispensable for Mouse Healthspan. *Cell Metabolism*, *32*(1), 87-99.e6. https://doi.org/10.1016/j.cmet.2020.05.002

Guccini, I., Revandkar, A., D'Ambrosio, M., Colucci, M., Pasquini, E., Mosole, S., Troiani, M., Brina, D., Sheibani-Tezerji, R., Elia, A. R., Rinaldi, A., Pernigoni, N., Rüschoff, J. H., Dettwiler, S., De Marzo, A. M., Antonarakis, E. S., Borrelli, C., Moor, A. E., Garcia-Escudero, R., … Alimonti, A. (2021). Senescence Reprogramming by TIMP1 Deficiency Promotes Prostate Cancer Metastasis. *Cancer Cell*, *39*(1), 68-82.e9. https://doi.org/10.1016/j.ccell.2020.10.012

Guelen, L., Pagie, L., Brasset, E., Meuleman, W., Faza, M. B., Talhout, W., Eussen, B. H., De Klein, A., Wessels, L., De Laat, W., & Van Steensel, B. (2008). Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions. *Nature*, *453*(7197), 948–951. https://doi.org/10.1038/nature06947

Guey, B., Wischnewski, M., Decout, A., Makasheva, K., Kaynak, M., Sakar, M. S., Fierz, B., & Ablasser, A. (2020). BAF restricts cGAS on nuclear DNA to prevent innate immune activation. *Science*, *369*(6505), 823–828. https://doi.org/10.1126/science.aaw6421

Guldner, H. H., Lakomek, H. J., & Bautz, F. A. (1984). Human anti-centromere sera recognise a 19.5 kD non-histone chromosomal protein from HeLa cells. *Clinical and Experimental Immunology*, *58*(1), 13–20.

Gupta, A., Hunt, C. R., Hegde, M. L., Chakraborty, S., Udayakumar, D., Horikoshi, N., Singh, M., Ramnarain, D. B., Hittelman, W. N., Namjoshi, S., Asaithamby, A., Hazra, T. K., Ludwig, T., Pandita, R. K., Tyler, J. K., & Pandita, T. K. (2014). MOF Phosphorylation by ATM Regulates 53BP1-Mediated Double-Strand Break Repair Pathway Choice. *Cell Reports*, *8*(1), 177–189. https://doi.org/10.1016/j.celrep.2014.05.044

Gutierrez-Arcelus, M., Lappalainen, T., Montgomery, S. B., Buil, A., Ongen, H., Yurovsky, A., Bryois, J., Giger, T., Romano, L., Planchon, A., Falconnet, E., Bielser, D., Gagnebin, M., Padioleau, I., Borel, C., Letourneau, A., Makrythanasis, P., Guipponi, M., Gehrig, C., … Dermitzakis, E. T. (2013). Passive and active DNA methylation and the interplay with genetic variation in gene regulation. *eLife*, *2*, e00523. https://doi.org/10.7554/eLife.00523

Hara, M., Ariyoshi, M., Sano, T., Nozawa, R.-S., Shinkai, S., Onami, S., Jansen, I., Hirota, T., & Fukagawa, T. (2023). Centromere/kinetochore is assembled through CENP-C oligomerization. *Molecular Cell*, *83*(13), 2188-2205.e13. https://doi.org/10.1016/j.molcel.2023.05.023

Harris, H. L., Gu, H., Olshansky, M., Wang, A., Farabella, I., Eliaz, Y., Kalluchi, A., Krishna, A., Jacobs, M., Cauer, G., Pham, M., Rao, S. S. P., Dudchenko, O., Omer, A., Mohajeri, K., Kim, S., Nichols, M. H., Davis, E. S., Gkountaroulis, D., … Rowley, M. J. (2023). Chromatin alternates between A and B compartments at kilobase scale for subgenic organization. *Nature Communications*, *14*(1), 3303. https://doi.org/10.1038/s41467-023-38429-1

Hasan, N., & Ahuja, N. (2019). The Emerging Roles of ATP-Dependent Chromatin Remodeling Complexes in Pancreatic Cancer. *Cancers*, *11*(12), 1859. https://doi.org/10.3390/cancers11121859

Hasson, D., Panchenko, T., Salimian, K. J., Salman, M. U., Sekulic, N., Alonso, A., Warburton, P. E., & Black, B. E. (2013). The octamer is the major form of CENP-A nucleosomes at human centromeres. *Nature Structural & Molecular Biology*, *20*(6), 687–695. https://doi.org/10.1038/nsmb.2562

Hatton, I. A., Galbraith, E. D., Merleau, N. S. C., Miettinen, T. P., Smith, B. M., & Shander, J. A. (2023). The human cell count and size distribution. *Proceedings of the National Academy of Sciences*, *120*(39), e2303077120. https://doi.org/10.1073/pnas.2303077120

Hauf, S., Roitinger, E., Koch, B., Dittrich, C. M., Mechtler, K., & Peters, J.-M. (2005). Dissociation of Cohesin from Chromosome Arms and Loss of Arm Cohesion during Early Mitosis Depends on Phosphorylation of SA2. *PLoS Biology*, *3*(3), e69. https://doi.org/10.1371/journal.pbio.0030069

He, Q., Au, B., Kulkarni, M., Shen, Y., Lim, Kah. J., Maimaiti, J., Wong, Cheng. Kit., Luijten, Monique. N. H., Chong, H. C., Lim, E. H., Rancati, G., Sinha, I., Fu, Z., Wang, X., Connolly, John. E., & Crasta, K. C. (2018). Chromosomal instability-induced senescence potentiates cell non-autonomous tumourigenic effects. *Oncogenesis*, *7*(8), 62. https://doi.org/10.1038/s41389-018-0072-4

Hédouin, S., Grillo, G., Ivkovic, I., Velasco, G., & Francastel, C. (2017). CENP-A chromatin disassembly in stressed and senescent murine cells. *Scientific Reports*, *7*(1), 42520. https://doi.org/10.1038/srep42520

Helman, A., Klochendler, A., Azazmeh, N., Gabai, Y., Horwitz, E., Anzi, S., Swisa, A., Condiotti, R., Granit, R. Z., Nevo, Y., Fixler, Y., Shreibman, D., Zamir, A., Tornovsky-Babeay, S., Dai, C., Glaser, B., Powers, A. C., Shapiro, A. M. J., Magnuson, M. A., … Ben-Porath, I. (2016). p16Ink4a-induced senescence of pancreatic beta cells enhances insulin secretion. *Nature Medicine*, *22*(4), 412–420. https://doi.org/10.1038/nm.4054

Hernandez-Segura, A., Nehme, J., & Demaria, M. (2018). Hallmarks of Cellular Senescence. *Trends in Cell Biology*, *28*(6), 436–453. https://doi.org/10.1016/j.tcb.2018.02.001

Heun, P., Erhardt, S., Blower, M. D., Weiss, S., Skora, A. D., & Karpen, G. H. (2006). Mislocalization of the Drosophila Centromere-Specific Histone CID Promotes Formation of Functional Ectopic Kinetochores. *Developmental Cell*, *10*(3), 303–315. https://doi.org/10.1016/j.devcel.2006.01.014

Hoffmann, S., Dumont, M., Barra, V., Ly, P., Nechemia-Arbely, Y., McMahon, M. A., Hervé, S., Cleveland, D. W., & Fachinetti, D. (2016). CENP-A Is Dispensable for Mitotic

Centromere Function after Initial Centromere/Kinetochore Assembly. *Cell Reports*, *17*(9), 2394–2404. https://doi.org/10.1016/j.celrep.2016.10.084

Hoffmann, S., Izquierdo, H. M., Gamba, R., Chardon, F., Dumont, M., Keizer, V., Hervé, S., McNulty, S. M., Sullivan, B. A., Manel, N., & Fachinetti, D. (2020). A genetic memory initiates the epigenetic loop necessary to preserve centromere position. *The EMBO Journal*, *39*(20), e105505. https://doi.org/10.15252/embj.2020105505

Horvath, S., & Raj, K. (2018). DNA methylation-based biomarkers and the epigenetic clock theory of ageing. *Nature Reviews Genetics*, *19*(6), 371–384. https://doi.org/10.1038/s41576-018-0004-3

Howman, E. V., Fowler, K. J., Newson, A. J., Redward, S., MacDonald, A. C., Kalitsis, P., & Choo, K. H. A. (2000). Early disruption of centromeric chromatin organization in centromere protein A (*Cenpa*) null mice. *Proceedings of the National Academy of Sciences*, *97*(3), 1148–1153. https://doi.org/10.1073/pnas.97.3.1148

Huang, R., & Zhou, P.-K. (2021). DNA damage repair: Historical perspectives, mechanistic pathways and clinical translation for targeted cancer therapy. *Signal Transduction and Targeted Therapy*, *6*(1), 254. https://doi.org/10.1038/s41392-021-00648-7

Huang, W., Hickson, L. J., Eirin, A., Kirkland, J. L., & Lerman, L. O. (2022). Cellular senescence: The good, the bad and the unknown. *Nature Reviews Nephrology*, *18*(10), 611–627. https://doi.org/10.1038/s41581-022-00601-z

Hudson, D. F., Fowler, K. J., Earle, E., Saffery, R., Kalitsis, P., Trowell, H., Hill, J., Wreford, N. G., De Kretser, D. M., Cancilla, M. R., Howman, E., Hii, L., Cutts, S. M., Irvine, D. V., & Choo, K. H. A. (1998). Centromere Protein B Null Mice are Mitotically and Meiotically Normal but Have Lower Body and Testis Weights. *The Journal of Cell Biology*, *141*(2), 309–319. https://doi.org/10.1083/jcb.141.2.309

International Human Genome Sequencing Consortium, Whitehead Institute for Biomedical Research, Center for Genome Research:, Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., … Morgan, M. J. (2001). Initial sequencing and analysis of the human genome. *Nature*, *409*(6822), 860–921. https://doi.org/10.1038/35057062

Jack Y. Ghannam; Jason Wang; Arif Jan. (2023). *Biochemistry, DNA Structure*. StatPearls Publishing. www.ncbi.nlm.nih.gov/books/NBK538241/

Janicki, S. M., Tsukamoto, T., Salghetti, S. E., Tansey, W. P., Sachidanandam, R., Prasanth, K. V., Ried, T., Shav-Tal, Y., Bertrand, E., Singer, R. H., & Spector, D. L. (2004). From Silencing to Gene Expression. *Cell*, *116*(5), 683–698. https://doi.org/10.1016/S0092-8674(04)00171-0

Jansen, L. E. T., Black, B. E., Foltz, D. R., & Cleveland, D. W. (2007). Propagation of centromeric chromatin requires exit from mitosis. *The Journal of Cell Biology*, *176*(6), 795–805. https://doi.org/10.1083/jcb.200701066

Jerkovic´, I., & Cavalli, G. (2021). Understanding 3D genome organization by multidisciplinary methods. *Nature Reviews Molecular Cell Biology*, *22*(8), 511–528. https://doi.org/10.1038/s41580-021-00362-w

Jiang, H., Xue, X., Panda, S., Kawale, A., Hooy, R. M., Liang, F., Sohn, J., Sung, P., & Gekara, N. O. (2019a). Chromatin-bound cGAS is an inhibitor of DNA repair and hence accelerates genome destabilization and cell death. *The EMBO Journal*, *38*(21), e102718. https://doi.org/10.15252/embj.2019102718

Jiang, S., & Guo, Y. (2020). Epigenetic Clock: DNA Methylation in Aging. *Stem Cells International*, *2020*, 1–9. https://doi.org/10.1155/2020/1047896

Jin, Z., & Liu, Y. (2018). DNA methylation in human diseases. *Genes & Diseases*, *5*(1), 1–8. https://doi.org/10.1016/j.gendis.2018.01.002

Jones, M. J., Goodman, S. J., & Kobor, M. S. (2015). DNA methylation and healthy human aging. *Aging Cell*, *14*(6), 924–932. https://doi.org/10.1111/acel.12349

Jung, M., & Pfeifer, G. P. (2015). Aging and DNA methylation. *BMC Biology*, *13*(1), 7. https://doi.org/10.1186/s12915-015-0118-4

Jung, Y.-S., Qian, Y., & Chen, X. (2010). Examination of the expanding pathways for the regulation of p21 expression and activity. *Cellular Signalling*, *22*(7), 1003–1012. https://doi.org/10.1016/j.cellsig.2010.01.013

Kadoch, C., Williams, R. T., Calarco, J. P., Miller, E. L., Weber, C. M., Braun, S. M. G., Pulice, J. L., Chory, E. J., & Crabtree, G. R. (2017). Dynamics of BAF–Polycomb complex opposition on heterochromatin in normal and oncogenic states. *Nature Genetics*, *49*(2), 213–222. https://doi.org/10.1038/ng.3734

Kang, C., & Elledge, S. J. (2016). How autophagy both activates and inhibits cellular senescence. *Autophagy*, *12*(5), 898–899. https://doi.org/10.1080/15548627.2015.1121361

Kang, C., Xu, Q., Martin, T. D., Li, M. Z., Demaria, M., Aron, L., Lu, T., Yankner, B. A., Campisi, J., & Elledge, S. J. (2015). The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4. *Science*, *349*(6255), aaa5612. https://doi.org/10.1126/science.aaa5612

Kapoor, M., Montes De Oca Luna, R., Liu, G., Lozano, G., Cummings, C., Mancini, M., Ouspenski, I., Brinkley, B. R., & May, G. S. (1998). The cenpB gene is not essential in mice. *Chromosoma*, *107*(8), 570–576. https://doi.org/10.1007/s004120050343

Kartikasari, A. E. R., Huertas, C. S., Mitchell, A., & Plebanski, M. (2021). Tumor-Induced Inflammatory Cytokines and the Emerging Diagnostic Devices for Cancer Detection and Prognosis. *Frontiers in Oncology*, *11*, 692142. https://doi.org/10.3389/fonc.2021.692142

Kasinathan, S., & Henikoff, S. (2018). Non-B-Form DNA Is Enriched at Centromeres. *Molecular Biology and Evolution*, *35*(4), 949–962. https://doi.org/10.1093/molbev/msy010

Kato, H., Jiang, J., Zhou, B.-R., Rozendaal, M., Feng, H., Ghirlando, R., Xiao, T. S., Straight, A. F., & Bai, Y. (2013). A Conserved Mechanism for Centromeric Nucleosome Recognition by Centromere Protein CENP-C. *Science*, *340*(6136), 1110–1113. https://doi.org/10.1126/science.1235532

Kciuk, M., Gielecińska, A., Mujwar, S., Kołat, D., Kałuzińska-Kołat, Ż., Celik, I., & Kontek, R. (2023). Doxorubicin—An Agent with Multiple Mechanisms of Anticancer Activity. *Cells*, *12*(4), 659. https://doi.org/10.3390/cells12040659

Keiten-Schmitz, J., Schunck, K., & Müller, S. (2020). SUMO Chains Rule on Chromatin Occupancy. *Frontiers in Cell and Developmental Biology*, *7*, 343. https://doi.org/10.3389/fcell.2019.00343

Kipling, D. (1997). Centromeres, CENP-B and Tigger too. *Trends in Genetics*, *13*(4), 141–145. https://doi.org/10.1016/S0168-9525(97)01098-6

Klare, K., Weir, J. R., Basilico, F., Zimniak, T., Massimiliano, L., Ludwigs, N., Herzog, F., & Musacchio, A. (2015). CENP-C is a blueprint for constitutive centromere–associated network assembly within human kinetochores. *Journal of Cell Biology*, *210*(1), 11–22. https://doi.org/10.1083/jcb.201412028

Ko, A., Han, S. Y., Choi, C. H., Cho, H., Lee, M.-S., Kim, S.-Y., Song, J. S., Hong, K.-M., Lee, H.-W., Hewitt, S. M., Chung, J.-Y., & Song, J. (2018). Oncogene-induced senescence mediated by c-Myc requires USP10 dependent deubiquitination and stabilization of

p14ARF. *Cell Death & Differentiation*, *25*(6), 1050–1062. https://doi.org/10.1038/s41418-018-0072-0

Kochin, V., Shimi, T., Torvaldson, E., Adam, S. A., Goldman, A., Pack, C.-G., Melo-Cardenas, J., Imanishi, S. Y., Goldman, R. D., & Eriksson, J. E. (2014). Interphase phosphorylation of lamin A. *Journal of Cell Science*, jcs.141820. https://doi.org/10.1242/jcs.141820

Kosar, M., Bartkova, J., Hubackova, S., Hodny, Z., Lukas, J., & Bartek, J. (2011). Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16 ^{ink4a}. *Cell Cycle*, *10*(3), 457–468. https://doi.org/10.4161/cc.10.3.14707

Koyanagi, E., Kakimoto, Y., Minamisawa, T., Yoshifuji, F., Natsume, T., Higashitani, A., Ogi, T., Carr, A. M., Kanemaki, M. T., & Daigaku, Y. (2022). Global landscape of replicative DNA polymerase usage in the human genome. *Nature Communications*, *13*(1), 7221. https://doi.org/10.1038/s41467-022-34929-8

Kranzusch, P. J., Lee, A. S.-Y., Berger, J. M., & Doudna, J. A. (2013). Structure of Human cGAS Reveals a Conserved Family of Second-Messenger Enzymes in Innate Immunity. *Cell Reports*, *3*(5), 1362–1368. https://doi.org/10.1016/j.celrep.2013.05.008

Kujirai, T., Zierhut, C., Takizawa, Y., Kim, R., Negishi, L., Uruma, N., Hirai, S., Funabiki, H., & Kurumizaka, H. (2020). Structural basis for the inhibition of cGAS by nucleosomes. *Science*, *370*(6515), 455–458. https://doi.org/10.1126/science.abd0237

Kwon, J., & Bakhoum, S. F. (2020). The Cytosolic DNA-Sensing cGAS–STING Pathway in Cancer. *Cancer Discovery*, *10*(1), 26–39. https://doi.org/10.1158/2159-8290.CD-19-0761

Kwon, Y., Kim, J. W., Jeoung, J. A., Kim, M.-S., & Kang, C. (2017). Autophagy Is Pro-Senescence When Seen in Close-Up, but Anti-Senescence in Long-Shot. *Molecules and Cells*, *40*(9), 607–612. https://doi.org/10.14348/molcells.2017.0151

Lahaye, X., Gentili, M., Silvin, A., Conrad, C., Picard, L., Jouve, M., Zueva, E., Maurin, M., Nadalin, F., Knott, G. J., Zhao, B., Du, F., Rio, M., Amiel, J., Fox, A. H., Li, P., Etienne, L., Bond, C. S., Colleaux, L., & Manel, N. (2018). NONO Detects the Nuclear HIV Capsid to Promote cGAS-Mediated Innate Immune Activation. *Cell*, *175*(2), 488-501.e22. https://doi.org/10.1016/j.cell.2018.08.062

Lanigan, F., Geraghty, J. G., & Bracken, A. P. (2011). Transcriptional regulation of cellular senescence. *Oncogene*, *30*(26), 2901–2911. https://doi.org/10.1038/onc.2011.34

Lara-Gonzalez, P., Pines, J., & Desai, A. (2021). Spindle assembly checkpoint activation and silencing at kinetochores. *Seminars in Cell & Developmental Biology*, *117*, 86–98. https://doi.org/10.1016/j.semcdb.2021.06.009

Lee, B. Y., Han, J. A., Im, J. S., Morrone, A., Johung, K., Goodwin, E. C., Kleijer, W. J., DiMaio, D., & Hwang, E. S. (2006). Senescence-associated β-galactosidase is lysosomal β-galactosidase. *Aging Cell*, *5*(2), 187–195. https://doi.org/10.1111/j.1474-9726.2006.00199.x

Lee, C.-H., Wu, J., & Li, B. (2013). Chromatin Remodelers Fine-Tune H3K36me-Directed Deacetylation of Neighbor Nucleosomes by Rpd3S. *Molecular Cell*, *52*(2), 255–263. https://doi.org/10.1016/j.molcel.2013.08.024

Lennarz, W. J., & Lane, M. D. (2004). *Encyclopedia of biological chemistry* (1st ed). Elsevier.

Leung, W., Simoneau, A., Saxena, S., Jackson, J., Patel, P. S., Limbu, M., Vindigni, A., & Zou, L. (2023). ATR protects ongoing and newly assembled DNA replication forks through distinct mechanisms. *Cell Reports*, *42*(7), 112792. https://doi.org/10.1016/j.celrep.2023.112792

Li, C. M., Lingeman, R. G., Haratipour, P., Gu, L., Jossart, J., Perry, J. J. P., Hickey, R. J., & Malkas, L. H. (2023). S Phase. In *Encyclopedia of Cell Biology* (pp. 266–284). Elsevier. https://doi.org/10.1016/B978-0-12-821618-7.00195-4

Li, H., Wang, W., Liu, X., Paulson, K. E., Yee, A. S., & Zhang, X. (2010). Transcriptional factor HBP1 targets P16INK4A, upregulating its expression and consequently is involved in Ras-induced premature senescence. *Oncogene*, *29*(36), 5083–5094. https://doi.org/10.1038/onc.2010.252

Li, M., Fang, X., Baker, D. J., Guo, L., Gao, X., Wei, Z., Han, S., Van Deursen, J. M., & Zhang, P. (2010). The ATM–p53 pathway suppresses aneuploidy-induced tumorigenesis. *Proceedings of the National Academy of Sciences*, *107*(32), 14188–14193. https://doi.org/10.1073/pnas.1005960107

Li, T., Huang, T., Du, M., Chen, X., Du, F., Ren, J., & Chen, Z. J. (2021). Phosphorylation and chromatin tethering prevent cGAS activation during mitosis. *Science*, *371*(6535), eabc5386. https://doi.org/10.1126/science.abc5386

Li, X., Li, X., Xie, C., Cai, S., Li, M., Jin, H., Wu, S., Cui, J., Liu, H., & Zhao, Y. (2022). cGAS guards against chromosome end-to-end fusions during mitosis and facilitates replicative senescence. *Protein & Cell*, *13*(1), 47–64. https://doi.org/10.1007/s13238-021-00879-y

Lin, Y.-Y., Müller, P., Karagianni, E., Hepp, N., Mueller-Planitz, F., Vanderlinden, W., & Lipfert, J. (2024). Epigenetic Histone Modifications H3K36me3 and H4K5/8/12/16ac Induce Open Polynucleosome Conformations via Different Mechanisms. *Journal of Molecular Biology*, *436*(16), 168671. https://doi.org/10.1016/j.jmb.2024.168671

Litovchick, L., Florens, L. A., Swanson, S. K., Washburn, M. P., & DeCaprio, J. A. (2011). DYRK1A protein kinase promotes quiescence and senescence through DREAM complex assembly. *Genes & Development*, *25*(8), 801–813. https://doi.org/10.1101/gad.2034211

Liu, H., Zhang, H., Wu, X., Ma, D., Wu, J., Wang, L., Jiang, Y., Fei, Y., Zhu, C., Tan, R., Jungblut, P., Pei, G., Dorhoi, A., Yan, Q., Zhang, F., Zheng, R., Liu, S., Liang, H., Liu, Z., … Ge, B. (2018). Nuclear cGAS suppresses DNA repair and promotes tumorigenesis. *Nature*, *563*(7729), 131–136. https://doi.org/10.1038/s41586-018-0629-6

Llewellyn, J., Mallikarjun, V., Appleton, E., Osipova, M., Gilbert, H. T. J., Richardson, S. M., Hubbard, S. J., & Swift, J. (2023). Loss of regulation of protein synthesis and turnover underpins an attenuated stress response in senescent human mesenchymal stem cells. *Proceedings of the National Academy of Sciences*, *120*(14), e2210745120. https://doi.org/10.1073/pnas.2210745120

Lockhead, S., Moskaleva, A., Kamenz, J., Chen, Y., Kang, M., Reddy, A. R., Santos, S. D. M., & Ferrell, J. E. (2020). The Apparent Requirement for Protein Synthesis during G2 Phase Is due to Checkpoint Activation. *Cell Reports*, *32*(2), 107901. https://doi.org/10.1016/j.celrep.2020.107901

Lodewijk, I., Nunes, S. P., Henrique, R., Jerónimo, C., Dueñas, M., & Paramio, J. M. (2021). Tackling tumor microenvironment through epigenetic tools to improve cancer immunotherapy. *Clinical Epigenetics*, *13*(1), 63. https://doi.org/10.1186/s13148-021-01046-0

Logsdon, G. A., Barrey, E. J., Bassett, E. A., DeNizio, J. E., Guo, L. Y., Panchenko, T., Dawicki-McKenna, J. M., Heun, P., & Black, B. E. (2015). Both tails and the centromere targeting domain of CENP-A are required for centromere establishment. *Journal of Cell Biology*, *208*(5), 521–531. https://doi.org/10.1083/jcb.201412011

Lu, Y., Zhao, M., Chen, L., Wang, Y., Liu, T., & Liu, H. (2024). cGAS: Action in the nucleus. *Frontiers in Immunology*, *15*, 1380517. https://doi.org/10.3389/fimmu.2024.1380517

Lum, K. K., Song, B., Federspiel, J. D., Diner, B. A., Howard, T., & Cristea, I. M. (2018). Interactome and Proteome Dynamics Uncover Immune Modulatory Associations of the

Pathogen Sensing Factor cGAS. *Cell Systems*, *7*(6), 627-642.e6. https://doi.org/10.1016/j.cels.2018.10.010

Maehara, K., Takahashi, K., & Saitoh, S. (2010). CENP-A Reduction Induces a p53-Dependent Cellular Senescence Response To Protect Cells from Executing Defective Mitoses. *Molecular and Cellular Biology*, *30*(9), 2090–2104. https://doi.org/10.1128/MCB.01318-09

Maloney, K. A., Sullivan, L. L., Matheny, J. E., Strome, E. D., Merrett, S. L., Ferris, A., & Sullivan, B. A. (2012). Functional epialleles at an endogenous human centromere. *Proceedings of the National Academy of Sciences*, *109*(34), 13704–13709. https://doi.org/10.1073/pnas.1203126109

Manuelidis, L. (1978). Chromosomal localization of complex and simple repeated human DNAs. *Chromosoma*, *66*(1), 23–32. https://doi.org/10.1007/BF00285813

Manuelidis, L., & Wu, J. C. (1978). Homology between human and simian repeated DNA. *Nature*, *276*(5683), 92–94. https://doi.org/10.1038/276092a0

Martins, N. M. C., Bergmann, J. H., Shono, N., Kimura, H., Larionov, V., Masumoto, H., & Earnshaw, W. C. (2016). Epigenetic engineering shows that a human centromere resists silencing mediated by H3K27me3/K9me3. *Molecular Biology of the Cell*, *27*(1), 177–196. https://doi.org/10.1091/mbc.E15-08-0605

Martins, N. M. C., Cisneros-Soberanis, F., Pesenti, E., Kochanova, N. Y., Shang, W.-H., Hori, T., Nagase, T., Kimura, H., Larionov, V., Masumoto, H., Fukagawa, T., & Earnshaw, W. C. (2020). H3K9me3 maintenance on a human artificial chromosome is required for segregation but not centromere epigenetic memory. *Journal of Cell Science*, *133*(14), jcs242610. https://doi.org/10.1242/jcs.242610

Masumoto, H., Masukata, H., Muro, Y., Nozaki, N., & Okazaki, T. (1989). A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. *The Journal of Cell Biology*, *109*(5), 1963–1973. https://doi.org/10.1083/jcb.109.5.1963

Matsuoka, S., Ballif, B. A., Smogorzewska, A., McDonald, E. R., Hurov, K. E., Luo, J., Bakalarski, C. E., Zhao, Z., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S. P., & Elledge, S. J. (2007). ATM and ATR Substrate Analysis Reveals Extensive Protein Networks Responsive to DNA Damage. *Science*, *316*(5828), 1160–1166. https://doi.org/10.1126/science.1140321

McKinley, K. L., & Cheeseman, I. M. (2016). The molecular basis for centromere identity and function. *Nature Reviews Molecular Cell Biology*, *17*(1), 16–29. https://doi.org/10.1038/nrm.2015.5

McNulty, S. M., & Sullivan, B. A. (2018). Alpha satellite DNA biology: Finding function in the recesses of the genome. *Chromosome Research*, *26*(3), 115–138. https://doi.org/10.1007/s10577-018-9582-3

McVey, S. L., Cosby, J. K., & Nannas, N. J. (2021). Aurora B Tension Sensing Mechanisms in the Kinetochore Ensure Accurate Chromosome Segregation. *International Journal of Molecular Sciences*, *22*(16), 8818. https://doi.org/10.3390/ijms22168818

Melters, D. P., Pitman, M., Rakshit, T., Dimitriadis, E. K., Bui, M., Papoian, G. A., & Dalal, Y. (2019). Intrinsic elasticity of nucleosomes is encoded by histone variants and calibrated by their binding partners. *Proceedings of the National Academy of Sciences*, *116*(48), 24066–24074. https://doi.org/10.1073/pnas.1911880116

Menolfi, D., Lee, B. J., Zhang, H., Jiang, W., Bowen, N. E., Wang, Y., Zhao, J., Holmes, A., Gershik, S., Rabadan, R., Kim, B., & Zha, S. (2023). ATR kinase supports normal proliferation in the early S phase by preventing replication resource exhaustion. *Nature Communications*, *14*(1), 3618. https://doi.org/10.1038/s41467-023-39332-5

Michalski, S., De Oliveira Mann, C. C., Stafford, C. A., Witte, G., Bartho, J., Lammens, K., Hornung, V., & Hopfner, K.-P. (2020). Structural basis for sequestration and autoinhibition of cGAS by chromatin. *Nature*, *587*(7835), 678–682. https://doi.org/10.1038/s41586-020-2748-0

Millán-Zambrano, G., Burton, A., Bannister, A. J., & Schneider, R. (2022). Histone post-translational modifications—Cause and consequence of genome function. *Nature Reviews Genetics*, *23*(9), 563–580. https://doi.org/10.1038/s41576-022-00468-7

Mitchell, A. R., Beauchamp, R. S., & Bostock, C. J. (1979). A study of sequence homologies in four satellite DNAs of man. *Journal of Molecular Biology*, *135*(1), 127–149. https://doi.org/10.1016/0022-2836(79)90344-9

Mitra, S., Bodor, D. L., David, A. F., Abdul-Zani, I., Mata, J. F., Neumann, B., Reither, S., Tischer, C., & Jansen, L. E. T. (2020). Genetic screening identifies a SUMO protease dynamically maintaining centromeric chromatin. *Nature Communications*, *11*(1), 501. https://doi.org/10.1038/s41467-019-14276-x

Moore, L. D., Le, T., & Fan, G. (2013). DNA Methylation and Its Basic Function. *Neuropsychopharmacology*, *38*(1), 23–38. https://doi.org/10.1038/npp.2012.112

Moroi, Y., Peebles, C., Fritzler, M. J., Steigerwald, J., & Tan, E. M. (1980). Autoantibody to centromere (kinetochore) in scleroderma sera. *Proceedings of the National Academy of Sciences*, *77*(3), 1627–1631. https://doi.org/10.1073/pnas.77.3.1627

Morrison, E. A., Bowerman, S., Sylvers, K. L., Wereszczynski, J., & Musselman, C. A. (2018). The conformation of the histone H3 tail inhibits association of the BPTF PHD finger with the nucleosome. *eLife*, *7*, e31481. https://doi.org/10.7554/eLife.31481

Muñoz-Galván, S., Lucena-Cacace, A., Perez, M., Otero-Albiol, D., Gomez-Cambronero, J., & Carnero, A. (2019). Tumor cell-secreted PLD increases tumor stemness by senescence-mediated communication with microenvironment. *Oncogene*, *38*(8), 1309–1323. https://doi.org/10.1038/s41388-018-0527-2

Nagl, N. G., Wang, X., Patsialou, A., Van Scoy, M., & Moran, E. (2007). Distinct mammalian SWI/SNF chromatin remodeling complexes with opposing roles in cell-cycle control. *The EMBO Journal*, *26*(3), 752–763. https://doi.org/10.1038/sj.emboj.7601541

Nagpal, H., Ali-Ahmad, A., Hirano, Y., Cai, W., Halic, M., Fukagawa, T., Sekulić, N., & Fierz, B. (2023). CENP-A and CENP-B collaborate to create an open centromeric chromatin state. *Nature Communications*, *14*(1), 8227. https://doi.org/10.1038/s41467-023-43739-5

Narita, M., Narita, M., Krizhanovsky, V., Nuñez, S., Chicas, A., Hearn, S. A., Myers, M. P., & Lowe, S. W. (2006). A Novel Role for High-Mobility Group A Proteins in Cellular Senescence and Heterochromatin Formation. *Cell*, *126*(3), 503–514. https://doi.org/10.1016/j.cell.2006.05.052

Narita, M., Nuñez, S., Heard, E., Narita, M., Lin, A. W., Hearn, S. A., Spector, D. L., Hannon, G. J., & Lowe, S. W. (2003). Rb-Mediated Heterochromatin Formation and Silencing of E2F Target Genes during Cellular Senescence. *Cell*, *113*(6), 703–716. https://doi.org/10.1016/S0092-8674(03)00401-X

Narita, M., Young, A. R. J., Arakawa, S., Samarajiwa, S. A., Nakashima, T., Yoshida, S., Hong, S., Berry, L. S., Reichelt, S., Ferreira, M., Tavaré, S., Inoki, K., Shimizu, S., & Narita, M. (2011). Spatial Coupling of mTOR and Autophagy Augments Secretory Phenotypes. *Science*, *332*(6032), 966–970. https://doi.org/10.1126/science.1205407

Naughton, C., Huidobro, C., Catacchio, C. R., Buckle, A., Grimes, G. R., Nozawa, R.-S., Purgato, S., Rocchi, M., & Gilbert, N. (2022). Human centromere repositioning activates transcription and opens chromatin fibre structure. *Nature Communications*, *13*(1), 5609. https://doi.org/10.1038/s41467-022-33426-2

Navarro, A. P., & Cheeseman, I. M. (2021). Kinetochore assembly throughout the cell cycle. *Seminars in Cell & Developmental Biology*, *117*, 62–74. https://doi.org/10.1016/j.semcdb.2021.03.008

Nechemia-Arbely, Y., Miga, K. H., Shoshani, O., Aslanian, A., McMahon, M. A., Lee, A. Y., Fachinetti, D., Yates, J. R., Ren, B., & Cleveland, D. W. (2019). DNA replication acts as an error correction mechanism to maintain centromere identity by restricting CENP-A to centromeres. *Nature Cell Biology*, *21*(6), 743–754. https://doi.org/10.1038/s41556-019-0331-4

Noubissi, F. K., McBride, A. A., Leppert, H. G., Millet, L. J., Wang, X., & Davern, S. M. (2021). Detection and quantification of γ-H2AX using a dissociation enhanced lanthanide fluorescence immunoassay. *Scientific Reports*, *11*(1), 8945. https://doi.org/10.1038/s41598-021-88296-3

Nurk, S., Koren, S., Rhie, A., Rautiainen, M., Bzikadze, A. V., Mikheenko, A., Vollger, M. R., Altemose, N., Uralsky, L., Gershman, A., Aganezov, S., Hoyt, S. J., Diekhans, M., Logsdon, G. A., Alonge, M., Antonarakis, S. E., Borchers, M., Bouffard, G. G., Brooks, S. Y., … Phillippy, A. M. (2022). The complete sequence of a human genome. *Science*, *376*(6588), 44–53. https://doi.org/10.1126/science.abj6987

Ogrodnik, M., Carlos Acosta, J., Adams, P. D., d'Adda Di Fagagna, F., Baker, D. J., Bishop, C. L., Chandra, T., Collado, M., Gil, J., Gorgoulis, V., Gruber, F., Hara, E., Jansen-Dürr, P., Jurk, D., Khosla, S., Kirkland, J. L., Krizhanovsky, V., Minamino, T., Niedernhofer, L. J., … Demaria, M. (2024). Guidelines for minimal information on cellular senescence experimentation in vivo. *Cell*, *187*(16), 4150–4175. https://doi.org/10.1016/j.cell.2024.05.059

Ohzeki, J., Nakano, M., Okada, T., & Masumoto, H. (2002). CENP-B box is required for de novo centromere chromatin assembly on human alphoid DNA. *The Journal of Cell Biology*, *159*(5), 765–775. https://doi.org/10.1083/jcb.200207112

Okada, T., Ohzeki, J., Nakano, M., Yoda, K., Brinkley, W. R., Larionov, V., & Masumoto, H. (2007). CENP-B Controls Centromere Formation Depending on the Chromatin Context. *Cell*, *131*(7), 1287–1300. https://doi.org/10.1016/j.cell.2007.10.045

Olan, I., Handa, T., & Narita, M. (2023). Beyond SAHF: An integrative view of chromatin compartmentalization during senescence. *Current Opinion in Cell Biology*, *83*, 102206. https://doi.org/10.1016/j.ceb.2023.102206

Ong, J. Y., & Torres, J. Z. (2020). Phase Separation in Cell Division. *Molecular Cell*, *80*(1), 9–20. https://doi.org/10.1016/j.molcel.2020.08.007

Orlic, D., & Sharkis, S. J. (1997). The elusive stem cell: How close are we? *Stem Cells*, *15*(S2), 283–284. https://doi.org/10.1002/stem.5530150836

Otake, K., Ohzeki, J., Shono, N., Kugou, K., Okazaki, K., Nagase, T., Yamakawa, H., Kouprina, N., Larionov, V., Kimura, H., Earnshaw, W. C., & Masumoto, H. (2020a). CENP-B creates alternative epigenetic chromatin states permissive for CENP-A or heterochromatin assembly. *Journal of Cell Science*, *133*(15), jcs243303. https://doi.org/10.1242/jcs.243303

Padeken, J., Methot, S. P., & Gasser, S. M. (2022). Establishment of H3K9-methylated heterochromatin and its functions in tissue differentiation and maintenance. *Nature Reviews Molecular Cell Biology*, *23*(9), 623–640. https://doi.org/10.1038/s41580-022-00483-w

Pai, C.-C., Deegan, R. S., Subramanian, L., Gal, C., Sarkar, S., Blaikley, E. J., Walker, C., Hulme, L., Bernhard, E., Codlin, S., Bähler, J., Allshire, R., Whitehall, S., & Humphrey, T. C. (2014). A histone H3K36 chromatin switch coordinates DNA double-strand break repair pathway choice. *Nature Communications*, *5*(1), 4091. https://doi.org/10.1038/ncomms5091

Palmer, D. K., O'Day, K., Trong, H. L., Charbonneau, H., & Margolis, R. L. (1991). Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proceedings of the National Academy of Sciences*, *88*(9), 3734–3738. https://doi.org/10.1073/pnas.88.9.3734

Palmer, D., O'Day, K., Wener, M., Andrews, B., & Margolis, R. (1987). A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *The Journal of Cell Biology*, *104*(4), 805–815. https://doi.org/10.1083/jcb.104.4.805

Panagopoulos, A., & Altmeyer, M. (2021). The Hammer and the Dance of Cell Cycle Control. *Trends in Biochemical Sciences*, *46*(4), 301–314. https://doi.org/10.1016/j.tibs.2020.11.002

Panchenko, T., Sorensen, T. C., Woodcock, C. L., Kan, Z., Wood, S., Resch, M. G., Luger, K., Englander, S. W., Hansen, J. C., & Black, B. E. (2011). Replacement of histone H3 with CENP-A directs global nucleosome array condensation and loosening of nucleosome superhelical termini. *Proceedings of the National Academy of Sciences*, *108*(40), 16588–16593. https://doi.org/10.1073/pnas.1113621108

Pardue, M. L., & Gall, J. G. (1970). Chromosomal Localization of Mouse Satellite DNA. *Science*, *168*(3937), 1356–1358. https://doi.org/10.1126/science.168.3937.1356

Parry, A. J., & Narita, M. (2016). Old cells, new tricks: Chromatin structure in senescence. *Mammalian Genome*, *27*(7–8), 320–331. https://doi.org/10.1007/s00335-016-9628-9

Pathare, G. R., Decout, A., Glück, S., Cavadini, S., Makasheva, K., Hovius, R., Kempf, G., Weiss, J., Kozicka, Z., Guey, B., Melenec, P., Fierz, B., Thomä, N. H., & Ablasser, A. (2020). Structural mechanism of cGAS inhibition by the nucleosome. *Nature*, *587*(7835), 668–672. https://doi.org/10.1038/s41586-020-2750-6

Payea, M., Gorospe, M., & Basisty, N. (2022). Measurement of Protein Turnover Rates in Senescent and Non-Dividing Cultured Cells with Metabolic Labeling and Mass Spectrometry. *Journal of Visualized Experiments*, *182*, 63835. https://doi.org/10.3791/63835

Perez-Castro, A. V., Shamanski, F. L., Meneses, J. J., Lovato, T. L., Vogel, K. G., Moyzis, R. K., & Pedersen, R. (1998). Centromeric Protein B Null Mice Are Viable with No Apparent Abnormalities. *Developmental Biology*, *201*(2), 135–143. https://doi.org/10.1006/dbio.1998.9005

Pesenti, M. E., Raisch, T., Conti, D., Walstein, K., Hoffmann, I., Vogt, D., Prumbaum, D., Vetter, I. R., Raunser, S., & Musacchio, A. (2022). Structure of the human inner kinetochore CCAN complex and its significance for human centromere organization. *Molecular Cell*, *82*(11), 2113-2131.e8. https://doi.org/10.1016/j.molcel.2022.04.027

Pommier, Y., Leo, E., Zhang, H., & Marchand, C. (2010). DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs. *Chemistry & Biology*, *17*(5), 421–433. https://doi.org/10.1016/j.chembiol.2010.04.012

Rayess, H., Wang, M. B., & Srivatsan, E. S. (2012). Cellular senescence and tumor suppressor gene p16. *International Journal of Cancer*, *130*(8), 1715–1725. https://doi.org/10.1002/ijc.27316

Ren, W., Fan, H., Grimm, S. A., Guo, Y., Kim, J. J., Yin, J., Li, L., Petell, C. J., Tan, X.-F., Zhang, Z.-M., Coan, J. P., Gao, L., Cai, L., Detrick, B., Çetin, B., Cui, Q., Strahl, B. D., Gozani, O., Wang, Y., … Song, J. (2020). Direct readout of heterochromatic H3K9me3 regulates DNMT1-mediated maintenance DNA methylation. *Proceedings of the National Academy of Sciences*, *117*(31), 18439–18447. https://doi.org/10.1073/pnas.2009316117

Retis-Resendiz, A. M., González-García, I. N., León-Juárez, M., Camacho-Arroyo, I., Cerbón, M., & Vázquez-Martínez, E. R. (2021). The role of epigenetic mechanisms in the regulation of gene expression in the cyclical endometrium. *Clinical Epigenetics*, *13*(1), 116. https://doi.org/10.1186/s13148-021-01103-8

Rodier, F., Muñoz, D. P., Teachenor, R., Chu, V., Le, O., Bhaumik, D., Coppé, J.-P., Campeau, E., Beauséjour, C. M., Kim, S.-H., Davalos, A. R., & Campisi, J. (2011). DNA-SCARS: Distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *Journal of Cell Science*, *124*(1), 68–81. https://doi.org/10.1242/jcs.071340

Roger, L., Tomas, F., & Gire, V. (2021). Mechanisms and Regulation of Cellular Senescence. *International Journal of Molecular Sciences*, *22*(23), 13173. https://doi.org/10.3390/ijms222313173

Rosandić, M., Paar, V., Basar, I., Glunčić, M., Pavin, N., & Pilaš, I. (2006). CENP-B box and pJα sequence distribution in human alpha satellite higher-order repeats (HOR). *Chromosome Research*, *14*(7), 735–753. https://doi.org/10.1007/s10577-006-1078-x

Rossiello, F., Jurk, D., Passos, J. F., & d'Adda Di Fagagna, F. (2022). Telomere dysfunction in ageing and age-related diseases. *Nature Cell Biology*, *24*(2), 135–147. https://doi.org/10.1038/s41556-022-00842-x

Sadaie, M., Salama, R., Carroll, T., Tomimatsu, K., Chandra, T., Young, A. R. J., Narita, M., Pérez-Mancera, P. A., Bennett, D. C., Chong, H., Kimura, H., & Narita, M. (2013). Redistribution of the Lamin B1 genomic binding profile affects rearrangement of heterochromatic domains and SAHF formation during senescence. *Genes & Development*, *27*(16), 1800–1808. https://doi.org/10.1101/gad.217281.113

Saito, Y., Yamamoto, S., & Chikenji, T. S. (2024). Role of cellular senescence in inflammation and regeneration. *Inflammation and Regeneration*, *44*(1), 28. https://doi.org/10.1186/s41232-024-00342-5

Salama, R., Sadaie, M., Hoare, M., & Narita, M. (2014). Cellular senescence and its effector programs. *Genes & Development*, *28*(2), 99–114. https://doi.org/10.1101/gad.235184.113

Saldivar, J. C., Hamperl, S., Bocek, M. J., Chung, M., Bass, T. E., Cisneros-Soberanis, F., Samejima, K., Xie, L., Paulson, J. R., Earnshaw, W. C., Cortez, D., Meyer, T., & Cimprich, K. A. (2018). An intrinsic S/G_2 checkpoint enforced by ATR. *Science*, *361*(6404), 806–810. https://doi.org/10.1126/science.aap9346

Salinas-Saavedra, M., Febrimarsa, Krasovec, G., Horkan, H. R., Baxevanis, A. D., & Frank, U. (2023). Senescence-induced cellular reprogramming drives cnidarian whole-body regeneration. *Cell Reports*, *42*(7), 112687. https://doi.org/10.1016/j.celrep.2023.112687

Santaguida, S., Richardson, A., Iyer, D. R., M'Saad, O., Zasadil, L., Knouse, K. A., Wong, Y. L., Rhind, N., Desai, A., & Amon, A. (2017). Chromosome Mis-segregation Generates Cell-Cycle-Arrested Cells with Complex Karyotypes that Are Eliminated by the Immune System. *Developmental Cell*, *41*(6), 638-651.e5. https://doi.org/10.1016/j.devcel.2017.05.022

Schrock, M. S., Stromberg, B. R., Scarberry, L., & Summers, M. K. (2020). APC/C ubiquitin ligase: Functions and mechanisms in tumorigenesis. *Seminars in Cancer Biology*, *67*, 80–91. https://doi.org/10.1016/j.semcancer.2020.03.001

Schueler, M. G., Higgins, A. W., Rudd, M. K., Gustashaw, K., & Willard, H. F. (2001). Genomic and Genetic Definition of a Functional Human Centromere. *Science*, *294*(5540), 109–115. https://doi.org/10.1126/science.1065042

Segurado, M., & Tercero, J. A. (2009). The S-phase checkpoint: Targeting the replication fork. *Biology of the Cell*, *101*(11), 617–627. https://doi.org/10.1042/BC20090053

Seibert, M., Krüger, M., Watson, N. A., Sen, O., Daum, J. R., Slotman, J. A., Braun, T., Houtsmuller, A. B., Gorbsky, G. J., Jacob, R., Kracht, M., Higgins, J. M. G., & Schmitz, M. L. (2019). CDK1-mediated phosphorylation at H2B serine 6 is required for mitotic chromosome segregation. *Journal of Cell Biology*, *218*(4), 1164–1181. https://doi.org/10.1083/jcb.201806057

Seki, A., Coppinger, J. A., Jang, C.-Y., Yates, J. R., & Fang, G. (2008). Bora and the Kinase Aurora A Cooperatively Activate the Kinase Plk1 and Control Mitotic Entry. *Science*, *320*(5883), 1655–1658. https://doi.org/10.1126/science.1157425

Serra, F., Nieto-Aliseda, A., Fanlo-Escudero, L., Rovirosa, L., Cabrera-Pasadas, M., Lazarenkov, A., Urmeneta, B., Alcalde-Merino, A., Nola, E. M., Okorokov, A. L., Fraser, P., Graupera, M., Castillo, S. D., Sardina, J. L., Valencia, A., & Javierre, B. M. (2024). P53 rapidly restructures 3D chromatin organization to trigger a transcriptional response. *Nature Communications*, *15*(1), 2821. https://doi.org/10.1038/s41467-024-46666-1

Shah, P. P., Donahue, G., Otte, G. L., Capell, B. C., Nelson, D. M., Cao, K., Aggarwala, V., Cruickshanks, H. A., Rai, T. S., McBryan, T., Gregory, B. D., Adams, P. D., & Berger, S. L. (2013). Lamin B1 depletion in senescent cells triggers large-scale changes in gene expression and the chromatin landscape. *Genes & Development*, *27*(16), 1787–1799. https://doi.org/10.1101/gad.223834.113

Sharma, G. G., So, S., Gupta, A., Kumar, R., Cayrou, C., Avvakumov, N., Bhadra, U., Pandita, R. K., Porteus, M. H., Chen, D. J., Cote, J., & Pandita, T. K. (2010). MOF and Histone H4 Acetylation at Lysine 16 Are Critical for DNA Damage Response and Double-Strand Break Repair. *Molecular and Cellular Biology*, *30*(14), 3582–3595. https://doi.org/10.1128/MCB.01476-09

Sheekey, E., & Narita, M. (2023a). P53 in senescence – it's a marathon, not a sprint. *The FEBS Journal*, *290*(5), 1212–1220. https://doi.org/10.1111/febs.16325

Sheekey, E., & Narita, M. (2023b). P53 in senescence – it's a marathon, not a sprint. *The FEBS Journal*, *290*(5), 1212–1220. https://doi.org/10.1111/febs.16325

Shimi, T., Butin-Israeli, V., Adam, S. A., Hamanaka, R. B., Goldman, A. E., Lucas, C. A., Shumaker, D. K., Kosak, S. T., Chandel, N. S., & Goldman, R. D. (2011). The role of nuclear lamin B1 in cell proliferation and senescence. *Genes & Development*, *25*(24), 2579–2593. https://doi.org/10.1101/gad.179515.111

Shogren-Knaak, M., Ishii, H., Sun, J.-M., Pazin, M. J., Davie, J. R., & Peterson, C. L. (2006). Histone H4-K16 Acetylation Controls Chromatin Structure and Protein Interactions. *Science*, *311*(5762), 844–847. https://doi.org/10.1126/science.1124000

Sikder, S., Baek, S., McNeil, T., & Dalal, Y. (2023). *Unraveling the mechanism of centromere inactivation in human aging*. https://doi.org/10.1101/2023.12.30.573721

Skibbens, R. V. (2019). Condensins and cohesins – one of these things is not like the other! *Journal of Cell Science*, *132*(3), jcs220491. https://doi.org/10.1242/jcs.220491

Slobodnyuk, K., Radic, N., Ivanova, S., Llado, A., Trempolec, N., Zorzano, A., & Nebreda, A. R. (2019). Autophagy-induced senescence is regulated by p38α signaling. *Cell Death & Disease*, *10*(6), 376. https://doi.org/10.1038/s41419-019-1607-0

Smoak, E. M., Stein, P., Schultz, R. M., Lampson, M. A., & Black, B. E. (2016). Long-Term Retention of CENP-A Nucleosomes in Mammalian Oocytes Underpins Transgenerational Inheritance of Centromere Identity. *Current Biology*, *26*(8), 1110–1116. https://doi.org/10.1016/j.cub.2016.02.061

Song, J.-X., Villagomes, D., Zhao, H., & Zhu, M. (2022). cGAS in nucleus: The link between immune response and DNA damage repair. *Frontiers in Immunology*, *13*, 1076784. https://doi.org/10.3389/fimmu.2022.1076784

Srinivasan, M., Fumasoni, M., Petela, N. J., Murray, A., & Nasmyth, K. A. (2020). Cohesion is established during DNA replication utilising chromosome associated cohesin rings as well as

those loaded de novo onto nascent DNAs. *eLife*, *9*, e56611. https://doi.org/10.7554/eLife.56611

Srivastava, S., Zasadzińska, E., & Foltz, D. R. (2018). Posttranslational mechanisms controlling centromere function and assembly. *Current Opinion in Cell Biology*, *52*, 126–135. https://doi.org/10.1016/j.ceb.2018.03.003

Stanton, B. Z., Hodges, C., Calarco, J. P., Braun, S. M. G., Ku, W. L., Kadoch, C., Zhao, K., & Crabtree, G. R. (2017). Smarca4 ATPase mutations disrupt direct eviction of PRC1 from chromatin. *Nature Genetics*, *49*(2), 282–288. https://doi.org/10.1038/ng.3735

Stevens, D., Gassmann, R., Oegema, K., & Desai, A. (2011). Uncoordinated Loss of Chromatid Cohesion Is a Common Outcome of Extended Metaphase Arrest. *PLoS ONE*, *6*(8), e22969. https://doi.org/10.1371/journal.pone.0022969

Storer, M., Mas, A., Robert-Moreno, A., Pecoraro, M., Ortells, M. C., Di Giacomo, V., Yosef, R., Pilpel, N., Krizhanovsky, V., Sharpe, J., & Keyes, W. M. (2013). Senescence Is a Developmental Mechanism that Contributes to Embryonic Growth and Patterning. *Cell*, *155*(5), 1119–1130. https://doi.org/10.1016/j.cell.2013.10.041

Sullivan, K. F., Hechenberger, M., & Masri, K. (1994). Human CENP-A contains a histone H3 related histone fold domain that is required for targeting to the centromere. *The Journal of Cell Biology*, *127*(3), 581–592. https://doi.org/10.1083/jcb.127.3.581

Sun, H., Huang, Y., Mei, S., Xu, F., Liu, X., Zhao, F., Yin, L., Zhang, D., Wei, L., Wu, C., Ma, S., Wang, J., Cen, S., Liang, C., Hu, S., & Guo, F. (2021). A Nuclear Export Signal Is Required for cGAS to Sense Cytosolic DNA. *Cell Reports*, *34*(1), 108586. https://doi.org/10.1016/j.celrep.2020.108586

Sun, L., Wu, J., Du, F., Chen, X., & Chen, Z. J. (2013). Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway. *Science*, *339*(6121), 786–791. https://doi.org/10.1126/science.1232458

Suzuki, N., Nakano, M., Nozaki, N., Egashira, S., Okazaki, T., & Masumoto, H. (2004). CENP-B Interacts with CENP-C Domains Containing Mif2 Regions Responsible for Centromere Localization. *Journal of Biological Chemistry*, *279*(7), 5934–5946. https://doi.org/10.1074/jbc.M306477200

Swanson, E. C., Manning, B., Zhang, H., & Lawrence, J. B. (2013a). Higher-order unfolding of satellite heterochromatin is a consistent and early event in cell senescence. *Journal of Cell Biology*, *203*(6), 929–942. https://doi.org/10.1083/jcb.201306073

Swift, M. L., Beishline, K., Flashner, S., & Azizkhan-Clifford, J. (2021). DSB repair pathway choice is regulated by recruitment of 53BP1 through cell cycle-dependent regulation of Sp1. *Cell Reports*, *34*(11), 108840. https://doi.org/10.1016/j.celrep.2021.108840

Takasugi, M., Yoshida, Y., Hara, E., & Ohtani, N. (2023a). The role of cellular senescence and SASP in tumour microenvironment. *The FEBS Journal*, *290*(5), 1348–1361. https://doi.org/10.1111/febs.16381

Takata, H., Masuda, Y., & Ohmido, N. (2023). CRISPR imaging reveals chromatin fluctuation at the centromere region related to cellular senescence. *Scientific Reports*, *13*(1), 14609. https://doi.org/10.1038/s41598-023-41770-6

Takizawa, Y., Ho, C.-H., Tachiwana, H., Matsunami, H., Kobayashi, W., Suzuki, M., Arimura, Y., Hori, T., Fukagawa, T., Ohi, M. D., Wolf, M., & Kurumizaka, H. (2020). Cryo-EM Structures of Centromeric Tri-nucleosomes Containing a Central CENP-A Nucleosome. *Structure*, *28*(1), 44-53.e4. https://doi.org/10.1016/j.str.2019.10.016

Tan, Q., Niu, K., Zhu, Y., Chen, Z., Li, Y., Li, M., Wei, D., Balajee, A. S., Fang, H., & Zhao, Y. (2021). RNF8 ubiquitinates RecQL4 and promotes its dissociation from DNA double strand breaks. *Oncogenesis*, *10*(3), 24. https://doi.org/10.1038/s41389-021-00315-0

Tanaka, Y. (2001). Crystal structure of the CENP-B protein-DNA complex: The DNA-binding domains of CENP-B induce kinks in the CENP-B box DNA. *The EMBO Journal*, *20*(23), 6612–6618. https://doi.org/10.1093/emboj/20.23.6612

Tanaka, Y., Tachiwana, H., Yoda, K., Masumoto, H., Okazaki, T., Kurumizaka, H., & Yokoyama, S. (2005). Human Centromere Protein B Induces Translational Positioning of Nucleosomes on α-Satellite Sequences. *Journal of Biological Chemistry*, *280*(50), 41609–41618. https://doi.org/10.1074/jbc.M509666200

Tardif, K. D., Rogers, A., Cassiano, J., Roth, B. L., Cimbora, D. M., McKinnon, R., Peterson, A., Douce, T. B., Robinson, R., Dorweiler, I., Davis, T., Hess, M. A., Ostanin, K., Papac, D. I., Baichwal, V., McAlexander, I., Willardsen, J. A., Saunders, M., Christophe, H., … Williams, B. L. (2011). Characterization of the Cellular and Antitumor Effects of MPI-0479605, a Small-Molecule Inhibitor of the Mitotic Kinase Mps1. *Molecular Cancer Therapeutics*, *10*(12), 2267–2275. https://doi.org/10.1158/1535-7163.MCT-11-0453

Tawaramoto, M. S., Park, S.-Y., Tanaka, Y., Nureki, O., Kurumizaka, H., & Yokoyama, S. (2003). Crystal Structure of the Human Centromere Protein B (CENP-B) Dimerization

Domain at 1.65-Å Resolution. *Journal of Biological Chemistry*, *278*(51), 51454–51461. https://doi.org/10.1074/jbc.M310388200

Thompson, T., Tovar, C., Yang, H., Carvajal, D., Vu, B. T., Xu, Q., Wahl, G. M., Heimbrook, D. C., & Vassilev, L. T. (2004). Phosphorylation of p53 on Key Serines Is Dispensable for Transcriptional Activation and Apoptosis. *Journal of Biological Chemistry*, *279*(51), 53015–53022. https://doi.org/10.1074/jbc.M410233200

Thornton, T. M., & Rincon, M. (2009). Non-Classical P38 Map Kinase Functions: Cell Cycle Checkpoints and Survival. *International Journal of Biological Sciences*, 44–52. https://doi.org/10.7150/ijbs.5.44

Toledo, L. I., Altmeyer, M., Rask, M.-B., Lukas, C., Larsen, D. H., Povlsen, L. K., Bekker-Jensen, S., Mailand, N., Bartek, J., & Lukas, J. (2013). ATR Prohibits Replication Catastrophe by Preventing Global Exhaustion of RPA. *Cell*, *155*(5), 1088–1103. https://doi.org/10.1016/j.cell.2013.10.043

Tóth, K. F., Knoch, T. A., Wachsmuth, M., Frank-Stöhr, M., Stöhr, M., Bacher, C. P., Müller, G., & Rippe, K. (2004). Trichostatin A-induced histone acetylation causes decondensation of interphase chromatin. *Journal of Cell Science*, *117*(18), 4277–4287. https://doi.org/10.1242/jcs.01293

Trakarnphornsombat, W., & Kimura, H. (2023a). Live-cell tracking of γ-H2AX kinetics reveals the distinct modes of ATM and DNA-PK in the immediate response to DNA damage. *Journal of Cell Science*, *136*(8), jcs260698. https://doi.org/10.1242/jcs.260698

Trimarchi, J. M., & Lees, J. A. (2002). Sibling rivalry in the E2F family. *Nature Reviews Molecular Cell Biology*, *3*(1), 11–20. https://doi.org/10.1038/nrm714

Trivedi, P., Palomba, F., Niedzialkowska, E., Digman, M. A., Gratton, E., & Stukenberg, P. T. (2019). The inner centromere is a biomolecular condensate scaffolded by the chromosomal passenger complex. *Nature Cell Biology*, *21*(9), 1127–1137. https://doi.org/10.1038/s41556-019-0376-4

Tsai, L. J., Lopezcolorado, F. W., Bhargava, R., Mendez-Dorantes, C., Jahanshir, E., & Stark, J. M. (2020). RNF8 has both KU-dependent and independent roles in chromosomal break repair. *Nucleic Acids Research*, *48*(11), 6032–6052. https://doi.org/10.1093/nar/gkaa380

Unterholzner, L., & Dunphy, G. (2019). cGAS-independent STING activation in response to DNA damage. *Molecular & Cellular Oncology*, *6*(4), 1558682. https://doi.org/10.1080/23723556.2018.1558682

Vukušić, K., & Tolić, I. M. (2021). Anaphase B: Long-standing models meet new concepts. *Seminars in Cell & Developmental Biology*, *117*, 127–139. https://doi.org/10.1016/j.semcdb.2021.03.023

Wang, R. W., Viganò, S., Ben-David, U., Amon, A., & Santaguida, S. (2021). Aneuploid senescent cells activate NF-κB to promote their immune clearance by NK cells. *EMBO Reports*, *22*(8), e52032. https://doi.org/10.15252/embr.202052032

Wang, Z. (2021). Regulation of Cell Cycle Progression by Growth Factor-Induced Cell Signaling. *Cells*, *10*(12), 3327. https://doi.org/10.3390/cells10123327

Weaver, I. C. G., Korgan, A. C., Lee, K., Wheeler, R. V., Hundert, A. S., & Goguen, D. (2017). Stress and the Emerging Roles of Chromatin Remodeling in Signal Integration and Stable Transmission of Reversible Phenotypes. *Frontiers in Behavioral Neuroscience*, *11*. https://doi.org/10.3389/fnbeh.2017.00041

Weinberg, R. A. (2013). *The Biology of Cancer* (0 ed.). W.W. Norton & Company. https://doi.org/10.1201/9780429258794

Wiley, C. D., & Campisi, J. (2016). From Ancient Pathways to Aging Cells—Connecting Metabolism and Cellular Senescence. *Cell Metabolism*, *23*(6), 1013–1021. https://doi.org/10.1016/j.cmet.2016.05.010

Wilkinson, G. S., Adams, D. M., Haghani, A., Lu, A. T., Zoller, J., Breeze, C. E., Arnold, B. D., Ball, H. C., Carter, G. G., Cooper, L. N., Dechmann, D. K. N., Devanna, P., Fasel, N. J., Galazyuk, A. V., Günther, L., Hurme, E., Jones, G., Knörnschild, M., Lattenkamp, E. Z., … Horvath, S. (2021). DNA methylation predicts age and provides insight into exceptional longevity of bats. *Nature Communications*, *12*(1), 1615. https://doi.org/10.1038/s41467-021-21900-2

Willard, H. F. (1985). Chromosome-specific organization of human alpha satellite DNA. *American Journal of Human Genetics*, *37*(3), 524–532.

Wilson, V. L., & Jones, P. A. (1983). DNA Methylation Decreases in Aging But Not in Immortal Cells. *Science*, *220*(4601), 1055–1057. https://doi.org/10.1126/science.6844925

Yamakoshi, K., Takahashi, A., Hirota, F., Nakayama, R., Ishimaru, N., Kubo, Y., Mann, D. J., Ohmura, M., Hirao, A., Saya, H., Arase, S., Hayashi, Y., Nakao, K., Matsumoto, M., Ohtani, N., & Hara, E. (2009). Real-time in vivo imaging of p16Ink4a reveals cross talk with p53. *Journal of Cell Biology*, *186*(3), 393–407. https://doi.org/10.1083/jcb.200904105

Yang, F., Teves, S. S., Kemp, C. J., & Henikoff, S. (2014). Doxorubicin, DNA torsion, and chromatin dynamics. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, *1845*(1), 84–89. https://doi.org/10.1016/j.bbcan.2013.12.002

Yang, G., Chen, Y., Wu, J., Chen, S.-H., Liu, X., Singh, A. K., & Yu, X. (2020). Poly(ADP-ribosyl)ation mediates early phase histone eviction at DNA lesions. *Nucleic Acids Research*, *48*(6), 3001–3013. https://doi.org/10.1093/nar/gkaa022

Yang, H., Wang, H., Ren, J., Chen, Q., & Chen, Z. J. (2017). cGAS is essential for cellular senescence. *Proceedings of the National Academy of Sciences*, *114*(23). https://doi.org/10.1073/pnas.1705499114

Yao, H., Wallace, J., Peterson, A. L., Scaffa, A., Rizal, S., Hegarty, K., Maeda, H., Chang, J. L., Oulhen, N., Kreiling, J. A., Huntington, K. E., De Paepe, M. E., Barbosa, G., & Dennery, P. A. (2023). Timing and cell specificity of senescence drives postnatal lung development and injury. *Nature Communications*, *14*(1), 273. https://doi.org/10.1038/s41467-023-35985-4

Yasmineh, W. G., & Yunis, J. J. (1974). Localization of repeated DNA sequences in CsCl gradients by hybridization with complementary RNA. *Experimental Cell Research*, *88*(2), 340–344. https://doi.org/10.1016/0014-4827(74)90249-3

Ye, X., Zerlanko, B., Kennedy, A., Banumathy, G., Zhang, R., & Adams, P. D. (2007). Downregulation of Wnt Signaling Is a Trigger for Formation of Facultative Heterochromatin and Onset of Cell Senescence in Primary Human Cells. *Molecular Cell*, *27*(2), 183–196. https://doi.org/10.1016/j.molcel.2007.05.034

Yoda, K., Ando, S., Okuda, A., Kikuchi, A., & Okazaki, T. (1998). *In vitro* assembly of the CENP-B/α-satellite DNA/core histone complex: CENP-B causes nucleosome positioning. *Genes to Cells*, *3*(8), 533–548. https://doi.org/10.1046/j.1365-2443.1998.00210.x

Young, A. R. J., Narita, M., Ferreira, M., Kirschner, K., Sadaie, M., Darot, J. F. J., Tavaré, S., Arakawa, S., Shimizu, S., Watt, F. M., & Narita, M. (2009). Autophagy mediates the mitotic senescence transition. *Genes & Development*, *23*(7), 798–803. https://doi.org/10.1101/gad.519709

Xu, D., Tian, Y., Xia, Q., & Ke, B. (2021). The cGAS-STING Pathway: Novel Perspectives in Liver Diseases. *Frontiers in Immunology*, *12*, 682736. https://doi.org/10.3389/fimmu.2021.682736

Zhang, D., Tang, Z., Huang, H., Zhou, G., Cui, C., Weng, Y., Liu, W., Kim, S., Lee, S., Perez-Neut, M., Ding, J., Czyz, D., Hu, R., Ye, Z., He, M., Zheng, Y. G., Shuman, H. A., Dai, L., Ren, B., … Zhao, Y. (2019). Metabolic regulation of gene expression by histone lactylation. *Nature*, *574*(7779), 575–580. https://doi.org/10.1038/s41586-019-1678-1

Zhang, H., & Cohen, S. N. (2004). Smurf2 up-regulation activates telomere-dependent senescence. *Genes & Development*, *18*(24), 3028–3040. https://doi.org/10.1101/gad.1253004

Zhang, R., Poustovoitov, M. V., Ye, X., Santos, H. A., Chen, W., Daganzo, S. M., Erzberger, J. P., Serebriiskii, I. G., Canutescu, A. A., Dunbrack, R. L., Pehrson, J. R., Berger, J. M., Kaufman, P. D., & Adams, P. D. (2005). Formation of MacroH2A-Containing Senescence-Associated Heterochromatin Foci and Senescence Driven by ASF1a and HIRA. *Developmental Cell*, *8*(1), 19–30. https://doi.org/10.1016/j.devcel.2004.10.019

Zhao, F., Kim, W., Kloeber, J. A., & Lou, Z. (2020). DNA end resection and its role in DNA replication and DSB repair choice in mammalian cells. *Experimental & Molecular Medicine*, *52*(10), 1705–1714. https://doi.org/10.1038/s12276-020-00519-1

Zhao, S., Allis, C. D., & Wang, G. G. (2021). The language of chromatin modification in human cancers. *Nature Reviews Cancer*, *21*(7), 413–430. https://doi.org/10.1038/s41568-021-00357-x

Zheng, C., & Hayes, J. J. (2003). Structures and interactions of the core histone tail domains. *Biopolymers*, *68*(4), 539–546. https://doi.org/10.1002/bip.10303

Zhong, L., Hu, M.-M., Bian, L.-J., Liu, Y., Chen, Q., & Shu, H.-B. (2020). Phosphorylation of cGAS by CDK1 impairs self-DNA sensing in mitosis. *Cell Discovery*, *6*(1), 26. https://doi.org/10.1038/s41421-020-0162-2

Zhou, W., Mohr, L., Maciejowski, J., & Kranzusch, P. J. (2021). cGAS phase separation inhibits TREX1-mediated DNA degradation and enhances cytosolic DNA sensing. *Molecular Cell*, *81*(4), 739-755.e7. https://doi.org/10.1016/j.molcel.2021.01.024

Zhu, H., Blake, S., Kusuma, F. K., Pearson, R. B., Kang, J., & Chan, K. T. (2020). Oncogene-induced senescence: From biology to therapy. *Mechanisms of Ageing and Development*, *187*, 111229. https://doi.org/10.1016/j.mad.2020.111229

RÉSUMÉ

Les stress cellulaires provoquent la sénescence, une sortie irréversible du cycle cellulaire, qui favorise à son tour la réponse inflammatoire. L'un des premiers événements de la sénescence est la formation d'une distension de l'ADN satellite associée à la sénescence (SADS), au cours de laquelle l'ADN satellite se déploie massivement. L'origine de la SADS et son rôle dans la promotion de la sénescence sont inconnus. Pour répondre à ces questions, j'ai étudié un type d'ADN satellite appelé « α-satellite », présent au niveau des centromères, des loci chromosomiques essentiels à la ségrégation précise des chromatides sœurs. Ici, l'ADN α-satellite se replie en structures secondaires uniques qui jouent un rôle dans la fonctionnalité des centromères. En étudiant les mécanismes de formation du SADS et leurs conséquences, j'ai découvert comment les protéines centromériques influencent la formation du SADS et ont un impact sur le phénotype de sénescence en aval. De plus, j'ai découvert que le SADS peut se produire par l'activation de p53, mais que son rôle est dispensable lors de dommages à l'ADN. La décompactation centromérique est également influencée par des changements épigénétiques tels que les modifications des histones et l'abondance des histones, qui affectent des processus tels que la transcription centromérique. J'ai approfondi mes recherches sur le lien entre les SADS et l'immunité innée en étudiant le rôle du cGAS, un activateur de l'immunité innée, pendant la sénescence. Ces travaux, qui mettent en évidence la dynamique des centromères au début de la sénescence, permettront de mieux comprendre la progression de conditions physiologiques et pathologiques telles que le vieillissement et le cancer.

MOTS CLÉS

Sénescence, centromère, dommages à l'ADN, épigénétique, immunité innée

ABSTRACT

Cellular stresses cause senescence, an irreversible exit from the cell cycle, in turn promoting inflammatory response. An early event in senescence is the formation of Senescence Associated Distension of Satellite DNA (SADS) where satellite DNA massively unfold. The origin of SADS and their function in promoting senescence is unknown. To answer these, I have studied a type of satellite DNA called α-satellite, that is present at centromeres, chromosomal loci essential to mediate accurate sister chromatids segregation. Here α-satellite DNA folds into unique secondary structures playing a role in centromere functionality. By investigating the mechanisms of SADS formation and their consequences, I have discovered how centromeric proteins influence SADS formation and impact downstream senescence phenotype. Additionally, I found that SADS can occur through p53 activation, but its role is dispensible during DNA damage. Centromere decompaction is further influenced by epigenetic changes like histone modifications and histone abundancy that affect processes like centromeric transcription. I have further explored how SADS is linked to innate immunity by investigating the role of cGAS, an innate immune activator, during senescence. This work highlighting centromere dynamics during early senescence will enhance our understanding of the progression of physio and pathological conditions like aging and cancer.

KEYWORDS

Senescence, centromere, DNA damage, epigenetics, innate immunity