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Replicative senescence-associated oxidative stress and Hog1 activation in telomerase negative *Saccharomyces cerevisiae* cells

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Équipe Biologie des télomères dirigée par Dr. Teixeira Teresa

Replicative senescence-associated oxidative stress and Hog1 activation in telomerase negative *Saccharomyces cerevisiae* cells

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To my Inner Child

To my Family

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I thought that this part was the easiest to write but turned out it was the most difficult!

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Abstract

Replicative senescence, the process by which cells cease to divide after a certain number of divisions, is triggered by a DNA damage response, resulting from telomeres shortening during each cellular division. Data obtained in different organisms show that mitochondrial dysfunctions and oxidative stress are features of replicative senescence. Yet, the production and regulation of reactive oxygen species (ROS) levels during replicative senescence in budding yeast are still elusive. The aim of my PhD was to quantify ROS levels during replicative senescence and delineate the pathways linking telomerase inactivation with changes in oxidative stress levels using *Saccharomyces cerevisiae* as a model organism. I demonstrated that oxidative stress increases during replicative senescence in budding yeast. The ortholog of the MAPK p38, Hog1, becomes activated during replicative senescence through its canonical MAPKK Pbs2, and likely contributes to ROS counteraction. My results suggest that Hog1's action is independent of Mec1, the major DNA damage checkpoint protein in budding yeast, which also likely counteracts the increase in ROS. Hog1 also likely functions independently of the telomeric long non-coding RNA TERRA. Moreover, I established the role of Hog1 in maintaining telomere length homeostasis and showed that the absence of Hog1 results in a marked cell mortality even in unchallenged conditions. Furthermore, my research revealed that autophagic processes do not impact replicative senescence onset in budding yeast. Collectively, data obtained during my thesis highlight that oxidative stress is a hallmark of replicative senescence in budding yeast and that Hog1 regulates telomeres homeostasis contributing to the link between telomeres and the ROS metabolism.

Keywords: Replicative Senescence, Oxidative Stress, Telomeres, Hog1, *Saccharomyces cerevisiae*

Résumé

La sénescence réplivative, le processus par lequel les cellules cessent de se diviser après un certain nombre de divisions, est déclenchée par une réponse aux dommages de l'ADN, qui résulte du raccourcissement des télomères au cours de chaque division cellulaire. Les données obtenues chez différents organismes montrent que les dysfonctionnements mitochondriaux et le stress oxydant sont des caractéristiques de la sénescence réplivative. Cependant, la production et la régulation des niveaux d'espèces réactives de l'oxygène (ROS) au cours de la sénescence réplivative chez la levure restent énigmatiques. L'objectif de ma thèse a été de quantifier les niveaux de ROS pendant la sénescence réplivative et de délimiter les voies reliant l'inactivation de la télomérase aux niveaux de ROS en utilisant *Saccharomyces cerevisiae* comme organisme modèle. J'ai démontré que le stress oxydant augmente pendant la sénescence réplivative chez *Saccharomyces cerevisiae*. L'orthologue de la MAPK p38, Hog1, s'active au cours de la sénescence réplivative par le biais de sa MAPKK canonique, Pbs2 et contribue probablement à la détoxification des ROS. Mes résultats suggèrent que l'action de Hog1 est indépendante de Mec1, qui probablement participe aussi à la détoxification des ROS. Hog1 semble aussi fonctionner indépendamment du long ARN non codant TERRA. De plus, j'ai établi un rôle indispensable de Hog1 dans le maintien de l'homéostasie de la taille des télomères et montré que la délétion de Hog1 entraîne une mortalité cellulaire marquée même en conditions de croissances normales. Mes recherches ont aussi révélé que les processus autophagiques n'ont pas d'impact sur l'établissement de la sénescence réplivative chez *S. cerevisiae*. Dans l'ensemble, les données obtenues au cours de ma thèse mettent en évidence que le stress oxydant est une caractéristique de la sénescence réplivative chez *Saccharomyces cerevisiae* et que Hog1 régule l'homéostasie des télomères contribuant au lien entre les télomères et le métabolisme des ROS.

Mots clés : Sénescence réplivative, Stress oxydant, Télomères, Hog1, *Saccharomyces cerevisiae*

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Abbreviations

A

A: Adenine

ARS: Autonomously Replicated Sequence

ALT: Alternative Lengthening of Telomeres

ATR: Ataxia-Telangiectasia and Rad3 related

ATM: Ataxia-Telangiectasia Mutated

ATP: Adenosine TriPhosphate

ANT: Adenylic Nucleotide Transporter

AHP1: Alkyl HydroPeroxide reductase

AP-1: Activator Protein 1

ATG8/32: AuTophagy related 8/32

B

B: Base

Bp: Base Pair

BIR: Break Induced Replication

BER: Base Excision Repair

bZIP: Basic leucine ZIPper

C

C: Cytosine

CDC13/28/42: Cell Division Cycle 13/28/42

CAT: CATalase

CoQ: COenzyme Q

Cytc: CYTochrome C

CII/III/IV: Complex II/III/IV

Cu: Copper

CTT1: CaTalase T 1

CTA1: CaTalase A 1
CRM1: Chromosome Region Maintenance 1
CD: Common Docking domain
CLA4: CLn Activity dependent 4
CREs: cAMP Response Elements
ChIP: Chromatin IMmunoprecipitation

D

DNA: DesoxyriboNucleic Acid
DSBs: Double Strand Breaks
DLD1: D-Lactate Dehydrogenase 1
DOT5: Disruptor Of Telomeric silencing 5
DUN1: DNA-damage UNinducible 1
Da: DAAlton
dNTPs: DeoxyNucleotide Triphosphates

E

EST1/2/3: Ever Shorter Telomeres 1/2/3
EXO1: EXOnuclease 1
ETC: Electron Transport Chain
ER: Endoplasmic Reticulum

F

FAD: Flavin Adenine Dinucleotide
Fe: Iron

G

G: Guanine
GUT2: Glycerol UTilization 2

GPx1/2/3: Glutathione Peroxidase 1/2/3
Grx1/2: Glutaredoxins 1/2
GTT1: GlutaThione Transferase 1
GSH: Glutathione
GPD1/2: Glycerol-3-Phosphate Dehydrogenase 1/2
GRE2 : Genes de Respuesta a Estres 2
GLR1: GLutathione Reductase 1

H

HOG1: High Osmolarity Glycerol 1
HR: Homologous Recombination
hTERT: Human Telomerase Reverse Transcriptase
hTR: Human Telomerase RNA
H₂O₂: Hydrogen peroxide
HSP: Heat Shock Protein
H: Hydrogen
HSF1: Heat Shock transcription Factor 1
HAP1/2/3/4/5: Heme Activator Protein 1/2/3/4/5
HRK1: Hygromycin Resistance Kinase 1
HOT1: High Osmolarity-induced Transcription 1
HoREs: Hog1 Responsive Elements

I

IMM: Inner Mitochondrial Membrane
IMS: Intermembrane Mitochondrial Space
ISC1: Inositol phosphoSphingolipid phospholipase C 1

L

LPS: Leptospiral lipopolysaccharide

M

MEC1: Mitosis Entry Checkpoint 1
MRE11: Meiotic REcombination 11
MRC1: Mediation of the Replication Checkpoint 1
mtDNA: Mitochondrial DNA
Mn: Manganese
MTH1: Human MutT homolog 1
MAPK: Mitogen Activated Protein Kinase
MAPKK: Mitogen Activated Protein Kinase Kinase
MAPKKK: Mitogen Activated Protein Kinase Kinase Kinase
MSB2: Multicopy Suppression of a Budding Effect
MSN2/4: Multicopy Suppressor of SNF1 mutation
MMS: Methyl Methane Sulfonate

N

NAD: Nicotinamide Adenine Dinucleotide
NHEJ: Non homologous End Joining
NOX: NADPH Oxidase
NES: Nuclear Export Signal
NDE1/2: NADH Dehydrogenase External 1/2
NDI1: NADH Dehydrogenase Internal 1
NBP2: Nap1 Binding Protein 2
NMD5: Nonsense-Mediated mRNA Decay 5
NUP1/2/60: NUclear Pore 1/2/20

O

O₂: Dioxygen
O₂^{•-}: Superoxide anion
OH[•]: Hydroxyl radical
OMM: Outer Mitochondrial Membrane

OXPHOS: OXidative PHOSphorylation

OXR: OXidative stress Response

OPY2: Overproduction-induced Pheromone-resistant Yeast 2

OE: Over Expression

P

PIF1: Petite Integration Frequency 1

P16/38/53: tumor Protein 16/38/53

PRx: Peroxiredoxins

Pi: Phosphate

PiC: PI Carrier

PSE1: Protein Secretion Enhancer 1

PRDX1: Peroxiredoxin 1

PBD-2: PBS2 Binding Domain

PBS2: Polymyxin B Sensitivity 2

PTP1/2/3: PhosphoTyrosine-specific Phosphatase 1/2/3

PTC1/2/3/4: Phosphatase type Two C 1/2/3/4

PKA: Protein Kinase A

PCD: Programmed Cell Death

PNC1: Pyrazinamidase and NiCotinamidase 1

PGS1: PhosphatidylGlycerolphosphate Synthase

R

ROS: Reactive Oxygen Species

RAP1: Repressor Activator site binding Protein 1

RIF1/2: Rap1 Interacting Factor 1/2

RNA: RiboNucleic Acid

RAD9/52/53: RADiation sensitive 9/52/53

RRMR3: rDNA Recombination Mutation

RO₂[•]: peroxy radicals

RO₂H: Hydroperoxides

RO[•]: alkoxy radical

RSC: Chromatin Structure Remodeling

RCK2: Radiation Sensitivity Complementing Kinase

Rt-qPCR: Reverse Transcription – Quantitative Polymerase Chain Reaction

S

S. cerevisiae: *Saccharomyces Cerevisiae*

SIR2/3/4: Silent Information Regulator 2/3/4

STN1: Suppressor of cdc Thirteen

STR: Subtelomeric Repeated Element

SGS1: Slow Growth Suppressor 1

SAE2: Sporulation in the Absence of spo Eleven

Sa-β-gal: Senescence Associated Beta-Galactosidase

SASP: Senescence Associated Secretory Phenotype

SOD: SuperOxide Dismutase

SKN7: Suppressor of Kre Null 7

SHO1: Synthetic high osmolarity glycerol 1

SLN1: Synthetic Lethal of N-end rule

SSK1/2/22: Suppressor of Sensor Kinase 1/2/22

STE11/20/50: STerile 11/20/50

SAM: Sterile Alpha Motifs

SAGA: Apt-Ada-Gnc5-Acetyltransferase

SPT4: Suppressor of Ty's 4

SKO1: Suppressor of Kinase Overexpression 1

SMP1: Second MEF2-like Protein 1

STREs: Stress Transcription Response Elements

SSN6: Suppressor of SNf1 6

STL1: Sugar Transporter Like protein 1

SFA1: Sensitive to FormAldehyde

SCF: Skp, Cullin, F-box

Ser: Serine

T

TPE: Telomere Position Effect

T: Thymine

TEN1: TELomeric pathway with STn1

TLC1: TeLomerase Component 1

TERRA: TELomeric Repeats containing RNA

TEL1: TELomere maintenance 1

TFs: Transcription Factors

TRX: Thioredoxins

TCA: TriCarboxylic Acid

TSA1: Thiol-Specific Antioxidant 1

TRF1/2: Telomeric Repeat binding Factor 1/2

TDR: Telomerase Deletion Response

Thr: Threonine

Tyr: Tyrosine

TM: TransMembrane

Tup1: dTMP-UPtake

TeloPCR: Telomere Polymerase Chain Reaction

U

U: Uracil

UV: UltraViolet

UB14: UBiquitin 14

W

WT: Wild Type

X

Xrs2: X-Ray Sensitive 2

Y

Yku 70/80 : Yeast KU protein 70/80

Yno1: Yeast NADPH Oxidase 1

YREs: YAP1 Response Elements

YAP1: Yeast AP-1

Ypd1: tYrosine Phosphatase Dependent 1

Z

Zn: Zinc

8

8-oxoG: 8-oxoGuanine

Introduction

In many Eukaryotes, cells, the fundamental building blocks of living organisms, have a limited capacity to divide, leading to aging and diseases, ultimately culminating in death. This limited capacity of proliferation is known as replicative senescence, wherein cells undergo irreversible growth arrest after a certain number of divisions. Replicative senescence is observed in somatic cells of humans for instance and is considered as a protective mechanism against uncontrolled cell proliferation, and tumorigenesis. The erosion of telomeres, which are protective caps at the end of eukaryotic chromosomes, has long been associated with replicative senescence. With each cell division, telomeres experience incomplete replication and shorten until they reach a critical length, signalling replicative senescence. The vast heterogeneity of replicative senescence emphasizes the importance of various cellular and molecular changes associated with this process. These changes include alterations in mitochondrial functions, increased production of reactive oxygen species (ROS), and the activation of specific signalling pathways. To bypass replicative senescence, germs cells or cancerous cells express telomerase, an enzyme that maintains telomere length. In contrast, some unicellular organisms, like budding yeast, rely on telomerase for their long-term viability. Experimental removal of telomerase in yeast leads to replicative senescence, similar to mammalian cells. There is an evolutionary conservation in the initial response to short telomeres triggering replicative senescence, as well as in the maintenance of other essential telomeric functions between budding yeast and mammalian cells. Despite these similarities, there is a lack of data regarding ROS levels during replicative senescence in *Saccharomyces cerevisiae*. During my thesis I was interested in studying ROS levels during replicative senescence in budding yeast and investigating potential pathways involved in ROS regulation during this process.

In this introduction, I will begin by introducing telomeres and their significance in maintaining chromosome ends. Next, I delve into the concept of replicative senescence, which arises from telomere attrition, and explore its remarkable heterogeneity. The involvement of mitochondrial dysfunction and oxidative stress is central to the understanding of replicative senescence. The third chapter encompasses the production and detoxification of ROS within the cell, along with research highlighting the increase in mitochondrial dysfunction and oxidative stress during senescence. Finally, I will discuss the high osmolarity glycerol 1 (Hog1) signalling pathway and its implication in ROS related mechanisms.

I- Chapter I: Telomeres

In eukaryotes, linear chromosomes, start and end with special nucleoproteic structures called telomeres, crucial for maintaining genome integrity and protecting essential genes (Zakian, 2012). Telomeres were discovered by the work of Muller and McClintock in the late 1930's even prior to the discovery of the Watson-Crick double helical structure of DNA which recognized DNA as the carrier of the genetic information. Muller and McClintock observed that the terminal parts of the chromosomes remain stable and do not undergo rearrangements or fusions after X-ray irradiation, unlike the internal broken end's regions of chromosome. Consequently, these ends were named telomeres (Terminal (*telos*) ends (*meros*)) and their primary function is to shield the chromosomes from fusions and degradations.

Yeast's ease of cultivation, powerful genetics and genomics techniques (genome size approx. 12,1 mega base), have positioned it at the forefront of telomere research. *Saccharomyces cerevisiae*, which serves as our model organism, has been extensively used to unravel numerous telomeric mechanisms (Wellinger & Zakian, 2012). In this chapter, I will provide an overview of telomeres structure, their indispensable role in chromosome function, and the maintenance mechanisms, with a particular focus on budding yeast.

1. Telomere functions

The structure of telomeres resembles the most hazardous DNA damaging events: double strand breaks (DSBs) (Zakian, 2012). Cells possess the ability to differentiate between the natural ends of chromosomes and the internal ends caused by DSBs. Telomeres play a crucial role in this process, safeguarding chromosome ends from checkpoint activation, fusions, and degradation. This protective function of telomeres is commonly referred to as the "capping function." Mechanistically, the capping function inhibits the DNA repair by homologous recombination (HR) or non-homologous end joining (NHEJ). This function enables the maintenance of genome integrity and the full replication of chromosomes to prevent the loss of vital genes necessary for cellular functioning. In addition, in budding yeast, the genes near telomeric ends are low expressed or silenced, a transcription regulation function known as telomere position effect (TPE) (Zakian, 2012). Telomeres are also essential for proper mitotic and meiotic divisions, chromosome positioning and dynamic, cell survival and proliferation (Teixeira & Gilson, 2005).

2. Telomere structure

2.1. Telomeric DNA

Telomeres are structures made up of a simple non-coding pattern of DNA repeated in tandem. The length, composition and number of these repeated sequences vary across species ranging from several tens of base pairs to several kilobases (Zakian, 2012). In addition, the number of repeats vary not only across species but also across individuals, cells, and even between chromosomes of the same cell. Mammalian telomeres are composed of TTAGGGn DNA tandem repeats and exhibit considerable variability in size, spanning several kilobases (Moyzis *et al*, 1988). *S. cerevisiae* telomeres are degenerated in nature, histone free and consist of 300 +/- 75 base pairs of C₁₋₃A/TG₁₋₃ repeats (Wellinger & Zakian, 2012) (Figure 1). Thus, one of the strands is designated as the G rich strand and the complementary as the C rich strand. In many organisms, telomere ends are not blunt: the G rich strand forms a 3' single strand overhang. In *S. cerevisiae* this overhang has a length between 5 to 10 nucleotides but varies throughout the cell cycle reaching approx. 40 nucleotides at the late S/G₂ phase (Soudet *et al*, 2014).

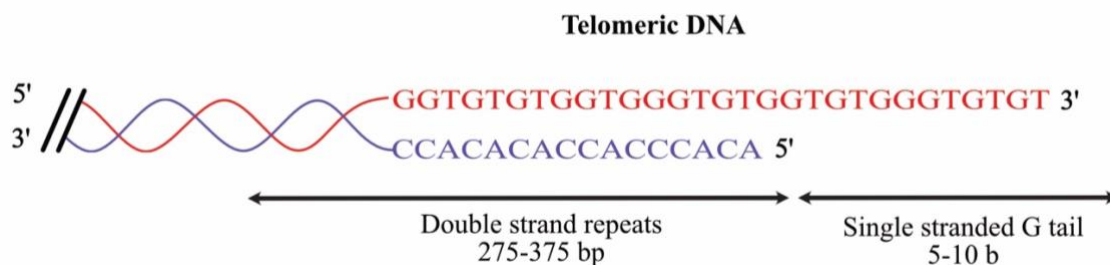


Figure 1 : Telomeric DNA in budding yeast.

Schematic representation of telomeric DNA in budding yeast. Telomeres consist of 300 +/- 75 base pairs of C₁₋₃A/TG₁₋₃ double stranded degenerated repeats. The G rich strand ends with a 3' single strand tail of 5 to 10 bases. In blue the C rich strand and in red the G rich strand. The bases represented are an example and vary between telomeres. Adapted from (Wellinger & Zakian, 2012).

2.2. Telomeric proteins and their functions

Telomeres are associated with a distinct set of proteins, the telosome, collectively referred to as the shelterin complex in mammalian cells (de Lange, 2018). These proteins work together to maintain telomere structure and regulate telomere length. Some of these proteins have functions beyond telomeres and can be found elsewhere in the genome. These crucial players

can be classified based on the regions of the telomere to which they bind, namely the double-stranded part and the 3' G tail (*Figure 2*).

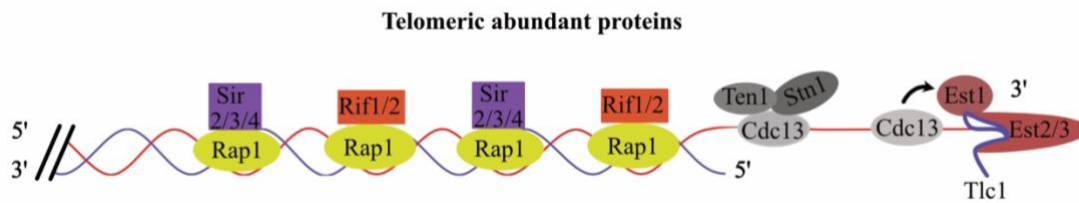


Figure 2 : Abundant proteins at budding yeast telomeres.

Rap1 binds double stranded telomeric DNA and recruits the Sir complex (Sir2,3,4) and Rif1/Rif2. The Yku complex is also found at telomeres possibly at the junction between the single stranded and the double stranded DNA but is not represented in this figure. Cdc13 binds the 3' overhang and composes the CST complex with Ten1 and Stn1. Cdc13 also interacts with Est1, to recruit to telomeres the other components of telomerase: Est2, Est3 and Tlc1. Adapted from (Pfeiffer & Lingner, 2013).

2.2.1. Double-stranded DNA binding proteins

Rap1, also known as Repressor Activator Protein 1, is a protein that plays a role in both repressing and activating gene expression (Shore & Nasmyth, 1987). It's the only protein conserved from the mammalian shelterin complex, its abundance at telomeres is directly related to telomeric length and it has binding sites distributed throughout the repetitive sequences of telomeres, occurring every 20 base pairs (Gilson *et al*, 1993). However, Rap1 is not exclusively associated with telomeres but can also be found within internal regions of the chromosome. It functions in various aspects of telomere biology, including telomere capping, telomere silencing, and regulation of telomere length (Marcand *et al*, 1997; Lustig *et al*, 1990). Rap1 plays a crucial role in interacting with and recruiting other telomeric proteins, such as Rif1/Rif2 and the Sir complex (Wellinger & Zakian, 2012). Rif1/Rif2 contribute to telomeres length regulation and to telomere capping. On the other hand, the Sir complex consists of Sir2, Sir3, and Sir4, where Sir3/4 interacts with Sir2, an NAD⁺ dependent histone deacetylase, to repress transcription. The Sir proteins are significant players in the TPE.

The Yku complex, comprising Yku70 and Yku80, is present at telomeres, despite its primary role in the NHEJ mechanism, which is somewhat paradoxical (Boulton & Jackson, 1998; Gravel *et al*, 1998). This complex plays critical functions at telomeres, including the regulation of telomere length, telomere capping, TPE, and the folding of telomeres.

2.2.2. 3'G tail binding proteins

The CST complex, which binds to the 3' overhang of telomeres, is composed of Cdc13, Stn1, and Ten1 (Gao *et al*, 2007). This complex plays a crucial role in safeguarding the C-strand of telomeres from 5' to 3' resection and also regulates the length of telomeres through the interaction with telomerase subunits.

2.3. Telomere high structural organization

In mammals, telomeres can fold into a structure known as t-loops (Griffith *et al*, 1999). These t-loops form when the single-stranded G tails invade the duplex region of the telomere. Stabilization of t-loops relies on DNA base pairing and requires specific telomeric proteins (Doksani *et al*, 2013). T-loops haven't been found at budding yeast telomeres. Instead, telomere folding in budding yeast has been proposed, and it is maintained through protein interactions involving the Yku complex (Strahl-Bolsinger *et al*, 1997; Marvin *et al*, 2009). Telomere folding in yeast is believed to be involved in TPE and in protecting the telomeric ends from recombination.

Another structure suspected to be present at telomeres are G4 quadruplexes which are formed when guanine-rich DNA sequences fold into a unique four-stranded structure (Venczel & Sen, 1993). However, the precise function of G4 quadruplexes at telomeres is still not fully understood.

3. Subtelomeres

Subtelomeres refer to the genomic regions located adjacent to the telomeres, positioned upstream in the chromosome (Figure 3). In budding yeast, these regions are characterized by their long length compared to telomeres and consist of heterogeneous and variable repeated elements, which are known to undergo rapid evolution. Unlike the core genome, subtelomeres do not typically contain genes that are essential for the survival of cells under optimal growth conditions. However, their remarkable plasticity enables yeast to adapt swiftly to diverse stress conditions (Brown *et al*, 2010; Louis, 1995; Bergström *et al*, 2014). This adaptability is facilitated by the presence of multigene families within the subtelomeres, which encode

functions associated with interactions with the environment. *S. cerevisiae* subtelomeric regions contain two classes of repeated elements, the Y' elements and the X elements and are covered by nucleosomes, which contribute to their structural organization (Pasquier & Wellinger, 2020). The Sir complex, is essential to compact subtelomeres and to facilitate gene silencing within these regions (Hocher & Taddei, 2020). Y' elements exist in two distinct sizes and are present in approximately half of the telomeres in budding yeast. These elements located upstream of the telomeric repeats, can be arranged in tandem repeats. In contrast, X elements are present at all telomeres and exhibit higher heterogeneity compared to Y' elements. If Y' elements are present on the same telomere, X elements juxtapose Y' elements. Autonomously replicating sequences (ARS) elements are present in Y' and X elements. Furthermore, subtelomeres contain specific binding sites for many transcription factors (TFs), especially at X elements, which can vary from one telomere to another (Wellinger & Zakian, 2012). A study has shown that 11% of *S. cerevisiae* TFs (Msn4, Phd1, Pdr1, Rox1, Yap5, Yap6, Xbp1, Mig1, Dal80, Gzf3, Nrg1, Uga3, Gal4, Mal33, Hap4, Atf2, Cup9, Dat1, Gat3, Rgm1, Yjl206cp, Ypr196wp) preferentially bind at subtelomeric genes and can interact with known telomeric proteins (Mak *et al.*, 2009). The binding of a subset of these 22 TFs is dynamic, depending on growth conditions and exposure to stress (such as rapamycin, butanol, and hydrogen peroxide), and correlates with gene expression. TFs also bind to distinct classes of subtelomeric genes (Mak *et al.*, 2009). Additionally, it has been shown that upon stress or metabolic change, some of these transcription factors can regulate TPE (Smith *et al.*, 2011). Other TFs, like Reb1, Tbf1, and Abf1, can recognize specific sites in subtelomeric sequences (Wellinger & Zakian, 2012). Reb1, also seems to affect negatively the transcription of the telomeric LncRNA TERRA (*see below*) (Bauer *et al.*, 2022).

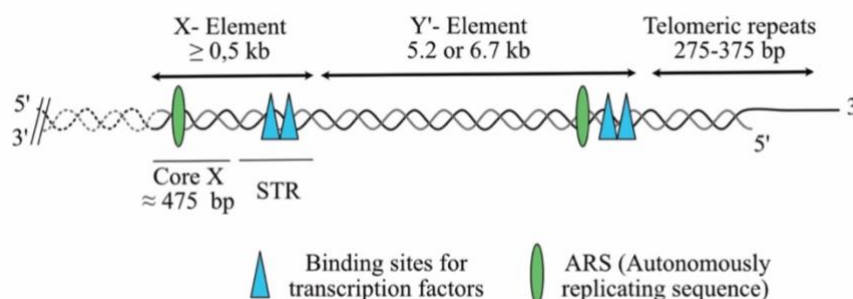


Figure 3 : Subtelomeric regions in budding yeast.

Schematic representation of subtelomeric X and Y' elements as well as the repeated terminal sequences. Telomere-proximal, Y' elements are

present in approx. half of telomeres in two possible sizes. Centromere-proximal, X elements contain the core X and the STR (subtelomeric repeated element). Binding sites for transcription factors and ARS are found in subtelomeric regions. Adapted from (Wellinger & Zakian, 2012).

4. Telomeric transcripts

Telomeres harbour epigenetic characteristics of heterochromatin. In budding yeast, while telomeric repeats themselves are free of histones, however the presence of the Sir complex at subtelomeres is associated with hypo-acetylation of histones H3 and H4 (Braunstein *et al*, 1996) and hypo-methylation of H3K4 (Santos-Rosa *et al*, 2004). Because of this heterochromatic signature, telomeres were previously believed to be transcriptionally silent, until the discovery of telomeric transcripts (Azzalin *et al*, 2007; Luke *et al*, 2008; Zeinoun *et al*, 2023). The transcription of telomeres is observed throughout the eukaryotic evolution. Several non-coding RNAs transcribed from regions near the chromosomal tip include ARRET, alphaARRET, subTERRA, and TERRA. Among these, TERRA has been the most studied. TERRA is transcribed from various subtelomeric regions adjacent to the terminal repeats of telomeres, resulting in an RNA population of heterogeneous size. It plays a crucial role in maintaining telomere homeostasis, as changes in TERRA expression can influence telomere length, interfere with the replicative machinery, and potentially lead to telomere dysfunction. Notably, TERRA has the ability to form R-loops, which are RNA-DNA hybrid structures associated with replicative senescence and ALT mechanisms. Recent studies have even suggested a potential link between TERRA and oxidative stress (Galigniana *et al*, 2020; Perez-Romero *et al*, 2018). Considering that both replicative senescence and oxidative stress are central themes in my thesis, we recently authored a review focusing on TERRA and its significance in preserving telomeres in budding yeast. Therefore, I will not further detail this section neither the role of TERRA in many facets of telomeres homeostasis (*Refer to the review attached in the annex section*).

5. Maintenance of telomeres

5.1. Telomere replication

Telomere replication takes place near the end of the S phase by the conventional semi-conservative DNA replication machinery (Pfeiffer & Lingner, 2013). Replicating telomeres poses unique challenges. The replication fork encounters difficulties at telomeric ends due to their repeated GC-rich sequences and the specific chromatin structure they adopt such as G-quadruplex. This contributes to a slowing down of the replication process (Lopes *et al*, 2011; Sfeir *et al*, 2009). In addition, telomeric transcription can generate conflicts between the

replication and transcription machineries and, special helicases are needed to facilitate telomere replication. In budding yeast, Rrmr3, a 5'3' helicase, has been shown to avoid fork stalling at telomeres during replication (Ivessa *et al*, 2002; Makovets *et al*, 2004). Furthermore, the DNA helicase Pif1 is important for the replication of G rich sequences which are also found at telomeres (Paeschke *et al*, 2011). An *In vitro* study has shown that while telomeric sequences *per se* do not inhibit the replication fork, Rap1, found throughout telomeric sequences inhibits many stages of DNA replication which can be partially resolved by the helicase Pif1 (Douglas & Diffley, 2021).

During semi-conservative replication, telomeres are also replicated by the leading and lagging strand synthesis machinery. The C-strand serves as the leading template, while the G-strand acts as the lagging template (Figure 4). Linear DNA poses a challenge for complete replication due to the unidirectional nature of DNA polymerases catalysis, which can only synthesize DNA in a 5' to 3' direction. Moreover, these replisome polymerases are unable to start *de novo* DNA synthesis and rely on RNA primers to initiate DNA synthesis. This creates a problem for the lagging strand synthesis during replication. When the primer of the last Okazaki fragment is removed at the end of the lagging strand, it is expected to leave a gap that cannot be filled by polymerases and thus a 3' overhang is restored. On the other hand, even if the 5' leading strand is fully replicated, resulting in a blunt end, its C-strand undergoes degradation and C-strand synthesis in a manner thought to be similar to the lagging strand synthesis, so that a 3' single-stranded overhang after each round of replication is regenerated also at the leading strand (Soudet *et al*, 2014; Cai & de Lange, 2023). Nevertheless, because at the leading strand template, the C strand is shorter than at the lagging strand template, the newly synthesized leading telomere is shorter than the parental telomere. This phenomenon is known as the end replication problem.

The regeneration of the 3' overhang is crucial for telomerase action (*discussed later*) and for the binding of telomeric proteins that confers telomeres protection against degradation and fusions. This cell cycle controlled degradation, known as the C-strand resection, occurs during the late S/G₂ phase and is intricately linked to telomeric replication (Dionne & Wellinger, 1998). Resection is facilitated by the Mre11-Rad50-Xrs2 (MRX) complex, the DNA helicase Sgs1 and the endonuclease Sae2 (Bonetti *et al*, 2009). Resection initiation shares striking similarities with the DSB response where Tel1 and MRX associate with telomeres at the time they replicate. However, only DSBs result in the activation of the DNA damage checkpoint

through the activation of Mec1 to stop cell division in order to repair the DNA damages before the cell proceeds in the next phase of the cell cycle (Melo & Toczyski, 2002; McGee *et al*, 2010).

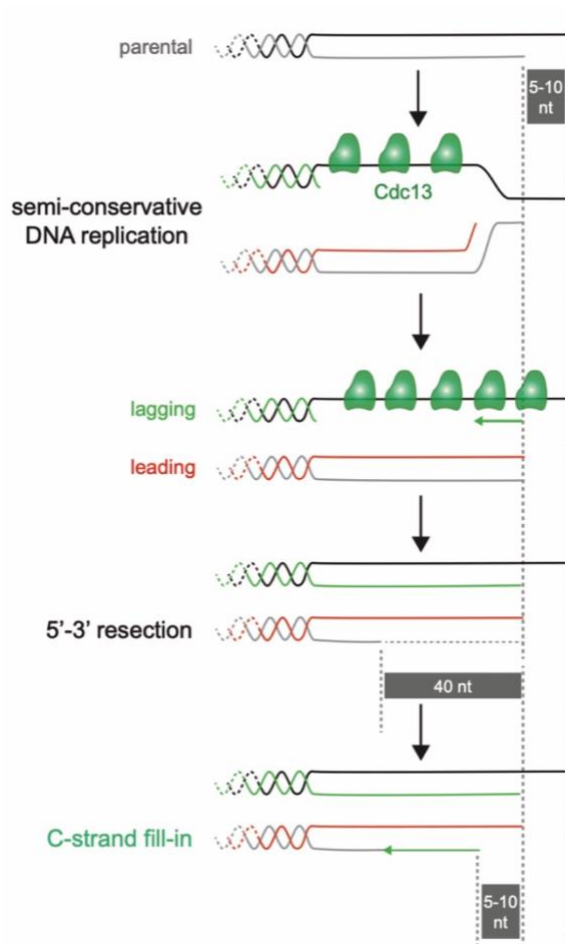


Figure 4 : Telomeres shortening after each round of replication in budding yeast.

*Telomeres shortening is the result of the DNA end replication problem due to the presence of a G overhang. The removal of the primer of the last Okazaki fragment is expected to leave a gap at the end of the lagging strand. The complete replication of the leading strand results in a blunt end which is resected resulting in a 3' overhang essential for telomeres functions. Adapted from (Soudet *et al*, 2014).*

5.2. Telomerase

In budding yeast, the average length of telomeres is maintained at approximately 300 bp through the activity of telomerase. Telomerase is a specialized enzyme with a reverse transcriptase activity that adds *de novo* DNA sequences to telomeres (Lue & Autexier, 2023). Budding yeast telomerase consists of many protein subunits and a long non-coding RNA: TLC1. TLC1 contains a short stretch that is complementary to the G-strand of the telomeric DNA and serves as a template for the iterative addition of telomeric repeats by the Est2 catalytic subunit (Singer & Gottschling, 1994). In total, Est1, the catalytic subunit Est2, Est3 and TLC1

are essential to form the telomerase holoenzyme *in vivo* (Lingner *et al*, 1997). The action of telomerase is tightly regulated throughout the cell cycle and occurs during the late S/G₂ phase, in coordination with the semi-conservative DNA replication (Pfeiffer & Lingner, 2013). Budding yeast has served as an excellent model organism to study telomerase due to the degenerated telomeres, which allow for the identification of *de novo* added telomeric sequences. Telomerase exhibits a preference for acting on short telomeres in budding yeast (Teixeira *et al*, 2004). This phenomenon has been attributed to a mechanism in which high levels of telomeric proteins, such as Rap1, found at long telomeres repress the action of telomerase. As telomeres shorten, the abundance of Rap1 molecules decreases, thereby relieving the inhibition on telomerase activity (Marcand *et al*, 1997). TERRA has also been proposed play the role of a scaffold tethering telomerase to the short telomeres from where its transcribed (Cusanelli *et al*, 2013) (*refer to review attached in the annex section*).

Telomerase is conserved in multicellular organisms and in human it's made up of the hTERT catalytic subunit and the hTR RNA subunit (Nandakumar & Cech, 2013). However, telomerase is not active in human somatic cells, which leads to the gradual shortening of telomeres until they reach a critical length where the cell stops to divide. This can be recapitulated in budding yeast, where in the absence of telomerase, cells exhibit the ever-shortening telomere phenotype and viability is reduced after many generations. This phenomenon known as replicative senescence will be further detailed in the chapter II.

II- Chapter II: Replicative Senescence

Cellular senescence is an irreversible growth arrest where the cell stops entering the cell cycle and dividing. It was first discovered in the 1960's by Hayflick who observed that human fibroblasts lose their capacity to proliferate after several generations *ex vivo*, in spite of being supplied by nutrients and growth factors (Hayflick & Moorhead, 1961; Hayflick, 1965). Senescence is the essence of antagonistic pleiotropy, an evolutionary biology concept, because it enhances early-life fitness and is detrimental for longevity (Campisi & d'Adda di Fagagna, 2007). Senescence is a protective mechanism against the development of cancer because it inhibits aberrant cell proliferation in young individuals. Conversely, it is also associated with aging and age-related diseases because it causes the loss of regenerative capacity and the accumulation of senescent cells in organs of aged people. There are two distinct types of senescence: premature senescence and replicative senescence. Premature senescence occurs independently of telomeres and telomerase, and can be induced by oncogenes, nutrients' stress, oxidative stress and chromatin perturbation (Campisi & d'Adda di Fagagna, 2007). On the other hand, replicative senescence is triggered by telomere erosion in the absence of telomerase and is the type of senescence that was first observed by Hayflick in human fibroblasts.

Replicative senescence can be naturally observed in somatic cells of some multicellular organisms where telomerase is physiologically repressed during development and differentiation. In humans, five dysfunctional telomeres (Kaul *et al*, 2012) are required to signal senescence through the p53 pathway (Herbig *et al*, 2004). Human senescent cells exhibit distinct characteristics that distinguish them from actively dividing cells. One prominent feature is their usual arrest in the G₁ phase of the cell cycle (Herbig *et al*, 2004). Senescent cells also display specific morphological changes and exhibit increased activity of an enzyme called β -galactosidase (SA- β -gal) (Dimri *et al*, 1995). Importantly, senescent cells remain metabolically active despite their cell cycle arrest and altered morphology. They acquire a Senescence-Associated Secretory Phenotype (SASP), which involves the secretion of various bioactive molecules, including cytokines, growth factors, and proteases (Coppé *et al*, 2008). The SASP can influence the behavior of neighboring cells, impact the surrounding microenvironment and even affect the cells that secrete these factors. It can have both beneficial and detrimental

effects, depending on the context. The SASP can contribute to tissue repair and regeneration but can also promote chronic inflammation and age-related pathologies.

In this chapter, I will provide an overview of the mechanism of replicative senescence, the escapers of senescence, and the immense heterogeneity of senescence, with a particular focus on budding yeast.

1. Mechanism of replicative senescence

In germ-line cells as in budding yeast, telomerase is constitutively active and needs to be inhibited in order to study replicative senescence. Many ways for inhibiting telomerase exist in budding yeast including deleting one of its components, or modulating its expression. Consequently, telomeres shorten 2-5 bp by generation until they reach a critical length where they activate the DNA damage checkpoint and stop to divide (Teixeira, 2013). This results in a proliferation decrease after four to seven days in budding yeast telomerase mutant (Lundblad & Szostak, 1989). In contrast to human cells, only one telomere of the total of 32 is sufficient to trigger replicative senescence in haploid budding yeast, and based on mathematical modelling it can be possibly short as 20 base pairs (Abdallah *et al*, 2009; Xu *et al*, 2013; Bourgeron *et al*, 2015a).

The shortest telomere in the cell leads to the checkpoint activation through the kinases Mec1 (the ortholog of mammalian ATR) and Tel1 (the ortholog of mammalian ATM) (Fallet *et al*, 2014; Abdallah *et al*, 2009; Mallory & Petes, 2000). As a consequence, cells arrest in the G₂/M phase of the cell cycle but remain metabolically active and increase in volume (IJpma & Greider, 2003). Published data suggest a two-step model for senescence mechanistic signalling beginning with a pre-senescence stage where telomeres are maintained by Tel1 then switching to senescence when Mec1 is localized at telomeres (Fallet *et al*, 2014; Abdallah *et al*, 2009) (Figure 5). Mec1 is essential for the DNA damage checkpoint and its recruitment is facilitated by the resection induced at short telomeres by nucleases such as Mre11 and Exo1. Rad53 the main checkpoint effector is not only hyperphosphorylated by Rad9 during senescence but its activation requires also Mrc1, a protein essential for replication stress (Grandin *et al*, 2005). Consequently, as telomeres shorten without telomerase, their identity undergoes alteration, leading to signalling pathways resembling those triggered by DSBs and replication stress

responses (Teixeira, 2013). However, in contrast to these latter stresses that signal a possibly repairable damage, replicative senescence is irreversible.

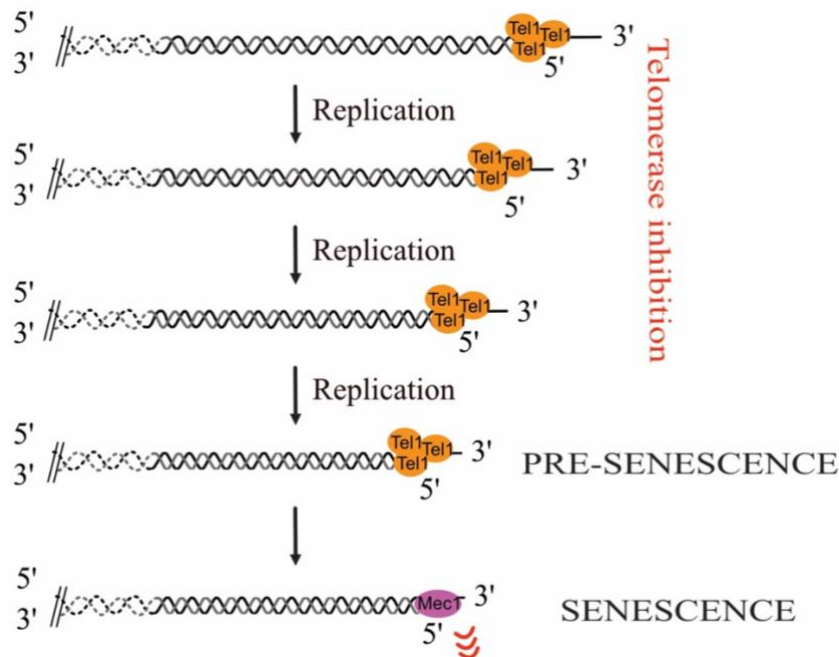


Figure 5 : Short telomeres triggers senescence in a two-step model.

Short telomeres are in a defined pre-senescent stage when Tel1 is recruited. It is only after the binding of Mec1 to single-stranded subtelomeric sequences that cells switch from a pre-senescent to a senescence state and the checkpoint is activated. Adapted from (Abdallah *et al*, 2009).

2. Survivors

When senescent cells are cultured for an extended period in fresh media, a subset of cells escape senescence and resume cellular divisions. These dividing cells, known as survivors, have the ability to repopulate the culture. Survivors maintain the length of their telomeres independently of telomerase activity. Instead, they rely on recombination mechanisms that are conserved from yeast to mammals, referred to as the ALT mechanism. In budding yeast, the emergence of survivors is a rare event, occurring at a frequency of approximately 2×10^{-5} cells (Kockler *et al*, 2021). Furthermore, this mechanism is reversible since the re-expression of telomerase can restore telomere equilibrium (Teng & Zakian, 1999; Makovets *et al*, 2008). There are two types of survivors that can arise from senescent cultures in budding yeast : Type I and Type II (Teng & Zakian, 1999; Chen *et al*, 2001). These pathways are not mutually exclusive, and Type II survivors tend to outgrow Type I survivors (Kockler *et al*, 2021).

Type I survivors, exhibit a distinct telomere phenotype characterized by the acquisition of multiple tandem Y' elements through replication-dependent recombination. Additionally, Type I survivors display shorter TG₁₋₃ terminal repeats at chromosomes ends. On the other hand, Type II survivors exhibit highly heterogeneous telomere lengths, with some instances showing extremely elongated TG₁₋₃ repeat arrays at chromosome ends. While the extension of TG₁₋₃ repeats by rolling circle replication of telomeric circles has been observed in survivor cells, studies have revealed that the majority of telomere extensions in these cells are primarily facilitated by a process known as break-induced replication (BIR) (Kockler *et al*, 2021; Lydeard *et al*, 2007; Aguilera *et al*, 2022; Churikov *et al*, 2014). This indicates that both Type I and Type II survivors arise from a unified BIR mechanism.

The reactivation of telomerase, leading to telomere elongation and sustained cell proliferation, is observed in approximately 90% of cancer cases (Shay & Bacchetti, 1997). However, there is a subset of tumors, around 10%, that are classified as telomerase-negative (Bryan *et al*, 1997). In these latter, telomere maintenance is achieved through the ALT mechanism, involving BIR similar to type II survivors in yeast (Sobinoff & Pickett, 2020). ALT allows cancer cells to maintain their telomeres and continue proliferating despite the absence of telomerase activity. It is worth noting that ALT mechanisms have also been implicated in cancer relapses following treatment with anti-telomerase drugs, highlighting their significance in cancer biology and therapeutic resistance (Hu *et al*, 2012). In addition, TERRA, the long non-coding RNA previously mentioned, has emerged as a key component in ALT mechanisms in both yeast and mammalian cells (Zeinoun *et al*, 2023).

3. Heterogeneity of senescence

Senescence is highly heterogeneous and this inherent difficulty limits the use of telomeres mean length to predict aging and diseases. The heterogeneity of senescence was first observed by variations in the lifespan of human cells cultures with different population doubling capacities (Smith & Hayflick, 1974; Smith & Whitney, 1980; Jones *et al*, 1985). Similar variations were later reported in budding yeast using a telomerase mutant which showed high morphological differences in size and shape of senescent cells indicating different growth potential (Lundblad & Szostak, 1989). These variations in the proliferation potential could be grouped in two categories: interclonal variations between cells from different telomerase defective clones, and intraclonal variations between cells stemming from the same telomerase

defective clone (Xu & Teixeira, 2019). However, proliferation potential is an endpoint measure that reflects the population as a whole, where the fittest cells are selected and take over the culture. As a result, it cannot provide much information about the heterogeneity in senescence, unlike single-cell approaches. Microfluidics can be used to track multiple independent lineages in a time-resolved manner from telomerase inactivation until cell death (Xu *et al*, 2015). This method has revealed significant variations in the number of generations (10 to 70) undergone by different cell lineages, confirming that differences in cell cycle duration and increased cell death contribute to the heterogeneity of senescence. Additionally, it has revealed two types of senescent cells: type A and type B (Figure 6). Type A cells experience a variable number of regular cell cycles before undergoing two to three prolonged cell cycles preceding cell death. On the other hand, type B cells exhibit nonterminal arrests followed by normal cell cycles after telomerase inactivation. These non-terminal cell cycle arrests add a new complex layer of heterogeneity to replicative senescence and may increase genomic instability (Coutelier *et al*, 2018).

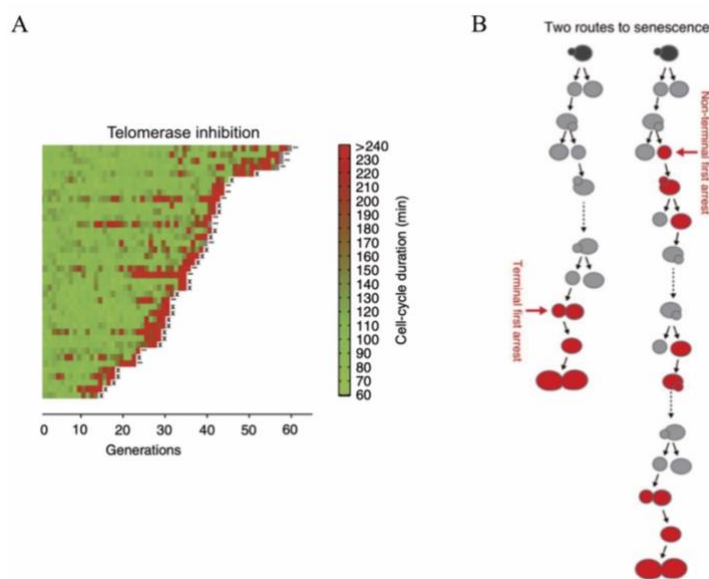


Figure 6 : Heterogeneity of replicative senescence observed by microfluidics.

(A) Display of telomerase inhibited cells lineages observed by microfluidics. Each line in the chart represents an independent lineage, and each square within the lines corresponds to the duration of the cell cycle for a specific cell. The colour scale is used to encode the cell-cycle duration: green indicates a normal cell cycle, while red indicates a longer cell cycle. The independent lineages show a variation in the number of generations they undergo confirming

that difference in the cell cycle duration and cell death contribute to the heterogeneity of senescence. Two types of senescent cells can be distinguished: Type A and Type B. Type A that undergo few prolonged cell cycles before cell death and Type B that undergo nonterminal arrest followed by normal cycles. An ellipse (...) indicates that cells were still alive at the end of the experiment and an X indicates cell death. The colour represents duration of the cell cycle.

(B) Scheme representing the two archetypes of telomerase negative lineages. Retrieved from (Xu & Teixeira, 2019; Xu *et al*, 2015).

Differences in the initial telomere's length distributions, the diversity of the shortest telomeres length between clones and the asymmetrical replication could explain most of the interclonal variations (Bourgeron *et al*, 2015b; Eugène *et al*, 2017; Xu *et al*, 2013). However, intraclonal variations suggests that factors other than the asymmetrical replication are involved in senescence. Studies in mammalian cells suggested that increased oxidative stress, genome wide changes in chromatin structure and mitochondrial dysfunctions contribute to senescence and can be at the origin of the heterogeneity observed in senescence (Ahmed & Lingner, 2018; Passos *et al*, 2007; Bahar *et al*, 2006; Sahin *et al*, 2011). Interestingly, budding yeast senescent cells activate genes involved in environmental stresses, oxidative stress and show alteration in mitochondrial morphology (Nautiyal *et al*, 2002). Thus, yeast could in principle be used as a model to study this metabolic alteration during senescence. This indicates that not only the initial response to replicative senescence is conserved between yeast and mammals but possibly also metabolic alterations. In addition, since budding yeast can be grown in fermentation or in oxidative conditions, one can expect that metabolic alterations could be more pronounced in one or the other condition. However, no further data exist on metabolic alterations and oxidative stress during replicative senescence in budding yeast.

III- Chapter III: Reactive Oxygen Species

ROS are highly reactive molecules that contain oxygen atoms and are produced as natural by-products of cellular metabolism. ROS can be generated during various physiological processes, including respiration, and immune response (Migdal & Serres, 2011). ROS are molecules characterized by their reactivity, which can exist in both radical and non-radical forms. It is important to distinguish between the radical nature of ROS and their general reactivity. Radicals are atoms or molecules that possess unpaired electrons in their orbit. In its stable state, dioxygen (O_2) is a radical with two unpaired electrons. However, its reactivity is limited to molecules that can supply one or two unpaired electrons with opposite spins to O_2 . This spin restriction restricts the reactivity of O_2 to few molecules. However, O_2 can be converted to a highly reactive intermediate called singlet oxygen, where the spin restriction is eliminated. Additionally, other reactive oxygen intermediates can be produced through the reduction of oxygen by one or two electrons. They represent different oxidation states of oxygen, including in order of reduction, the superoxide ion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^{\cdot}). Nevertheless, other reactive oxygen species can be produced like peroxy radicals (RO_2^{\cdot}), hydroperoxides (RO_2H) and alkoxy radicals (RO^{\cdot}).

ROS are known for their ability to cause oxidative damages to cellular components such as DNA, proteins and lipids (Checa & Aran, 2020). They have the potential to oxidize biomolecules and disrupt their normal functions, leading to cellular dysfunction, tissue damage, and various pathological conditions. However, in controlled time production and quantities, ROS also play important roles as signalling molecules in cellular processes such as cell signalling, immune response, and apoptosis (Checa & Aran, 2020). The cellular antioxidant defence systems, work to neutralize and eliminate excessive ROS, maintaining a delicate balance between ROS production and scavenging. When the balance is disrupted and ROS levels exceed the capacity of antioxidant defences, a condition called oxidative stress can occur. This is associated with numerous diseases and aging processes (Balaban *et al*, 2005). Thus, the balance between ROS generation and elimination is crucial for maintaining cellular homeostasis and overall health.

In this chapter, I will provide an overview of the production and detoxification of ROS then the link between oxidative damage, mitochondrial dysfunction and telomere dependent-

senescence. The last part will be focused on studies in mammalian models due to the lack of data in budding yeast.

1. ROS Production

1.1. Superoxide ion production by the mitochondria

Approximately two billion years ago, as oxygen began to accumulate in the Earth's atmosphere, aerobic organisms' evolution underwent selection for adaptation to enable the consumption of oxygen and elimination of produced reduced metabolites. O₂ plays a vital role in the energy production of various life forms. This energy generation, in the form of Adenosine Triphosphate (ATP), takes place through oxidative phosphorylation (OXPHOS), facilitated by electron transport chains (ETC) situated in the inner membrane of the mitochondria (Chaban *et al*, 2014).

1.1.1. Mitochondria

Mitochondria is a eukaryotic organelle that possesses two distinct membranes: an outer membrane (OMM) and an inner membrane (IMM), surrounding two compartments known as the intermembrane space (IMS) and the mitochondrial matrix (Malina *et al*, 2018). These membranes and compartments host a diverse array of proteins, which play roles in various cellular processes. These processes include the Krebs cycle, heme biosynthesis, maintenance of redox homeostasis and apoptotic cell death. Additionally, these proteins facilitate the exchange of metabolites between the mitochondria and the rest of the cell. Notably, the inner membrane forms intricate folding's called cristae within the mitochondrial matrix, further enhancing the functional complexity of mitochondria. The majority of mitochondrial proteins are encoded by nuclear DNA. However, mitochondria also harbor their own mitochondrial DNA (mtDNA) within the matrix, that in budding yeast, encodes seven proteins of the electron transport chain and oxidative phosphorylation, and one ribosomal protein. The mtDNA is histone free, has its own transcription machinery and is present by multiple copies in the cell depending on the organism. Mitochondria should not be regarded as individual organelles with fixed size and properties. Extensive evidence now confirms their dynamic nature through fusion

and fission processes, forming a dynamic mitochondrial network. This network's equilibrium, achieved by various molecular players, contributes to its functional characteristics.

1.1.2. Oxidative phosphorylation

As previously mentioned, mitochondria serve as the primary site for ATP generation through OXPHOS. However, in order to generate ATP, various metabolic pathways are involved. For instance, in aerobic conditions, the process typically begins with glycolysis followed by the Krebs cycle. During these pathways, electrons from nutrients are transferred to electron carriers such as NAD⁺ or FAD, leading to the production of NADH and FADH₂. These reduced molecules are crucial for the ATP generation through a series of steps within the mitochondrial ETC. OXPHOS involves a coupling mechanism between the ETC and a phosphorylation system (*Figure 7*) (Chaban *et al*, 2014).

In budding yeast, ETC consists of a complex II (succinate dehydrogenase), Gut2 (glycerol-3-phosphate dehydrogenase), complex III (ubiquinol-cytochrome c oxidoreductase) and a final complex IV (cytochrome C oxidase). Budding yeast, in contrast to mammalian cells, does not have a complex I but possesses external and internal NADH dehydrogenases (Nde1/2, Ndi) and a D-lactate dehydrogenase (Dld1). The respiratory chain facilitates the transfer of electrons and protons during cellular respiration. Complexes III, and IV are involved in both electron and proton transfer, while complex II and other dehydrogenases only participate in electron transfer. The mobile electron carriers in this process are the coenzyme Q and the cytochrome c. Coenzyme Q plays a crucial role in transporting electrons from all the dehydrogenases located prior to Complex III, leading them to reach this complex. On the other hand, cytochrome c acts as a mediator in transferring electrons from Complex III to Complex IV. Finally, electrons pass down through complex IV to the final electron acceptor dioxygen which results in formation of a water molecule. As electrons move through the chain, protons are simultaneously pumped from the matrix to the intermembrane space, creating an electrochemical gradient known as the proton-motive force. This force combines both electrical and chemical components, resulting from the accumulation of protons outside the matrix. The phosphorylating system, a crucial component of OXPHOS, harnesses the proton-motive force to drive the synthesis of ATP. The ATP synthase, facilitates the coupling of proton transfer along their electrochemical gradient with the synthesis of ATP from ADP and Pi. The phosphorylating system involves two transporters. The first one is the adenylic nucleotide transporter (ANT), which allows for the

electrogenic exchange of ADP and ATP across the inner mitochondrial membrane. The second one is the phosphate transporter (PiC), which enables the electroneutral transport of phosphate into the matrix simultaneously with a proton.

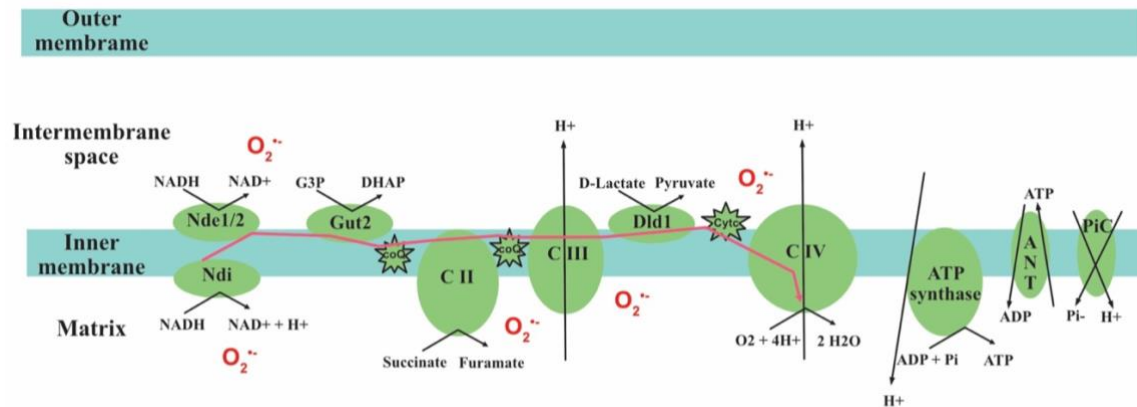


Figure 7 : Components of the OXPHOS system in budding yeast.

Mitochondria is composed of an outer and an inner membrane delimitating an intermembrane space and a mitochondrial matrix. OXPHOS system is composed from an electron transfer chain (ETC) and a phosphorylating system located on the inner membrane. The ETC is composed of external and internal NADH dehydrogenase (Nde and Ndi respectively), a glycerol-3-phosphate dehydrogenase (Gut2), a succinate dehydrogenase complex II (CII), a ubiquinol-cytochrome c oxidoreductase complex II or cytochrome BC1 complex (CIII), a D-lactate dehydrogenase (Dld1) and a cytochrome c oxidase complex IV (CIV). CoQ and Cyt c represent coenzyme Q and cytochrome c respectively and are the mobile electron carriers. H⁺ represents protons that are transferred to the intermembrane space by the CIII and the CIV. The phosphorylating system is composed of an ATP synthase, and two transporters: adenylic nucleotide transporter (ANT) and Phosphate transporter (PiC). O₂⁻ represents the superoxide radicals produced at different steps of electron transfer in the ETC. Adapted from (Mourier *et al*, 2008).

1.1.3. ROS production by the mitochondria through OXPHOS

Mitochondria play a pivotal role in the generation of reactive oxygen species (ROS), accounting for approximately 90% of ROS production in the cell (Balaban *et al*, 2005). Superoxide radicals, are generated through slippage events that occur at various points along the electron transport chain, where electrons leak from their carrier system directly reacting with oxygen leading to its incomplete reduction into water (Figure 7). In budding yeast, it has been shown that half of the superoxide radicals production comes from the external NADH dehydrogenase in addition to the involvement of the internal NADH dehydrogenase (Fang & Beattie, 2003). The other half would originate from the cytochrome bc1 complex (complex III). This does not exclude that other complexes in the ETC participate in ROS production. On the other hand, increased ROS production can also be detected by disrupting the electron flow by

inhibitors like antimycin A that inhibits the bc1 complex. Similar increase can also be measured when ADP becomes limited. In these cases, the electrons transferred before the blockage remain reduced and promote the transfer of electron to oxygen molecules (Herrero *et al*, 2008). Importantly, mitochondrial inhibitors could also have the opposite effects and reduce the generation of ROS as observed after the addition of myxothiazol or stigmatellin that blocks the oxidation of ubiquinol by the bc1 complex. It is important to note that superoxide production occurs within the normal physiological conditions of the cell and between 0.2% and 2% of the total oxygen consumption could be transformed into ROS (Balaban *et al*, 2005). This highlights the delicate balance between ROS production and cellular homeostasis within the mitochondria. Even if oxygen is transformed into superoxide radicals, $O_2^{\cdot-}$ exhibits relatively weak reactivity compared to other ROS when it comes to biomolecules. The superoxide radical shows particular reactivity towards iron, and its interaction with iron-sulphur centres is primarily responsible for its toxicity (Fridovich, 1997). Indeed, $O_2^{\cdot-}$ released to the IMS and matrix from complex III and to the matrix from complex II and IV damage proteins by iron-sulphur clusters interactions (Fridovich, 1997; Muller *et al*, 2004). However, $O_2^{\cdot-}$ can give rise to other forms of ROS that are more reactive and thus exert indirect high toxicity.

1.2. Hydrogen peroxide and hydroxyl radical production

In physiological conditions, the superoxide anion can rapidly form H_2O_2 by dismutation which can be spontaneous or catalysed by metalloproteins called superoxide dismutase (SOD) (Figure 8) (Migdal & Serres, 2011). This would maintain a low level of superoxide radicals ($O_2^{\cdot-}$) but will generate hydrogen peroxide. H_2O_2 is non radical but can react with ferrous ions Fe^{2+} forming Fe^{3+} and producing hydroxyl radicals (OH^{\cdot}) and hydroxyl anions (OH^-) which is known as the Fenton reaction (Figure 8) (Migdal & Serres, 2011). The reactivity of H_2O_2 is much more restricted than the hydroxyl radical (OH^{\cdot}) which is undoubtedly the most reactive compound. This latter reactivity is so high that it limits its diffusion, confining the reaction site of OH to its site of formation. Therefore, the formation of the hydroxyl radical (OH^{\cdot}) must be prevented through the elimination of the superoxide ion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) by specific enzymatic systems (*discussed later*). Hydroxyl radical (OH^{\cdot}), reacts in a non-specific way with cellular components through different types of reactivity and is involved in initiating chain reactions responsible for lipid peroxidation (Halliwell & Gutteridge, 1984). This

is very toxic for the cell because it can rapidly modify DNA and protein forming adducts and it causes genome instability (Dizdaroglu & Jaruga, 2012).

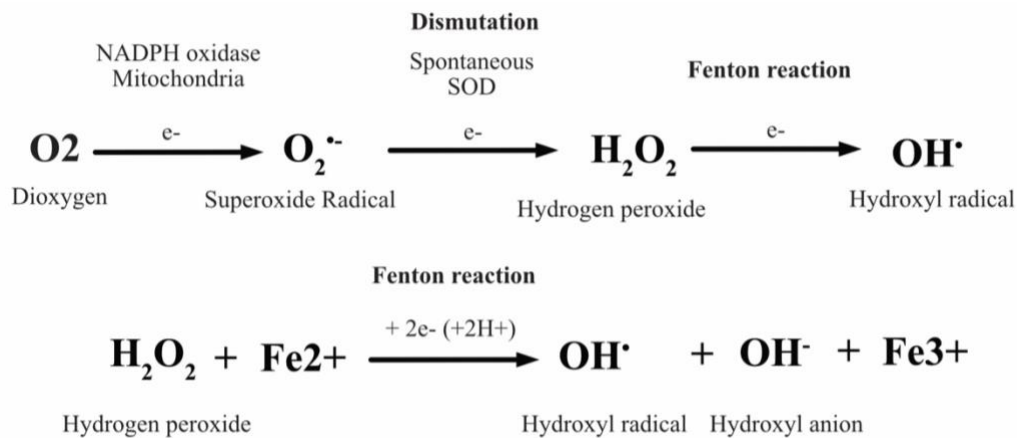


Figure 8 : The reduction of oxygen and formation of reactive oxygen species.

Oxygen can be reduced and be a source of superoxide radical through two major sources in the cell: NADPH oxidase and mitochondria. Superoxide radicals then would undergo spontaneous or SOD dependent dismutation and form hydrogen peroxide. This latter reacts with Fe^{2+} in a reaction known by the Fenton reaction and form hydroxyl radical.

1.3. Other sources of ROS generation

In mammalian cells, superoxide anion ($\text{O}_2^{\cdot-}$) can also be generated by the activity of NOX (NADPH oxidase) enzymes that catalyse the production of ROS by transferring electrons from NADPH to molecular oxygen. NOX can be found in various cells, including immune cells, and are involved in numerous physiological processes, such as immune responses, signalling pathways and cellular homeostasis (Sumimoto, 2008). Due to its function in mammalian cells, NADPH oxidase was thought to be restricted to multicellular organisms, however Yno1 (yeast NADPH oxidase 1), an ortholog of NOX was identified in budding yeast (Rinnerthaler *et al*, 2012). Yno1, is located in the endoplasmic reticulum and contributes to approximately 20% of ROS production during the exponential growth phase independently of the mitochondria. Interestingly, Yno1 is suggested to regulate actin cytoskeleton and the overexpression of Yno1 leads to cell death by apoptosis. In addition to NOX, other sources, present in the cytosol and within different organelles, can produce ROS. For instance, the xanthine oxidase, the enzymes of the endoplasmic reticulum, the enzymes of the arachidonic acid pathway, peroxisomes, lysosomes, and the nucleus are among these sources (Migdal & Serres, 2011). ROS can also be

generated during altered cellular environments by some heavy metals, genotoxic agents including UV irradiation and alkylating agents (Ikner & Shiozaki, 2005).

2. Antioxidant defences

The cellular response varies depending on the dose of ROS encountered by the cell (Ayer *et al*, 2014). At physiological levels, ROS play a positive role as signalling molecules. When exposed to low doses, cells undergo adaptation and become more resistant to oxidative stress. As the dose of ROS increases, cells activate response systems for detoxification and repair, while also arresting the cell cycle. However, at higher doses, apoptosis, a programmed cell death process, is initiated. Antioxidants play a vital role in maintaining redox homeostasis by facilitating the reduction and the elimination of ROS (Herrero *et al*, 2008) (Figure 9). The concentration of hydrogen peroxide is regulated through two different types of catalytical activities depending or not on the reducing activities of NADH or NADPH. Superoxide dismutase's and catalases catalyse the dismutation of superoxide ions into hydrogen peroxide and the subsequent dismutation of hydrogen peroxide into water without relying of NADH or NADPH but on the redox properties of a metal group associated with these enzymes. Peroxidases catalyse the reduction of hydrogen peroxide to water and organic peroxides to their corresponding alcohols, utilizing the reducing power of NADPH or NADH. The activity of these enzymes depends on the presence of reactive thiols or selenothiols and on enzymes which act as redox regulators. In addition, the redox state of the cell which represent the balance of its oxidation/reduction system is crucial for ROS responses.

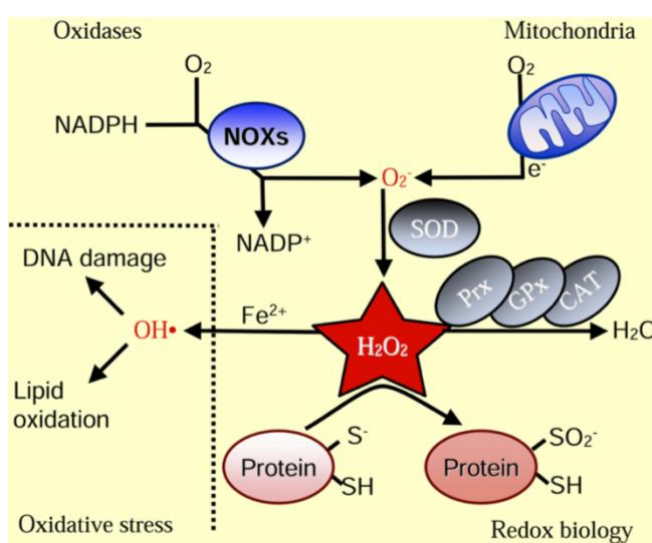


Figure 9 : ROS detoxification and toxicity.

Intracellular superoxide radicals originate primarily from the oxidation of NADPH by NOXs enzymes and as a by-product of mitochondrial oxidative phosphorylation. Superoxide anions are rapidly converted to hydrogen peroxide spontaneously or by Superoxide dismutase's (SOD). H_2O_2 plays a role in redox signalling by oxidizing cysteines residues of proteins. In order to limit excessive H_2O_2 that can be toxic, it's converted to H_2O by antioxidants such as catalases (CAT), peroxiredoxins (PRx) and glutathione peroxidase (GPx). H_2O_2 reacts

with Fe_{2+} to form hydroxyl radical which is highly toxic and causes irreversible damages to cellular macromolecules and DNA. Adapted from (Schieber & Chandel, 2014).

2.1. Superoxide dismutase

Superoxide dismutase (SOD) is an enzyme found in the majority of aerobic organisms. Its function is to facilitate the conversion of two molecules of $O_2^{\cdot-}$ into H_2O_2 and O_2 through a dismutation mechanism that relies on the presence of a transition metal. There are three types of SOD, classified based on the specific transition metal involved: iron-dependent superoxide dismutase (Fe-SOD), manganese-dependent superoxide dismutase (Mn-SOD), and copper/zinc-dependent superoxide dismutase (Cu/Zn-SOD) (Fridovich, 1997). In budding yeast, two SOD exist and differ in localization and the metal co-factor used. Sod1 is a copper/zinc superoxide dismutase and is located in the cytoplasm and mitochondrial IMS, while Sod2 is a manganese-dependent superoxide dismutase and is located in the mitochondrial matrix (Wallace *et al*, 2004; Luk *et al*, 2005). Both Sod enzymes are important in detoxifying superoxide anions generated by the ETC but Sod2 does not play a significant role in the defence of added oxidants. Sod2 is vital for protection against the oxidation of mtDNA (Piper, 1999). Superoxide dismutases, therefore, prevent the toxicity associated with the presence of superoxide ions. In doing so, they contribute to the production of another potentially toxic compound, hydrogen peroxide. Other activities, such as catalases and peroxidases, come into play in response.

2.2. Catalases

Catalases are homotetrameric proteins whose catalytic activity relies on the presence of a haem group. They reduce two molecules of H_2O_2 through dismutation into water and O_2 , utilizing the catalytic power of iron. Budding yeast possess two catalases: a cytoplasmic catalase T, Ctt1 (Hartig & Ruis, 1986), and a peroxisomal catalase A, Cta1 (Cohen *et al*, 1988). The activity of the peroxisomal catalase seems to be dispensable. In the absence of the cytoplasmic catalase exponential growing cells are not more sensitive to hydrogen peroxide. However, In the absence of these two catalases the survival of budding yeast is compromised after an exposition to high concentration of hydrogen peroxide and the adaptive response is impaired (Izawa *et al*, 1996). CTT1 expression is induced under several stresses including oxidative and osmotic stress (Martínez-Pastor *et al*, 1996a). Nevertheless, a study showed that the catalase activity becomes essential in yeast only when other antioxidants are lacking which suggest that thiol peroxidases appear to be the essential activities for controlling peroxide concentration in this organism (Grant *et al*, 1998).

2.3. Peroxidases

Peroxidases are small proteins that facilitate the conversion of peroxides (ROOH) into their corresponding alcohols (ROH) by catalysing a one-electron reduction. This process effectively reduces hydrogen peroxide to water. The active site of thiol peroxidases contains a reactive cysteine residue capable of nucleophilic attack on the peroxide molecule (Ursini *et al*, 1995). Peroxidase activity is dependent on the availability of electron donors in the form of thiols. Two distinct classes of peroxidases have been identified based on their specific electron donors: glutathione peroxidases (GPXs) use glutathione (GSH) as an electron donor, while TRX peroxidases, also known as peroxiredoxins (PRX), rely on thioredoxin (TRXs) as their reducing agents. Following oxidation, these electron donors are regenerated by thioredoxin reductase and glutathione reductase enzymes, which are dependent on NADH. This renewal process ensures the continuous cycle of peroxidase activity. In certain cases, glutathione can also play a significant role in supporting the activity of peroxiredoxins (Rhee *et al*, 2005).

In budding yeast, there are three known glutathione peroxidases (GPXs) named Gpx1, Gpx2, and Gpx3. Gpx2 is the only glutathione peroxidase upregulated transcriptionally upon an oxidative stress (Tsuzi *et al*, 2004) and rely on the TRX system (Tanaka *et al*, 2005). However, Gpx3 appears to be the most crucial, exhibiting the highest activity *in vitro* and displaying the strongest phenotype when is mutated (Avery & Avery, 2001; Inoue *et al*, 1999). Gpx3 also plays the role of redox transducer in the Yap1 pathway, which is the primary regulator of the oxidative stress response (Delaunay *et al*, 2002) (*this pathway will be discussed later*). The precise subcellular localization of these peroxidases in budding yeast remains uncertain. Apart from these three GPX proteins, budding yeast possesses other enzymes with GPX activity against hydrogen peroxide. This includes the two glutaredoxins (Grx1 and Grx2) (Collinson *et al*, 2002) and the glutathione transferase Gtt1 (Garcerá *et al*, 2006). In addition to all of this, glutathione (GSH: reduced form; GSSG: oxidized form), is a key player thiol in the redox environment of many cell compartments and acts as a primary redox buffer (Ayer *et al*, 2014).

In budding yeast, there are five distinct PRXs present: Tsa1, Tsa2, and Ahp1, which are cytosolic; Prx1, which is mitochondrial; and Dot5, which is nuclear (Park *et al*, 2000). The first line of defence in budding yeast is *via* peroxiredoxins (Winterbourn & Hampton, 2008). Among these PRXs, Tsa1 exhibits the highest activity, while Tsa2 shows the highest inducibility

(Munhoz & Netto, 2004). The protective function of PRXs extends beyond peroxide reduction and includes defence against ROS generated by heavy metals. Intriguingly, a yeast strain lacking all five PRXs exhibits hypermutability, indicating a connection between elevated intracellular ROS levels and genome instability (Wong *et al*, 2004). Apart from their peroxidase activity, both Tsa1 and Tsa2 possess chaperone functions independent of their peroxidase activity (Jang *et al*, 2004). Additionally, Dot5 is involved in telomeric silencing and its overexpression leads to the activation of telomeric transcription, regardless of its peroxidase activity (Izawa *et al*, 2004). These findings highlight the multifaceted roles of PRXs in budding yeast, encompassing peroxide reduction, defence against heavy metal-induced ROS, genome stability, chaperone functions, and modulation of telomeric processes.

3. Antioxidative transcriptional response

The stress signal must be detected by a regulator that will convert this signal to a transcriptional response involved in protecting against the stress of origin. Budding yeast respond to oxidative stress by altering its transcriptional program in a complex way (Ikner & Shiozaki, 2005; Gasch *et al*, 2000; Temple *et al*, 2005). At the proteome level, under stress by hydrogen peroxide yeast cells upregulate the TRX and GSH-dependent systems and also the pentose phosphate pathway to provide reducing equivalents for the redoxin systems (Godon *et al*, 1998). Hydrogen peroxide and menadione (drug that generates superoxide anions) have a similar transcriptional response which is the strong induction of detoxifying genes such as superoxide dismutases, glutathione peroxidases, and thiol-specific antioxidants, as well as genes involved in oxidative and reductive reactions within the cell (thioredoxin, thioredoxin reductases, glutaredoxin, and glutathione reductase) (Gasch *et al*, 2000). Budding yeast also upregulates antioxidants genes such as Sod2, Cta1, Ctt1 and Ub14 substrates in order to detoxify ROS increase from mitochondrial respiration when grown on non-fermentable substrates, or after the diauxic shift where glucose is exhausted in the media and cells change metabolism to grow on non-fermentable substrates (Moradas-Ferreira & Costa, 2000). Importantly, deletion of certain antioxidants factors results in an inability of budding yeast growth by respiration and lower growth rate in fermentative conditions. The transcriptional regulation upon oxidative stress is essentially regulated by the transcription factor Yap1 and others like Skn7, Msn2/4.

3.1. Antioxidant transcription factors

Yap1 (Yeast Activator Protein 1) is a AP-1 like transcription factor in yeast that plays a key role in the response to oxidative stress (Rodrigues-Pousada *et al*, 2019). It regulates the expression of various genes involved in antioxidant defence and detoxification processes including enzymes from the thioredoxin and glutathione pathways (Temple *et al*, 2005). Yap1 activation in yeast is primarily triggered by the presence of ROS or other oxidative stress conditions. Yap1 is present in the cytoplasm but upon exposition to oxidative stress it translocates from the cytoplasm to the nucleus and triggers the transcription of genes (Kuge *et al*, 1997) (Figure 10). Yap1 export to the cytoplasm is mediated by Crm1 and import to the nucleus by the importin Pse1 (Rodrigues-Pousada *et al*, 2019). Yap1 activation is effective through the modification of its cysteine residues by oxidation. Under normal conditions, Yap1 is maintained in an inactive state through the formation of disulfide bonds between specific cysteine residues. However, when cells are exposed to oxidative stress, ROS can oxidize these cysteine residues, leading to the disruption of disulfide bonds and subsequent conformational changes in Yap1 to mask its nuclear export signal (NES). This conformational change allows Yap1 to translocate into the nucleus, where it binds to specific DNA sequences called Yap1 response elements (YREs) and activates the transcription of target genes involved in oxidative stress response and cellular defense mechanisms like *TRX2*, *GSH1* and *GSH2*.

SKN7 was identified in a search for mutants sensitive to hydrogen peroxide (Krems *et al*, 1995, 1996). Skn7 encoded by *SKN7* appears to have an overlapping but not identical roles in the oxidative response to Yap1 (Figure 10). Indeed, many antioxidants' genes regulated by Yap1 are also under the regulation of Skn7 with the exception of certain genes such as the ones involved in the GSH-dependent defence mechanisms (Brombacher *et al*, 2006; Lee *et al*, 1999). In addition, the control of the same genes by these two transcription factors is independent. For example *TRX2* can be directly bound by Yap1 and Skn7 in an independent manner (Ikner & Shiozaki, 2005). Skn7 and Yap1 are crucial for the adaptation response to hydrogen peroxide that comes with a generation of NADPH (Ng *et al*, 2008). Skn7 is unlikely to be a redox sensor since its contribution to oxidative stress response is through serine/threonine phosphorylation (He *et al*, 2009). However, the kinase responsible for Skn7 phosphorylation before its association with Yap1 is still not identified. Skn7, independently of Yap1, is crucial for the induction of heat shock genes upon oxidative stress. The heat shock transcription factor Hsf1

also can activate the expression of some genes in response to ROS inducing agents but not to hydroperoxides (Yamamoto *et al*, 2007). This would be important because heat stress is known to cause oxidative stress and cross protection has been observed where heat shock induces resistance to stress conditions like H₂O₂ and menadione (Temple *et al*, 2005).

Msn2 and Msn4 are homologous transcription factors that regulate the expression of over two hundred genes in response to many stimuli. Indeed, the double mutant *msn2 msn4* is sensitive to different stresses (Martínez-Pastor *et al*, 1996a). The transcription factors Msn2 and Msn4 play a crucial role in the response to environmental stresses by binding to and activating genes that contain a Stress Response Element (STRE: CCCCT) (Martínez-Pastor *et al*, 1996a) and regulate genes involved with oxidative damage repair (Hasan *et al*, 2002) (Figure 10). These stresses include peroxide stress, diauxic shift, osmotic stress, heat stress, and acid stress. In the case of hydrogen peroxide stress, the simultaneous deletion of *msn2* and *msn4* regulators significantly impacts the expression of about 180 genes (Gasch *et al*, 2000). Among these genes are important players such as catalase *CTT1*, several heat shock proteins (HSP), and various enzymes involved in sugar metabolism (Gasch *et al*, 2000). Interestingly, the double deletion of *msn2* and *msn4* leads to increased sensitivity to H₂O₂ in drop tests, but the mutant still retains the ability to adapt (Hasan *et al*, 2002). Msn2/4 is also partially responsible for the induction of *SOD2* and *CTT1* when cells are grown on a non-fermentable carbon source and during diauxic shift (Moradas-Ferreira & Costa, 2000). Of note, in the absence of either Msn2 or Msn4 the redox state of glutathione is not affected in contrast to Yap1 or Skn7 deletion (Ayer *et al*, 2012). Importantly, telomere length has been shown to exhibit distinct physiological responses to various environmental stresses mediated by some known telomeric proteins (Romano *et al*, 2013). Exposure to alcohols such as ethanol, methanol, and isopropanol, as well as acetic acid, leads to telomere elongation. Conversely, telomeres undergo shortening in response to caffeine, high temperatures, and low levels of hydroxyurea and oxidative stress (1, 2, and 3 mM) of H₂O₂, did not impact telomeres. It's noteworthy that environmental stress response genes were not induced upon the treatments stress. This is possibly due to measurements of gene expression being performed after prolonged exposure rather than short-term exposure.

Sod1, that was always known for catalysing the superoxide radical to hydrogen peroxide, was also identified as a transcription factor that regulates the expression of oxidative resistance and DNA damage repair genes (Tsang *et al*, 2014) (Figure 10). Indeed, it has been

shown that Mec1 through Dun1 phosphorylates Sod1 which translocates in the nucleus in yeast upon high exposure to H₂O₂. In the nucleus, Sod1 binds promoters and regulates gene expression important for resistance against oxidative stress but also DNA damage repair, DNA replication stress-resistance, general stress response and Cu/Fe homeostasis.

In addition to these transcription factors, Hap1, an oxygen/heme-responsive transcription factor and the Hap2/3/4/5 complex induce antioxidants genes when cells are grown aerobically or in the diauxic-shift (Moradas-Ferreira & Costa, 2000).

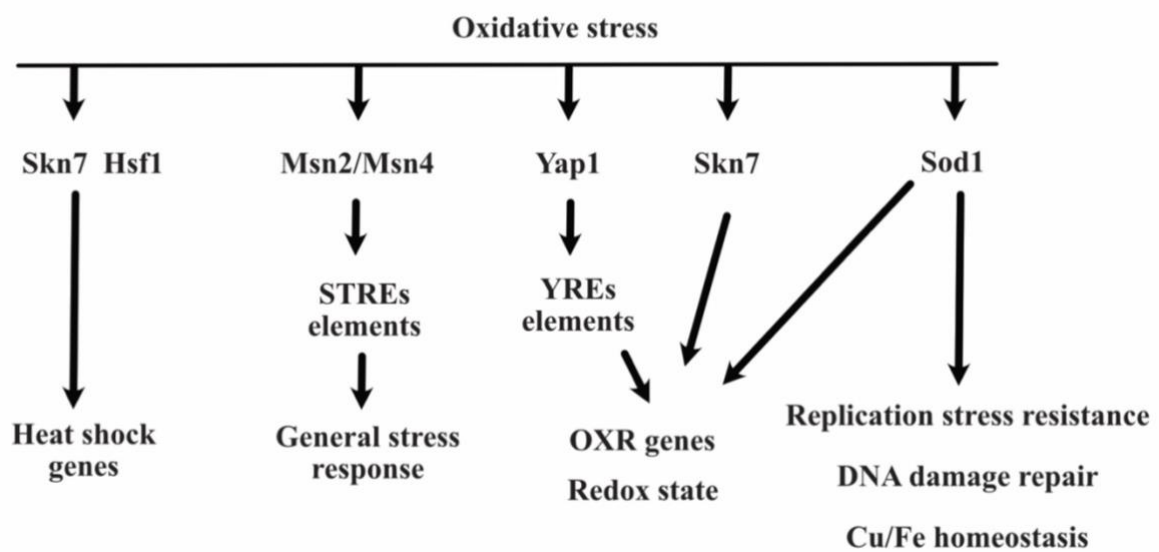


Figure 10 : Transcription factors involved in the oxidative stress detoxification response.

Oxidative stress promotes the activation of several transcription factors involved in the oxidative stress detoxification response. The complex Hsf1/Skn7 induced heat-shock genes. Msn2/4 through their binding on STREs elements induce the general stress response that includes antioxidants. Yap1 will bind the YREs elements and induces genes for the oxidative stress response genes (OXR). Skn7 in conjunction with Yap1 or independently participates also to the induction of the OXR genes. Yap1 and Skn7 are also crucial for the redox state balance of the cell. Sod1, through Mec1 activation, will induce genes for the resistance against oxidative stress, resistance of replication stress, DNA damage repair and metals homeostasis. Not all the transcription factors involved in the response against oxidative stress are represented. Adapted from (de la Torre-Ruiz et al, 2010).

4. Oxidative stress and replicative senescence

In 1955, Harman proposed the free radical theory of aging, suggesting that the accumulation of molecular and cellular damages caused by free radicals is responsible for the aging process (Harman, 1956). He made a connection between studies showing that irradiation of living

systems leads to mutations, cancer, and aging, all of which were associated with the production of free radicals. At that time, it was still unclear whether free radicals were produced within living systems. This theory gained substantial credibility 13 years later when Sod enzymes were discovered in living systems, highlighting the importance of *in vivo* ROS detoxifying enzymes and confirming the presence of ROS in cells (McCord & Fridovich, 1969). Then, in 1972, Harman expanded his theory to include the involvement of mitochondria in ROS production and its impact on the aging phenotype assigning the mitochondria as the biological clock of the cell (Harman, 1972). However, at that time, the validity of this theory was dismissed due to a prevailing notion stemming from early studies. These studies failed to demonstrate mitochondrial dysfunctions during senescence, leading to a generalization that oxidative damage is diluted throughout the senescence process. In addition, as discussed in Chapter II, by that time, replicative senescence had been discovered and was attributed to a counting program in which telomeres were assigned the role of a biological clock, contradicting Harman's theory. Importantly, the observation that replicative senescence is highly heterogeneous revealed that there is more than a counting telomeric mechanism and reconsidered mitochondrial dysfunctions and oxidative stress as key player (Sozou & Kirkwood, 2001). This heterogeneity may stem for stochastic events that leads to telomeres shortening in some cells triggered possibly by mitochondrial dysfunctions and oxidative stress.

4.1. Oxidative stress and telomeric damages

ROS can inflict significant damages to DNA, which can have profound consequences for the cell (*Figure 11*). The hydroxyl radical, in particular, poses a major threat to DNA integrity due to its strong oxidizing properties. ROS can cause oxidative damages to DNA bases and the deoxyribose backbone, resulting in the formation of various lesions (Cadet & Wagner, 2013). These lesions include modifications and chemical alterations to the bases, such as base oxidation, abasic sites and base adduct formation. Furthermore, oxidative stress can directly cause telomere loss by inducing single-strand breaks or lesions at telomeres, which can lead to replication fork collapses, blocks and arrests (von Zglinicki, 2002; Coluzzi *et al*, 2019). In addition, telomeres are more susceptible to oxidative damage than other part of nuclear DNA which is more resilient (Hewitt *et al*, 2012; Oikawa & Kawanishi, 1999; Qian *et al*, 2019).

Many types of oxidized bases have been identified with guanine being the most susceptible to oxidation (Cadet & Wagner, 2013). One specific product of guanine oxidation is 8-oxoguanine (8-oxoG). Due to the G-rich nature, telomeres are particularly prone to this oxidative damage that represent the most frequent telomeric DNA lesions. To repair such damage, the base excision repair mechanism (BER) comes into play and is essential for the stability of the genome and telomeres (Jacobs & Schär, 2012). However due to their single stranded overhang and higher order conformational structures, telomeric 8-oxoG modifications cannot be effectively repaired (Zhou *et al*, 2013). After an acute oxidative stress, it has been shown that 8-oxoG persists at telomeres, causes shortening and increases telomeric fusion leading to chromosome instability (Coluzzi *et al*, 2014). Furthermore, oxidized purines resulting from oxidative damages can block DNA replication and transcription, posing cytotoxic effects. For example, lesions from the oxidation of 8-oxoG can interfere with DNA replication and transcription (Kolbanovskiy *et al*, 2017; Henderson *et al*, 2003). Even if 8-oxoG are not lethal to the cell, it has a strong mutagenic potential and if not repaired before replication, it can lead to the misincorporation of an adenine (A) on the opposite strand, resulting in changes to the telomeric sequence (Maga *et al*, 2007; Markkanen, 2017). These changes in the telomeric composition due to base misincorporation can disrupt shelterin binding and affect telomeres length. Importantly, in budding yeast, the deletion of *OGG1*, a DNA glycosylase involved in BER, leads to an elevation in oxidized guanine levels at telomeres and concurrent telomere elongation (Lu & Liu, 2010). This elongation in telomere length is thought to be contingent on Rad52-mediated homologous recombination and/or telomerase, possibly through the dissociation of the negative regulators of telomerase Rap1-Rif1-Rif2 from telomeres. Notably, the deletion of *OGG1* delays replicative senescence in the absence of Est2, but this effect is not observed when combined with a Rad52 mutation. This underscores that the activation of recombination, in the absence of Ogg1, might rescue the loss in cell viability when telomerase is deactivated.

Free deoxyribonucleotides (dNTPs) are more prone to oxidative damages, than already incorporated bases in the DNA duplex. These oxidized dNTPs can be incorporated by DNA polymerases and telomerase at telomeric regions. *In vitro* studies have shown conflicting results, with some indicating that ROS-induced DNA damage can either promote or inhibit telomerase activity. When 8-oxoguanosine is present at the end of DNA substrates, it prevents telomerase from extending telomeres (Aeby *et al*, 2016). Additionally, if telomerase incorporates the oxidized nucleotide 8-oxo-dGTP, it can lead to premature chain termination

(Fouquerel *et al*, 2016). *In vivo* study using cancer cells, where *PRDX1* and *MTH1* antioxidants genes are deleted, demonstrates that oxidative stress inhibits the action of telomerase (Ahmed & Lingner, 2018). On the other hand, ROS can promote telomerase activity by destabilizing G-quadruplex structures (Fouquerel *et al*, 2016; Lee *et al*, 2017a). Moreover, ROS-induced telomeric damages can interfere with the binding of Trf1 and Trf2, which may contribute to telomere length increase (Opresko *et al*, 2005). This scenario was also observed in budding yeast where the deletion of the antioxidant gene *TSA1* reduces the binding of Rap1 and causes telomerase dependent telomere elongation (Lu *et al*, 2013).

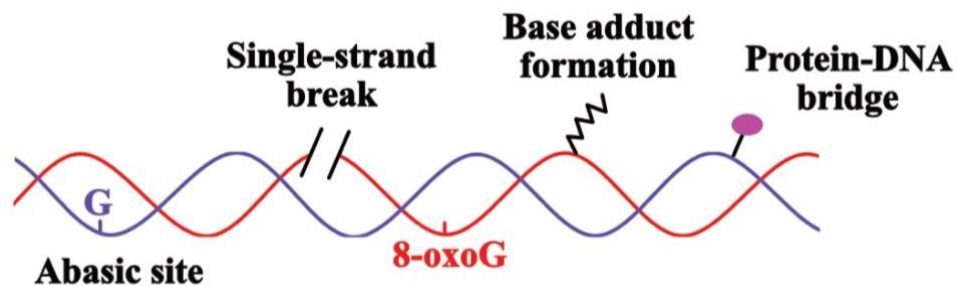


Figure 11 : Simplified scheme of ROS induced damages to DNA.

4.2. Mitochondrial dysfunctions, oxidative stress and replicative senescence

Many studies in different organisms highlighted the role of mitochondria in ROS production leading to telomeres dysfunctions during replicative senescence. *In vitro* studies on human senescent fibroblasts show that during replicative senescence mitochondria exhibit a significant increase in mass due to the upregulation of genes involved in mitochondrial biogenesis (Passos *et al*, 2007, 2010; Lee *et al*, 2002). The increase in mitochondrial mass can also be the result of an impairment in autophagic processes. The role of autophagy, the general process of defective organelles recycling in the cell, during senescence remains under debate due to conflicting results (Chapman *et al*, 2019). However, studies have reported a link between senescence induced pathways and the impairment of mitophagy, the specific type of autophagy responsible for the degradation of mitochondria (Chapman *et al*, 2019). Even if the number of mitochondria increases during replicative senescence, their functionality is compromised. Indeed, a decrease in mitochondrial membrane potential, an increase in mitochondrial uncoupling events and the activation of the retrograde response were observed in senescent fibroblasts (Passos *et al*, 2007, 2010; Lee *et al*, 2002). In addition, replicative senescence is accompanied by elevated levels of mitochondrial produced ROS (Passos *et al*, 2007, 2010;

Hutter *et al*, 2004; Allen *et al*, 1999; Lee *et al*, 2002), a decrease in cellular ATP (Passos *et al*, 2007; Hutter *et al*, 2004; Zwerschke *et al*, 2003), and an accumulation of oxidation products (Sitte *et al*, 2001, 2000). Another notable alteration in senescent cells is mitochondria hyperfusion, which occurs due to a disruption in the balance between fusion and fission events. This imbalance is characterized by a decrease in both fusion and fission processes, along with reduced expression of the proteins involved in regulating fission (Mai *et al*, 2010). Overall, *in vitro* studies using human senescent cells revealed changes in mitochondrial structure, dynamic, and functionality, in addition to an increase in oxidative stress and damages. In accordance, *in vivo* studies in mice and zebrafish show mitochondrial dysfunction and an increase in ROS levels during senescence (Passos *et al*, 2010; Sahin *et al*, 2011; El Maï *et al*, 2020).

Interestingly, an increase in mtDNA damages was also observed *in vitro* in senescent human fibroblasts (Passos *et al*, 2007). Mitochondrial damages could be caused by an increase in oxidative stress. Indeed mtDNA, which encodes essential subunits of the respiratory chain is highly susceptible to damage caused by ROS. There are several reasons for this. First, there is a close proximity between mtDNA and the ROS produced by the mitochondria themselves. Second, the repair mechanisms for mtDNA are relatively inefficient compared to those for nuclear DNA. Third, mtDNA is not protected by histones, which could make it more vulnerable to oxidative damage. These factors collectively make mitochondria more prone to ROS-induced damages, leading to mitochondrial dysfunction that can lead in a positive feedback loop to the production of more ROS. Several studies where oxidative stress have been induced by pro-oxidants or depleting antioxidants have provided evidence supporting this notion (Passos & von Zglinicki, 2005). However, whether mtDNA damages are the cause or the consequence of increased ROS levels is still elusive.

Studies have demonstrated that maintaining low oxidative stress conditions through cultivation in low oxygen levels (von Zglinicki *et al*, 1995), or overexpression of antioxidant enzymes (Serra *et al*, 2003; Forsyth *et al*, 2003) can better preserve average telomere length. Moreover, targeting antioxidants specifically to the mitochondria has been shown to counteract telomere shortening and extend lifespan in fibroblasts under mild oxidative stress conditions (Saretzki *et al*, 2003). Additionally, reducing ROS levels through mild uncoupling (Passos *et al*, 2007) and the use of nicotinamide (Kang *et al*, 2006) have also been found to prolong lifespan and prevent telomere dysfunction. Conversely, introducing an uncoupling agent has

been shown to increase ROS production, leading to telomere attrition, loss, and fusion in mouse embryos (Liu *et al*, 2002). However, longevity studies in organisms have not always showed that antioxidants delay or prevent aging (Schieber & Chandel, 2014). Nevertheless, even in the presence of telomerase, ROS induced by mitochondrial dysfunction can accelerate telomere shortening, further supporting the link between mitochondrial produced ROS and dysfunctional telomeres (Ahmed *et al*, 2008; Passos *et al*, 2007; Saretzki *et al*, 2003). Importantly, using a chemoptogenetic approach which specifically targets mitochondria and induces mitochondrial dysfunctions and subsequent ROS production, it has been shown that hydrogen peroxide can travel to the nucleus and leads to DNA double strand breaks specifically at telomeres and dysfunctional telomeres (Qian *et al*, 2019).

Taking a broader perspective, ROS could potentially act as a signalling mechanism to the nucleus, triggering cell proliferation limitations through telomere shortening. Telomeres, in turn, could function as sensors of mitochondrial dysfunction. Oxidative stress and mitochondrial dysfunctions have been shown to be delayed after the DDR response triggered by dysfunctional telomeres in human and mice senescent cells (Passos *et al*, 2010). In addition, this study revealed that ROS participate in the establishment and stabilization of replicative senescence. Indeed, after the DDR response, a pathway involving many proteins including p53 maintain high ROS levels which in a positive feedback loop maintains the DDR and the strong cell cycle arrest.

In budding yeast no studies have been done regarding the change in oxidative stress levels during replicative senescence. Only one study reported changes in mitochondrial structure and function during senescence (Nautiyal *et al*, 2002). What is known so far is that the deletion of telomerase induces the telomerase deletion response (TDR) that consists of four major responses: DNA damage response, stress response, altered metabolic program and the induction of unique telomerase deletion signature genes. TDR results in energy production genes (oxidative phosphorylation genes) upregulation, the most when telomeres are the shortest, and this is specific to telomerase deletion compared to other DNA damage stress. Mitochondria proliferation was also observed in senescent cells compared to early senescent cells and survivors but it seems that respiration *per se* does not affect the dynamics of senescence (Nautiyal *et al*, 2002).

The mammalian MAPK p38 has emerged as a key player in both premature senescence and telomere dependent replicative senescence. P38 induces premature senescence triggered by various stressors, including oxidative stress, overexpression of oncogenes, and culture shock conditions. Activation of p38 by oncogenic Ras leads to premature senescence through the upregulation of p16 mRNA, which inhibits the cellular cycle (Wang *et al*, 2002). Additionally, p38 activation in response to oxidative stress induces premature senescence through the accumulation of lamin B1, altering nuclear architecture (Barascu *et al*, 2012). The role of p38 in premature senescence extends to culture shock-induced senescence and its inhibition increases cellular growth (Iwasa *et al*, 2003). P38 is also implicated in replicative senescence. In human fibroblasts, p38 undergoes phosphorylation and activation when telomeres are short in senescent cells. Inhibiting p38 extends lifespan, delaying the onset of senescence (Iwasa *et al*, 2003). The expression of hTERT results in the re-elongation of telomeres and the inactivation of p38, suggesting that p38 is activated by telomere shortening (Iwasa *et al*, 2003). Inhibiting P38 has also been demonstrated to reverse accelerated aging in Werner syndrome disorder fibroblasts (Davis *et al*, 2005). In rabbit chondrocytes, p38 is activated during telomere independent replicative senescence, and its inhibition partially delays the onset of senescence, extending lifespan (Kang *et al*, 2005). In addition, p38 has been shown to be an important player in the positive feedback loop that keeps ROS levels elevated after the DDR in response to dysfunctional telomeres (Passos *et al*, 2010). Hog1 the orthologue of p38 in budding yeast shares a lot of similarities with p38, however no studies exist for its role during replicative senescence. Therefore, an important part of my thesis was dedicated on studying if Hog1 plays a role in replicative senescence and more specifically in regulating ROS levels that may arise.

IV- Chapter IV: The Hog1 pathway

Mitogen-activated protein kinase (MAPK) cascades are a highly conserved signalling mechanism used by eukaryotic cells to respond to various environmental stimuli (Plotnikov *et al*, 2011). These cascades play a crucial role in regulating various cellular processes, such as proliferation, differentiation, cell death, and the maintenance of cellular homeostasis (Plotnikov *et al*, 2011). By transmitting signals from the cell surface to the nucleus, MAPK cascades act as vital coordinators of cellular responses, allowing organisms to adapt properly their behaviour to environmental changes.

In budding yeast, there are five distinct MAPKs, each associated with specific cellular processes (Gustin *et al*, 1998). These cascades include the pheromone response pathway, involved in mating; the filamentation/invasion pathway, responsible for filamentous growth and invasive behaviour; the cell integrity pathway, essential for maintaining cell wall integrity; the spore wall assembly pathway, involved in the formation of the spore wall during sporulation; the high osmolarity glycerol (HOG) pathway, associated with response to osmotic stress. Initially discovered in 1993, High Osmolarity Glycerol 1 (Hog1), is the MAPK involved in the response to hyperosmotic stress (Brewster *et al*, 1993). Subsequent research revealed an activation and involvement of Hog1 in response to various stresses beyond salt stress, including heat (Winkler *et al*, 2002), low external pH (Kapteyn *et al*, 2001), citric acid (Lawrence *et al*, 2004), low temperature (Panadero *et al*, 2006), methylglyoxal (Aguilera *et al*, 2005), arsenite (Sotelo & Rodríguez-Gabriel, 2006), acetic acid (Mollapour & Piper, 2006), zymolyase (García *et al*, 2009), oxidative stress (Singh, 2000), and others. Hog1 is a multifunctional protein that plays a role in several cellular processes acting in the cytoplasm and the nucleus (de Nadal & Posas, 2022).

In this chapter, I will provide an overview of the HOG1 signalling cascade, and the different functions of Hog1 during osmotic stress then I will focus on the studies that unveiled the role of Hog1 in oxidative stress as well as regulating autophagic processes in budding yeast due to their role during replicative senescence.

1. Hog1 structure

The *HOG1* sequence in budding yeast localizes on chromosome 7 and consists of a single large open reading frame that measures 1,3 kb. This open reading frame encodes a protein comprised of 435 amino acids, with a molecular weight of approximately 48 kDa. Within the subdomain VIII of Hog1, there are residues Thr-174 and Tyr-176, which are similar to other MAPKs and are known to be phosphorylated in response to extracellular signals. These phosphorylation events are essential for the activation of Hog1 and its subsequent activity in cellular signalling pathways. Hog1 protein domains and different important regions have been mapped by two different studies (*Figure 12*).

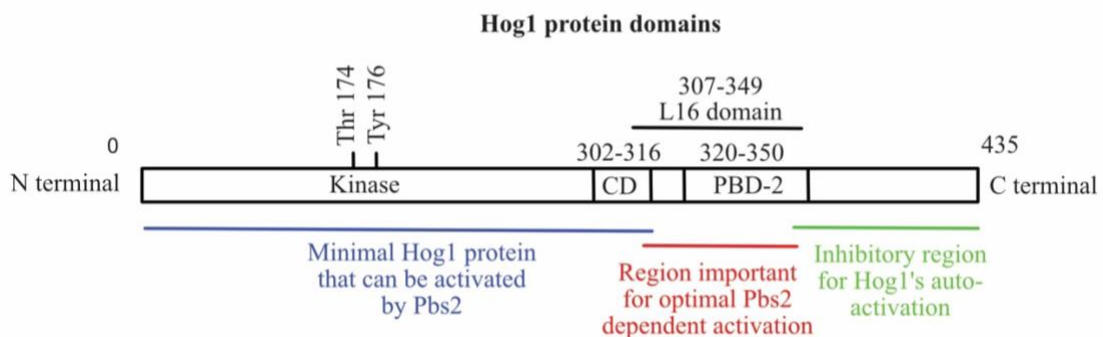


Figure 12 : Hog1 protein identified domains and regions.

Schematic map of the Hog1 protein domains and regions identified in two different studies. On the N-terminal side, the biggest domain is the kinase catalytic domain. Then the common docking domain (CD) important for interacting with different proteins and substrates like the MAPKK Pbs2 (activator), Ptp2 (inactivator) and Rck2 (substrate). The PBS2 binding domain (PBD-2) for binding with Pbs2 and Ptp2. The L16 domain, a conserved domain of MAPKs, that can be subject to conformational changes on protein interactions. Three important regions have also been identified for the minimal and optimal activity dependent on Pbs2 and an inhibitory region for Hog1 autophosphorylation. Thr 174 and Tyr 176 represent the threonine and tyrosine residues that need to be phosphorylated in order to activate Hog1. Adapted from (Murakami et al, 2008; Maayan et al, 2012).

2. HOG1 pathway: activation and regulation

2.1. Hog1 canonical cascade activation

A typical MAPK cascade involves a sequential series of three protein kinases that undergo phosphorylation and activation in response to a specific stimulus (Plotnikov *et al*, 2011). The central component, known as the MAPK, is activated through phosphorylation on specific threonine and tyrosine residues within its activation loop. This phosphorylation process is

facilitated by a dual-specificity MAPK kinase (MAPKK). Furthermore, the activation of the MAPKK itself relies on phosphorylation by another protein kinase called a MAPKK kinase (MAPKKK). Once fully activated, the MAPK can phosphorylate a variety of substrates, including transcription factors and cytosolic proteins. The Hog1 cascade can be activated by two branches: the Sln1 branch and the Sho1 branch. These two branches are functionally redundant but mechanistically different and appear to specialize in detecting distinct osmotic conditions (Maeda *et al*, 1994a; Van Wuytswinkel *et al*, 2000). When responding to a moderate increase in solute concentrations, Sln1 plays a more critical role in activating Hog1 (Maeda *et al*, 1995). Additionally, the activity of Sln1 is necessary for the expression of several reporter genes under high salt conditions (Van Wuytswinkel *et al*, 2000). Importantly, Sho1 is an inducible system with no basal activity. However, the Sln1 branch has a high basal activity that's restricted by a negative feedback system. The basal activity is essential for a higher efficiency and gives a faster response upon stress (Macia *et al*, 2009).

2.1.1. Sln1 branch of Hog1 pathway

The discovery of the transmembrane sensor protein Sln1 in 1994 (Maeda *et al*, 1994b) shed lights on the regulation of the first branch of the Hog1 pathway. Sln1 belongs to a specific subtype of sensors that possess both a histidine kinase and a receiver domain. This branch operates through a phosphorelay mechanism within the Sln1-Ypd1-Ssk1 system (Figure 13). Under normal growth conditions, Sln1 is activated, initiating a sequential transfer of a phosphate group from Sln1-His476 to Sln1-Asp1144, then to Ypd1-His64, and finally to Ssk1p-Asp554 (Posas *et al*, 1996). Phosphorylated form of Ssk1 cannot interact with the MAPKKKs of this branch and therefore Hog1 remains inactive. However, in response to osmotic stress, Sln1 is inactivated, leading to unphosphorylated Ssk1. The MAPKKKs of this branch are redundant proteins called Ssk2 and Ssk22 (Maeda *et al*, 1994a). When Ssk1 is inactivated, it binds to the N-terminal inhibitory domain of Ssk2/22, resulting in their activation by auto-phosphorylation. Phosphorylated Ssk2/22 then binds on a specific docking site in the N-terminal region of the MAPKK Pbs2 (Tatebayashi *et al*, 2003) and phosphorylates Pbs2, which in turn phosphorylates Hog1, leading to its activation. In addition to its role in activating Ssk2/22, Ssk1 also plays a negative regulatory role in the pathway (Horie *et al*, 2008). A study has revealed that Ssk1 primarily exists in a dimeric form in the cell, which can take three different forms: Ssk1-P/Ssk1-P, Ssk1-P/Ssk1-OH, and Ssk1-OH/Ssk1-OH. Dimers containing Ssk1-P are unable to activate Ssk2/22 and, if Ssk1-P is present in a dimer with Ssk1-OH, it

counteracts the activation of Ssk2/22. Only the unphosphorylated dimer, Ssk1-OH/Ssk1-OH, is able to bind two molecules of Ssk2/22 and activates them. However, *in vivo* confirmation of Ssk2/22 dimerization is still lacking. Under normal conditions, this negative regulatory role of Ssk1-P is crucial to maintain low levels of Ssk1-OH activation and preventing the inappropriate activation of Hog1 under normal conditions. It has been suggested that Sln1 acts as a turgor pressure sensor where its periplasmic region is essential for its function (Reiser *et al*, 2003; Tao *et al*, 1999). Sln1 activity has also been associated with the presence and absence of outer cell wall proteins (Narang *et al*, 2008). Moreover, how Sln1 senses the changes in osmotic stress and if phosphatases participate in Ssk1 dephosphorylation remain elusive.

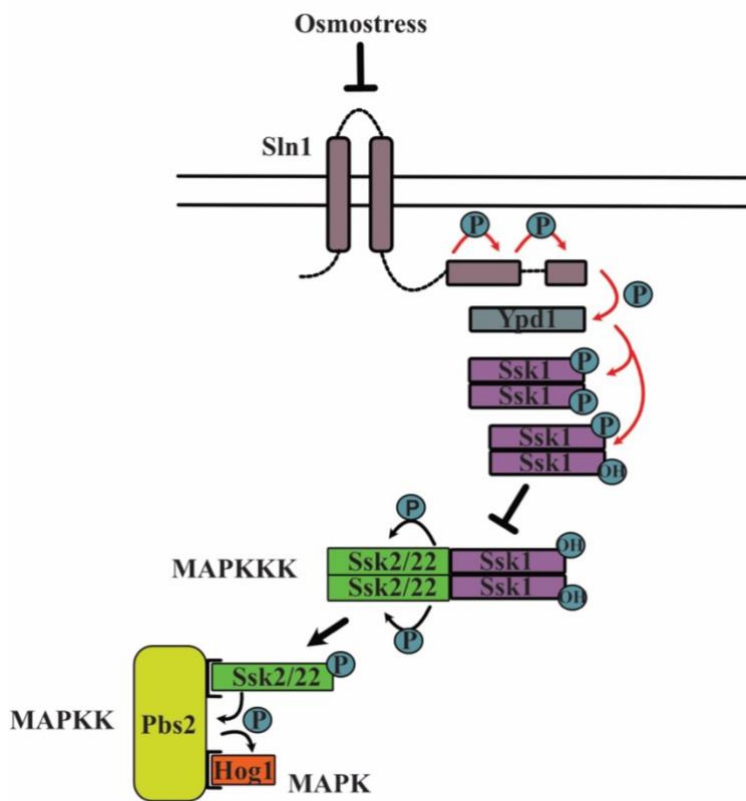


Figure 13 : Model of Sln1 branch in the Hog1 pathway signalling.

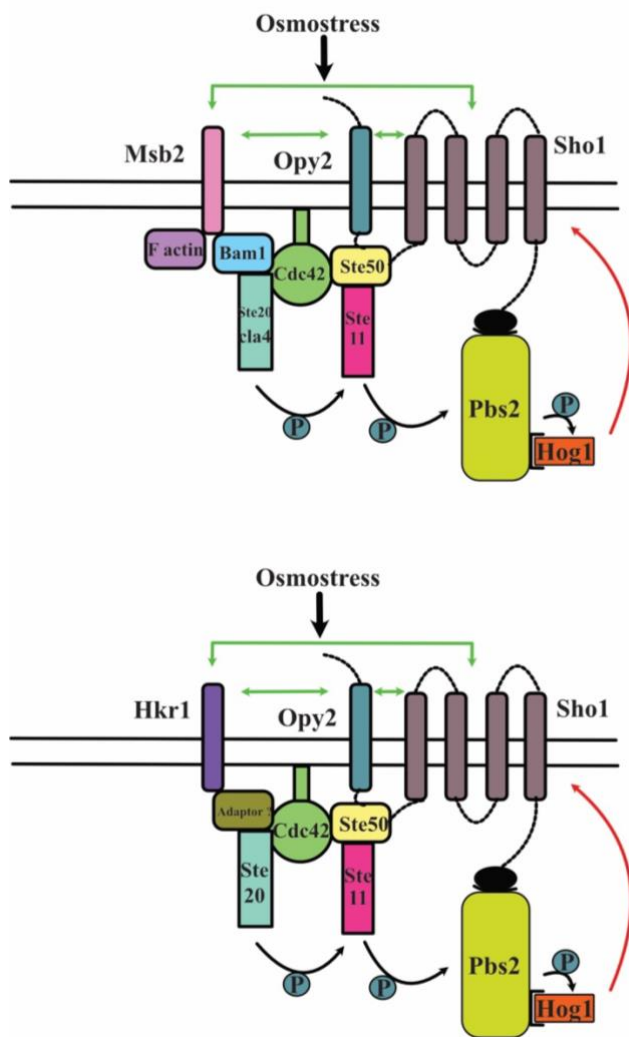
*In normal osmotic conditions, Sln1 auto-phosphorylated on His-576 transfers the phosphate group to Asp-1144. The phosphate group is then transferred to His-64 Ypd1 then finally to Asp-554 of Ssk1. Ssk1-P/Ssk1-P dimers and Ssk1-P/Ssk1-OH dimers cannot activate Ssk2/Ssk22. Upon osmotic stress, Sln1 detects turgor changes and is inhibited resulting in unphosphorylated Ssk1. Ssk1-OH/Ssk1-OH dimers will bind to Ssk2/22 and phosphorylates these two redundant MAPKKK. Ssk2/22 activated will then interact with Pbs2 and phosphorylate Pbs2. Pbs2 will then interact and activate Hog1 through phosphorylation. The dimerization of Ssk2/22 *in vivo* has not yet been identified.*

2.1.2. Sho1 branch of Hog1 pathway

A second branch of the Hog1 activation pathway was suspected when a double mutant *ssk2 ssk22* still resulted in the activation of Hog1 under osmotic stress conditions (Maeda *et al*, 1994a) (Figure 14). In 1995, the SH3-containing protein called Sho1 (Synthetic High Osmolarity Glycerol 1) was identified as an osmosensor composed of four transmembrane segments acting separately from the first branch (Maeda *et al*, 1995). This was supported by

findings that a triple mutant of *ssk2 ssk22 sho1* resulted in cells sensitive to osmotic stress and in the non-activation of Hog1 (Maeda *et al*, 1994a). Sho1 has the ability to interact with the proline rich motif in the N-terminal region of Pbs2, and this interaction is crucial for the activation of Pbs2 (Maeda *et al*, 1995). However, this interaction alone is not sufficient for Pbs2 to activate Hog1; Pbs2 requires phosphorylation. In this branch, a different MAPKKK called Ste11 is involved (Posas & Saito, 1997). Interestingly, Ste11 is shared between the Hog1 pathway and the pheromone response pathway, but there is no cross talk between these two pathways (Posas & Saito, 1997). Another protein called Ste50, which also plays a role in the pheromone response, is essential for this branch. Ste50 is constitutively associated with Ste11 and its binding to Ste11 is necessary for its activation (Posas *et al*, 1998). Ste50/Ste11 is anchored to the membrane by Opy2, a single transmembrane domain protein. Opy2 interacts through its C terminal fragment with the RA-like domain of Ste50 and will thus localize the Ste11 bound to Ste50 by their SAM domains to the membrane (Wu *et al*, 2006). Ste20 and Cla4 which are functionally redundant and Cdc42 are also essential for this branch (Tatebayashi *et al*, 2006). Sho1 will bind PBS2 targeting it to the membrane, where Cdc42-bound membrane localized Ste20/Cla4 phosphorylates Ste11 (Raitt *et al*, 2000) which will lead to the phosphorylation of Pbs2 and Hog1. Later, studies unveiled that Sho1 is an essential adaptor of this branch but may not sense the change in osmolarity by itself. The osmosensors of this branch are two mucin-like transmembrane proteins: Hkr1 and Msb2 that form a complex with Sho1 (Tatebayashi *et al*, 2007). Msb2 and Hkr1 are redundant and are suggested to directly monitor osmotic changes that will result in their interaction with Sho1 through their TM domain that will generate an intracellular signal. These two osmosensors activate Hog1 in different ways even if they are redundant (Tanaka *et al*, 2014). Ste11 is activated by Ste20/Cla4 through Msb2 but only by Ste20 through Hkr1. This is because Msb2 requires Bam1 that binds to both Ste20 and Cla4. The actin cytoskeleton also participates in the signalling through Msb2 but its function is still unclear. Nevertheless, Ste20 is also capable of binding Sho1 and contributes to Hog1 activation through Hkr1. The membrane bound proteins also interact: Msb2 and Hkr1 interact with Opy2 and Opy2 interacts with Sho1 (Yamamoto *et al*, 2016; Takayama *et al*, 2019). Negative feedback loop has been identified in this branch. Indeed, Hog1 can phosphorylate Sho1 at Ser-166 that will result in the loss of Sho1 oligomerization and diminishes the signal through this branch (Hao *et al*, 2007).

Figure 14: Model of Sho1 branch in the Hog1 pathway signalling.



The difference between the panels is whether *Msb2* or *Hkr1* stimulates *Sho1*. Upon osmotic stress, *Msb2* or/and *Hkr1* stimulate *Sho1* and interact with *Opy2* which also interacts with *Sho1*. *Msb2* interacts with *Bam1* which interacts with *Ste20/Cla4* and *Cdc42*-bound GTP. *Opy2* recruits *Ste50/Ste11* to the membrane. *Ste50* also interacts with *Cdc42* and *Sho1*. *Sho1* recruits *Pbs2* to the membrane which recruits *Hog1*. *Ste20/Cla4* activated by *Cdc42* will phosphorylate the auto-inhibitory domain of the MAPKKK *Ste11* and leads to its activation. *Ste11* phosphorylates residues in the activation loop of the MAPKK *Pbs2*. Lastly, *Hog1* will be phosphorylated by *Pbs2*. The green arrow represents the interactions between the membrane proteins. The red arrow represents the negative feedback loop; *Hog1* phosphorylates *Sho1* resulting in a diminished signal. Not all protein interactions are represented. Another model where *Ste20* binds also *Sho1* and contributes to *Hog1* activation was proposed but was not represented here. The sequential events as written in the description may not be in order. Adapted from (Tanaka et al, 2014).

2.2. Downstream *Pbs2* activation: *Hog1* phosphorylation

The *Sln1* and *Sho1* branches converge and lead to the activation and phosphorylation of *Pbs2* on two conserved residues Ser-514 and Thr-518. Mutating the phosphoacceptors sites of *Pbs2* to alanine causes osmosensitivity, in contrast to their mutation to aspartic acid that causes the constitutive activation of *Hog1* (Posas & Saito, 1997; Wurgler-Murphy et al, 1997). However, *Ste11* and *Ssk2/22* phosphorylate *Pbs2* differentially. *Ste11* only phosphorylate Thr-518 and *Ssk2/22* phosphorylate both residues. Monophosphorylated *Pbs2* can only phosphorylate *Hog1* in conditions of osmotic stress. This would prevent crosstalk's between the pheromone pathway and the high osmolarity pathway in the absence of osmotic stress and would limit the activation of *Hog1* by basal activity of MAPKKKs. In addition, *Pbs2* seems to function as a signal integrator that integrates different kinetic responses from the two upstream branches:

the dose response of the Sln1 branch and the duration response of the Sho1 branch (Macia *et al*, 2009).

Hog1 is activated by Pbs2 mediated phosphorylation on Thr-174 and Tyr176. Thr-174 phosphorylation seems to stabilize an active catalytic conformation of Hog1, and Tyr-176 phosphorylation serves more to amplify the activity of Hog1 in response to external stimuli (Bell & Engelberg, 2003). The expression level of Hog1 is independent of its MAPKK Pbs2 (Brewster *et al*, 1993) which seems to only be involved with its phosphorylation. The activation of Hog1 varies depending on the severity of the osmotic shock. In cases of low to mild osmotic shock, Hog1 undergoes rapid but transient activation, with the maximum tyrosine phosphorylation occurring at 300 mM of NaCl within one minute. This level is maintained for 15 to 20 minutes (Brewster *et al*, 1993). Conversely, severe osmotic stress can extend the activation of Hog1 for up to 100 minutes (Hohmann *et al*, 2007). Importantly, a study suggests that Hog1 basal activity which is not detected by immunoblotting techniques is required for some functions. Hog1 deletion results in a deregulation in Golgi compartments localized Mnn1 protein involved in the O-mannosylation (Reynolds *et al*, 1998). The basal activity of Hog1 is suggested to be controlled by MAPK-specific protein phosphatases (Wurgler-Murphy *et al*, 1997). Interestingly, an arsenite stress activates Hog1 by inhibiting phosphatases confirming a basal phosphorylation of Hog1 (Lee *et al*, 2019).

In addition to the well-established canonical pathway, several studies have suggested the existence of an alternative route for Hog1 activation under severe osmotic stress. For instance, in strains where *ssk2*, *ssk22*, and *stel1* are deleted, Pbs2 can still be activated (Van Wuytswinkel *et al*, 2000). Moreover, in osmoshocked *ssk1* mutant cells, Pbs2 can be phosphorylated by Ssk2 *in vitro* (Bettinger *et al*, 2007). Additionally, Ssk2 can be activated in *ssk1*, *ssk22*, and *stel1* triple mutant cells (Zhi *et al*, 2013). These findings indicate the presence of an alternative mechanism for Hog1 activation dependently on Pbs2 in response to severe osmotic stress. Importantly, Hog1 can also undergo autophosphorylation when strongly overexpressed upon high osmotic stress (1M of NaCl) but autophosphorylation is inhibited by a C terminal inhibition region (Maayan *et al*, 2012). However, if Hog1 is phosphorylated when there is a defective connection with osmosensors, it's not sufficient to induce the major response necessary for adaptation to osmostress (Vázquez-Ibarra *et al*, 2018).

2.3. *Hog1 and Pbs2 dephosphorylation*

The level of phosphorylation on threonine and tyrosine residues of MAPKs plays a crucial role in determining the overall signal output, and this phosphorylation is tightly regulated by phosphatases. In the case of Hog1, studies have identified Ptp2 and Ptp3 as the major tyrosine phosphatases responsible for dephosphorylating the tyrosine residue in Hog1 (Jacoby *et al*, 1997; Maeda *et al*, 1994b; Wurgler-Murphy *et al*, 1997) (Figure 15). While deletion of Ptp3 alone does not have a significant effect on Hog1 phosphorylation, its combined deletion with Ptp2 leads to a notable increase in tyrosine phosphorylated Hog1, indicating their cooperative role in tyrosine dephosphorylation (Wurgler-Murphy *et al*, 1997). The double mutant lacking both phosphatases shows constitutive phosphorylation of Hog1, even in the absence of osmotic stress, perhaps related to its basal activation. Furthermore, the expression of *PTP2* and *PTP3* is induced in response to osmotic stress in a Hog1-dependent manner, indicating that these phosphatases operate in a negative feedback loop to inactivate Hog1 (Jacoby *et al*, 1997). These findings suggest that Hog1 possesses the ability to regulate its own dephosphorylation, involving the interplay between Ptp2 and Ptp3 (Wurgler-Murphy *et al*, 1997). Ptp2 has been shown to be predominantly nuclear while Ptp3 appears to be cytoplasmic and excluded from the nucleus (Mattison *et al*, 1999).

In addition to tyrosine phosphatases, the serine/threonine phosphatases Ptc1, Ptc2, Ptc3 and Ptc4 play a role in regulating the Hog1 pathway (Warmka *et al*, 2001; Young *et al*, 2002; Shitamukai *et al*, 2004) (Figure 15). While Ptc1 is responsible for maintaining low levels of basal activity and regulates Hog1 during adaptation, Ptc2 and Ptc3 redundantly limit the maximal activity of Hog1. Ptc1 requires Nbp2 (Nap1 Binding protein 2) to be able to dephosphorylate Hog1. Nbp2 binds Pbs2 through its SH3 domain and Ptc1 through its N-terminal domain, Nbp2 thus mediates the binding between these two proteins. It was also suggested that Ptc1 dephosphorylates Hog1 bound by Pbs2 (Mapes & Ota, 2004). The three phosphatases Ptc1, Ptc2 and Ptc3 are present in both the nucleus and cytoplasm, and their localization remains unaffected by osmotic shock (Warmka *et al*, 2001; Young *et al*, 2002). Ptc1, Ptc2, Ptc3 and Ptc4 also dephosphorylate Pbs2 (Tatebayashi & Saito, 2023). Ptc1 mainly dephosphorylates the Thr-518 and participates with Ptc2, Ptc3 and Ptc4 in the dephosphorylation of Ser-514.

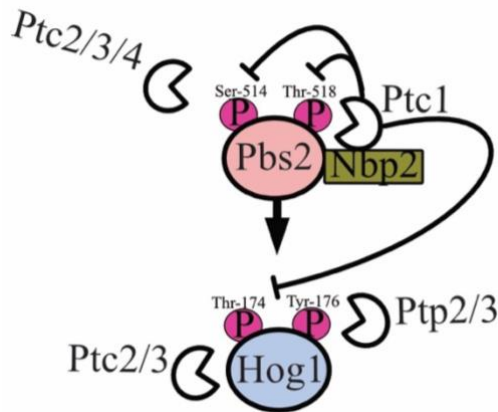


Figure 15: Phosphatases involved in the Hog1 pathway upon osmotic stress.

The Ptc1/2/3/4 phosphatases dephosphorylate Pbs2. Ptc1/2/3/4 dephosphorylate the Ser-514 while Ptc1 dephosphorylates mainly the Thr-518 through Nbp2. Ptc1 also dephosphorylates the Thr-174 of Hog1 together with Ptc2/3. Tyr-176 of Hog1 is dephosphorylated by Ptp2/3. Adapted from (Tatebayashi & Saito, 2023).

2.4. The lethality of Hog1 sustained phosphorylation

Numerous studies using mutants of the two branches of Hog1 pathway have demonstrated that Hog1 constitutive phosphorylation is lethal. Deletion of *sln1* is lethal but is rescued by the overexpression of phosphatases and Ssk1 or Hog1 deletion (Maeda *et al*, 1994b). Mutations in Sln1-His576, Sln1-Asp1144, and Ypd1-His64 result in the constitutive activation of Hog1 and cell death (Posas *et al*, 1996). The mutation in the N-terminal domain of the MAPKKK Ssk2/22 (Maeda *et al*, 1994a) and Ste11 (Posas & Saito, 1997) that constitutively activates them result in lethality and is abolished by Pbs2 or Hog1 deletion (Maeda *et al*, 1994a). Overexpression of phosphatases Ptc1, Ptc2 and Ptc3 rescues the lethality of sustained Hog1 activation, however, their deletion does not cause hyperactivation of Hog1 and growth defect. Interestingly, the overexpression of Hog1 is not lethal to the cells (Varela *et al*, 1995). The overexpression was only lethal in combination with a double mutant *ptp2 ptp3* but not in the respective single mutant (Wurgler-Murphy *et al*, 1997). A study isolated 9 mutants of Hog1 containing single point mutations that renders them hyperactive catalytically and biologically (Yaakov *et al*, 2003; Bell *et al*, 2001). These mutants can function independently of Pbs2 and endogenous Hog1. Six mutations are found in the L16 dimerization domain, two are located at the NH2 terminus and one just four amino acids from the phosphoacceptors Thr¹⁷⁴. All of these mutants showed a dramatic reduction of cell growth under optimal conditions and an increase in cell aggregation and flocculation. All of these results highlight the importance of the precise Hog1 regulation and the danger of its sustained activation. The lethality of Hog1 sustained activation will be further discussed later.

2.5. *Hog1* localization

The MAPK proteins lack classical nuclear import signals, thus non-conventional mechanisms are essential for their translocation in the nucleus. Even under non stressed conditions, Hog1 fusion with a GFP protein has been shown to shuttle between the nucleus and the cytoplasm. After its phosphorylation, Hog1 translocates transiently to the nucleus after an osmotic stress but all proteins working upstream this MAPK stay in the cytoplasm (Ferrigno *et al*, 1998; Reiser *et al*, 1999). Thus, in stressed conditions Hog1-P is concentrated in the nucleus and not in the cytoplasm. However, even if Pbs2 was not detected in the nucleus, it contains a nuclear exclusion signal that allows Pbs2 to accumulate in the nucleus if its hidden (Tatebayashi *et al*, 2003). Hog1 importation to the nucleus upon its activation requires *NMD5* that encodes importin B homolog and Ran-GSP1 a small GTP-binding protein. Export of Hog1 from the nucleus requires XPO1/CRMI a NES receptor. The localization of Hog1 in the cytoplasm or in the nucleus depends on various mechanisms (Reiser *et al*, 1999). Hog1 phosphorylation is crucial for its nuclear accumulation and the translocation of Hog1 into the nucleus correlates with the kinetics of its phosphorylation both peaking at 1 minute of mild osmotic stress then declining slowly after 15 minutes of maximum activation (Reiser *et al*, 1999; Mattison & Ota, 2000). A non phosphorylable form of Hog1, the deletion of Pbs2 or a dead kinase Pbs2 results in the retention of Hog1 in the cytoplasm after an osmotic stress. In contrast, the constitutive activation of MAPKKK induces the accumulation of Hog1 in the nucleus even in normal osmotic conditions. It has been also shown that the nuclear targets Msn2/Msn4 factors play a role in the retention of Hog1 in the nucleus and that the kinase activity of Hog1 is essential for its rapid nucleocytoplasmic transport (Westfall & Thorner, 2006). Even if the phosphorylation of Hog1 is crucial for its translocation, its dephosphorylation has been shown not to be important for its export (Mattison & Ota, 2000). Moreover, Ptp2 and Ptp3 play the role of anchors and regulate the localization of Hog1. Ptp2 is concentrated in the nucleus and acts as a nuclear anchor, whereas, Ptp3 tethers Hog1 to the cytoplasm. The C terminal part of Pbs2 also seems to play a role in the nuclear localization of Hog1 (Sharma & Mondal, 2005).

3. Hog1 multifunction's

Hog1 is a highly versatile protein with functions that extend beyond the simple induction of genes (de Nadal & Posas, 2022). The activation of several transcription factors by Hog1 will be discussed in this part, however, Hog1 exhibits other various important roles both in the cytoplasm and in the nucleus that are crucial for stress adaptation (*Figure 16*).

In the cytoplasm, Hog1 can regulate the function of transporters through direct phosphorylation (Proft & Struhl, 2004) and plays a role in the repositioning of the actin cytoskeleton upon osmotic stress (Brewster & Gustin, 1994). This MAPK regulates multiple stages of the cell cycle including G₁/S transition, G₂/M transition, and S phase essential for the proper stress response adaptation. This role of Hog1 regarding the cell cycle shed the light on its involvement in the transcriptional induction of LncRNAs in response to osmotic stress (Nadal-Ribelles *et al*, 2014). Of many LncRNAs which are dependent on Hog1 after stress, the antisens LncRNA of Cdc28, which encodes the yeast cyclin CDK1, is induced by Hog1. This induction promotes the gene expression of *CDC28* and allows the cells to re-enter efficiently the cell cycle after stress.

Hog1 regulates various steps in mRNA biogenesis, from initiation of transcription to mRNA export, to ensure optimal gene expression after stress. Studies have demonstrated that Hog1 directly interacts with RNA polymerase II and III (Alepuz *et al*, 2003; Nadal-Ribelles *et al*, 2012). Furthermore, Hog1 induces chromatin changes at stress-responsive elements (Nadal-Ribelles *et al*, 2012). Hog1 recruits the histone deacytelase Rpd3 (De Nadal *et al*, 2004), targets the RSC complex (Mas *et al*, 2009), the SAGA complex and the mediator complex to osmo-responsive genes (Zapater *et al*, 2007). Hog1 also plays a role in transcription elongations and is found throughout the coding regions of genes (Proft *et al*, 2006). Therefore, Hog1 acts as a selective transcription factor for genes involved in the osmostress response. In addition, Hog1 phosphorylates the elongation factor Spt4, which regulates the processivity of RNA polymerase II and is crucial for survival under high osmolarities (Silva *et al*, 2017). Hog1 also transiently inhibits translation in response to osmotic stress (Warringer *et al*, 2010; Uesono & Toh-E, 2002). Notably, translation regulation by phosphorylating Rck2, is even more dependent on Hog1 than transcription regulation after stress (Warringer *et al*, 2010; Bilsland-Marchesan *et al*, 2000; Teige *et al*, 2001). Moreover, Hog1 is involved in mRNA stability (Molin *et al*, 2009; Romero-Santacreu *et al*, 2009) and export (Regot *et al*, 2013). It associates with and

phosphorylates components of the inner nuclear pore complex, such as Nup1, Nup2, and Nup60, which are essential for growth and gene expression under osmostress conditions. Failure to properly regulate these components can lead to alterations in mRNA export (Regot *et al*, 2013).

Interestingly, Hog1 regulates transcriptional silencing at telomeres and has a global impact on heterochromatin localization (Mazor & Kupiec, 2009). Severe osmotic stress compromises the binding of Sir3 and Sir2 at telomeres, and their re-association is dependent on Hog1 and occurs rapidly after 30 minutes.

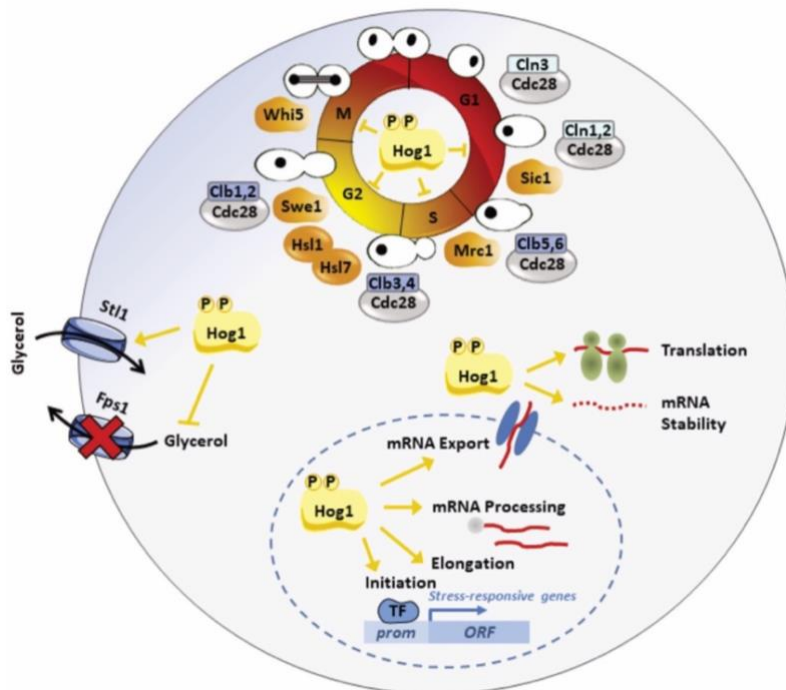


Figure 16: Hog1 orchestrates several cellular functions upon osmostress.

In the cytoplasm, Hog1 exerts its influence on multiple levels, including the regulation of transporters, cell cycle progression, translation, and mRNA stability. Hog1 regulates transporters by directly phosphorylating and modulating their activity. Moreover, Hog1 is involved in the transition between different cell cycle phases. It influences cell cycle regulators, such as cyclins and CDKs, to ensure proper progression through the cell cycle under

osmotic stress. This coordination between Hog1 and the cell cycle machinery enables cells to respond effectively to stress while maintaining cell cycle integrity. Additionally, Hog1 plays a role in translation and mRNA stability. Hog1 also translocates into the nucleus, where it induces gene expression through various mechanisms including direct interaction with transcription factors, chromatin remodelling, and recruitment of transcriptional co-regulators and plays a crucial role in mRNA metabolism. Hog1's actions in the nucleus contribute to the activation of stress-responsive genes, allowing cells to mount an appropriate response to osmotic stress. Furthermore, Hog1 is involved in mRNA metabolism at multiple levels within the nucleus. By modulating these processes, Hog1 ensures proper mRNA maturation, transport, and utilization, ultimately impacting gene expression and cellular adaptation to osmotic stress. Retrieved from (de Nadal & Posas, 2022).

3.1. Gene expression regulation through transcription factors

Studying the transcriptional response to osmotic stress has revealed that the majority of induced genes exhibit a strong or complete dependency on Hog1 (Posas *et al*, 2000). The concentration of NaCl and the duration of exposure play critical roles in determining the different responses and various levels of gene involvement. The initial investigations on Hog1 revealed multiple transcription factors that play a role in gene induction during osmotic stress. Among these transcription factors, Msn2/4, Hot1, Smp1, and Sko1 have been extensively studied (Figure 17). There are additional transcription factors regulated by Hog1 (Ruiz-Roig *et al*, 2012), although they will not be discussed in detail here.

3.1.1. Msn2/Msn4

Msn2/Msn4 are responsible for the transcriptional control of numerous genes in a Hog1-dependent manner during osmotic stress. Mutations in Hog1 have been shown to abolish the response mediated by Msn2/Msn4 through STREs specifically in osmotic stress, while the response to other stresses remains unaffected (Schüller *et al*, 1994). For example, deficiencies in these factors lead to reduced transcription of *CTT1* (Martínez-Pastor *et al*, 1996b) and *HSP12* (Varela *et al*, 1995), which are known to be induced through their STREs elements, in response to osmotic stress. It is worth noting that Msn2, but not Msn4, is specifically induced upon osmotic stress (Proft *et al*, 2005), and Msn2 can be directly phosphorylated by Hog1 (Vendrell *et al*, 2011). In addition, Msn2/Msn4 are potentially involved in the induction of Ptp2 upon osmotic stress and play a role in the nuclear retention of Hog1 (Jacoby *et al*, 1997; Reiser *et al*, 1999). However, a viable double mutant lacking both Msn2 and Msn4 has been observed under osmotic stress conditions and Msn2/Msn4 can accumulate in the nucleus following osmotic stress in a Hog1-independent manner (Görner *et al*, 1998).

3.1.2. Sko1

In 1999, Sko1 was initially identified as a downstream effector of the Hog1 pathway involved in the repression of the *ENAI* gene, which is crucial for the osmotic stress response (Proft & Serrano, 1999). Sko1 is a CREB (cAMP response element-binding protein) transcription factor that belongs to the bZIP (basic leucine zipper) family of repressors. It directly binds to CREs (cAMP response element) sequences in gene promoters, repressing their activity through the Ssn6-Tup1 corepressor complex. Sko1 possesses three phosphorylation

sites targeted by Hog1, and upon activation, these phosphorylation events disrupt the interaction between Sko1 and Tup1 (Proft *et al*, 2001; Pascual-Ahuir *et al*, 2001). During osmotic stress, the activity of Hog1 leads to the relief of gene repression by Sko1, causing Sko1 to be detected in the cytoplasm (Proft & Serrano, 1999; Pascual-Ahuir *et al*, 2001). However, Sko1 phosphorylation not only indirectly induces gene transcription but also plays a role in the recruitment of the SAGA histone acetylase and SWI/SNF nucleosome remodeling complex to promoters of genes induced by osmotic stress. This results in a switch from a repressed to an activated state, triggered by the phosphorylation by Hog1 (Proft & Struhl, 2002). Additionally, Sko1 can be regulated through PKA signalling, which affects its affinity to CREs motifs (Proft *et al*, 2001; Pascual-Ahuir *et al*, 2001). Sko1 regulates various factors *in vivo*, including stress defence proteins, regulators, and even other transcription factors (Proft *et al*, 2005). Notably, Sko1 can bind to the promoters of Msn2 and participates in its expression following osmotic stress. Moreover, the induction of Ptp3 after osmotic stress is fully dependent on Sko1. Importantly, under osmotic stress conditions, five genes (*GRE2*, *AHP1*, *SFA1*, *GLR1*, and *YML131w*) encoding oxidoreductases involved in the repair of oxidative damage are induced in a Hog1-Sko1-dependent manner (Rep *et al*, 2001).

3.1.3. Hot1

Hot1 is a transcriptional regulator that interacts with Hog1 and is phosphorylated in a Hog1-dependent manner upon osmotic shock (Rep *et al*, 1999). Hot1 plays a crucial role in the induction of enzymes involved in glycerol production, and its deletion leads to a significant reduction in the expression of *GPD1* and *GPD2* (Rep *et al*, 1999). A genome-wide analysis has demonstrated that the deletion of Hot1 affects nine genes during osmotic stress (Rep *et al*, 2000). However, only the complete induction of *STL1*, which encodes a putative sugar transporter, is abolished in the absence of Hot1 (Rep *et al*, 2000). In fact, there is evidence suggesting that Hot1 may be primarily responsible for the transcriptional regulation of the single gene *STL1* (Bai *et al*, 2015). Hot1 binds to the HoRE sequences (Hog1 Responsive Elements) specifically found in the promoter region of *STL1*, which are not present in other yeast promoters. In addition, Hot1 plays the role of an anchor and recruits Hog1 to osmo-induced genes which recruits the RNA polymerase II complex to promoters and thus plays a role in the initiation of transcription (Alepuz *et al*, 2003).

3.1.4. Smp1

Smp1 was identified as the fourth major transcription factor target of Hog1 in response to osmotic shock (de Nadal *et al.*, 2003). Upon osmotic shock, Hog1 interacts with and phosphorylates the C-terminal region of Smp1. Smp1 plays a critical role in controlling a subset of genes that are crucial for the osmotic response, including *STL1*. Deletion of Smp1 leads to increased sensitivity to osmotic stress. Furthermore, there is evidence suggesting that Smp1 mediates a role for Hog1 during the stationary phase of growth. Both the single mutants of *hog1* and *smp1* exhibit loss of viability during the stationary phase. This implies that Smp1 is involved in the regulation of gene expression and cell survival during this specific growth phase.

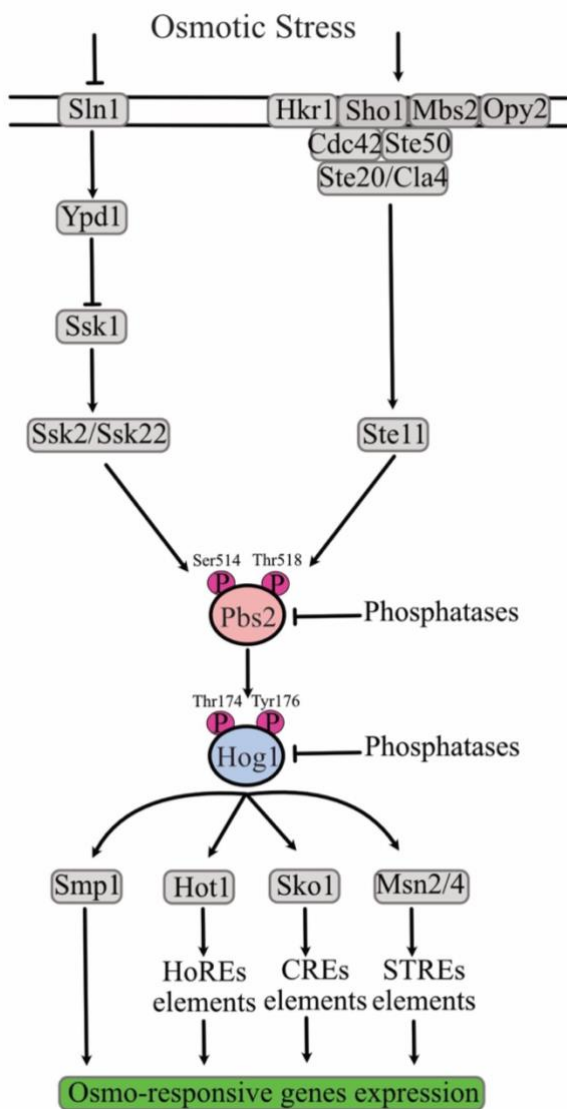


Figure 17: Hog1 controls gene expression through transcription factors upon osmostress.

Osmostress results in Hog1 activation through two different branches. Phosphorylated Hog1 translocates in the nucleus and then activates many transcription factors. These transcription factors induce the osmo-responsive genes expression essential for adaptation to high osmolarity through their binding on their respective elements or by other ways.

4. Hog1 and oxidative stress

Initially, Hog1 was believed to be primarily involved in the response to osmotic stress, with no apparent role in other stress conditions. Early studies indicated that Hog1 was not phosphorylated in response to a low concentration of H₂O₂ (0.4 mM) (Schüller *et al*, 1994) and did not translocate to the nucleus to influence gene expression (Reiser *et al*, 1999). In this part I will discuss subsequent research that provided substantial evidence supporting the essential role of Hog1 in oxidative stress.

4.1. Hog1 activation and localization upon oxidative stress

The involvement of the Hog1 pathway in the response to oxidative stress was firstly reported in 2000, by demonstrating the sensitivity of mutants in this pathway to H₂O₂ treatment (Singh, 2000). However, it was observed that the tyrosine phosphorylation of Hog1 was not induced after 10 minutes of 10 mM H₂O₂ treatment. This was consistent with another study that observed the lack of Hog1 phosphorylation following a 0.4 mM H₂O₂ treatment for 10 minutes (Schüller *et al*, 1994). These findings suggest that the phosphorylation of Hog1 might not be essential for its oxidative stress resistance phenotype (Singh, 2000). Subsequently, in 2004, it was demonstrated that Hog1 phosphorylation occurs in a H₂O₂ concentration-dependent manner (Haghnazari & Heyer, 2004). Hog1 activation begins at H₂O₂ concentrations of 0.5-1 mM and reaches its maximum activation between 5 and 7 mM H₂O₂. Above that Hog1-P is no longer detectable (Figure 18). This suggests that previous studies have employed either too low or too high H₂O₂ concentrations that did not allow for the detection of Hog1 phosphorylation. Hog1 was also observed to be phosphorylated and localize mainly in the cytoplasm when cells are treated with tBOOH, a pro-oxidant (Bilsland *et al*, 2004). In that case the peak of Hog1 phosphorylation occurs at 45-60 minutes. Later, a study reconfirmed that Hog1 is indeed phosphorylated upon 5mM of H₂O₂ from 5 to 30 minutes (Staleva *et al*, 2004). An additional study showed that Hog1 was the most phosphorylated at 3 mM of H₂O₂ and is detected from 5 minutes of treatment, reach the maximum at 10 minutes then diminished at 60 minutes (Lee *et al*, 2017b). It was also found that Hog1 is mainly cytoplasmic after its activation by H₂O₂ oxidative stress similarly to tBOOH. The absence of detectable Hog1 phosphorylation at high H₂O₂ concentrations, despite its requirement for cell viability, can be explained by two possible hypotheses (Singh, 2000; Haghnazari & Heyer, 2004). First, it is possible that there could be technical issues with the detection methods employed, such as difficulties in detecting

Hog1 phosphorylation due to post-translational modifications. These modifications could potentially affect the binding of antibodies. Alternatively, it is also plausible that Hog1 undergoes a different mode of activation or regulation at high levels of oxidative stress. This could involve alternative signalling pathways or additional modifications that may be independent from its phosphorylation. Accordingly, it has been shown in *Candida albicans* that after a nitrosative stress, Hog1 cysteines can be directly oxidized and are sufficient for its biological role independently of its phosphorylation (Herrero-de-Dios *et al*, 2018).

In addition, Hog1 activation is independent of checkpoint activation during oxidative stress (Haghnazari & Heyer, 2004). A mutant with kinase-defective Mec1 did not affect Hog1 phosphorylation following H₂O₂ treatment. The perfect additivity of these two phenotypes regarding sensitivity to oxidative stress further supports this distinction. This suggests that Hog1 may respond to cytoplasmic oxidative damage, while Mec1 responds to nuclear damage.

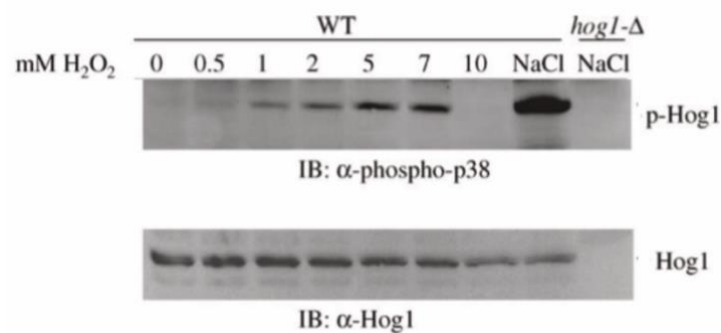


Figure 18: Hog1 phosphorylation upon different concentration of H₂O₂.

Hog1 is phosphorylated upon treatment with 0,5-1 mM to 7 mM of H₂O₂ for 10 minutes. The NaCl treatment is a positive control for Hog1 activation. The *hog1-Δ* strain serves as a negative control that shows the specificity of the signal. Retrieved from (Haghnazari & Heyer, 2004).

4.2. Hog1 signalling pathway in oxidative stress

First, a study showed that *Sln1 Ssk1* double mutant and Hog1 deletion results in H₂O₂ sensitivity in an epistatic manner (Singh, 2000). Sho1 mutant is slightly sensitive to H₂O₂, but the triple mutant *sho1 sln1 ssk2* is highly sensitive to oxidative stress. However, in a second study that focused on unravelling the precise pathway activation of Hog1 during oxidative stress, it was found that Sho1 is not involved in the oxidative stress response (Lee *et al*, 2017b). Spot assays with 4mM H₂O₂ revealed that *sho1Δ* and *ste11Δ* mutants were not sensitive to

oxidative stress, unlike the *pbs2Δ*, *hog1Δ*, *ssk1Δ*, and *ssk2Δ* single mutants. In contrast, the *ssk22Δ* mutant also did not show sensitivity to H₂O₂. Furthermore, Sho1, Ste11, and Ssk22 were not essential for Hog1 phosphorylation after oxidative stress, whereas Ssk1, Ssk2, and Pbs2 were required for Hog1 phosphorylation. Notably, Ssk2 could be activated independently of Ssk1, suggesting that under oxidative stress, Hog1 can be activated through alternative routes in addition to the canonical pathway observed during osmotic stress.

So far Hog1 activation by ROS was found only upon H₂O₂ treatment or diamide stress in budding yeast. Indeed, a *hog1* mutant has the same sensitivity than a wild type to UV treatment and gamma radiations which also induce oxidative lesions (Singh, 2000). In addition, the sensitivity of cells exposed to menadione, which also induces superoxide radicals is independent of Hog1 pathway. Accordingly, *sln1 ssk1* double mutant is not sensitive to a menadione treatment (Singh, 2000). In conclusion, current evidence suggests that the activation of Hog1 in response to H₂O₂ stress is primarily mediated through the Sln1-Ypd1-Ssk1-Ssk2-Pbs2 pathway, and Ssk2 serves as the MAPKKK that activates Hog1 specifically in response to oxidative stress (Figure 19). However, similar to osmotic stress, the activation of Hog1 through its branches may vary depending on the severity of the stress. By examining the response of Hog1 to different levels of H₂O₂, researchers can gain a deeper understanding of how the Hog1 pathway functions under oxidative stress conditions. This will allow for a more comprehensive analysis of Hog1's activation and signalling dynamics in response to varying degrees of stress severity.

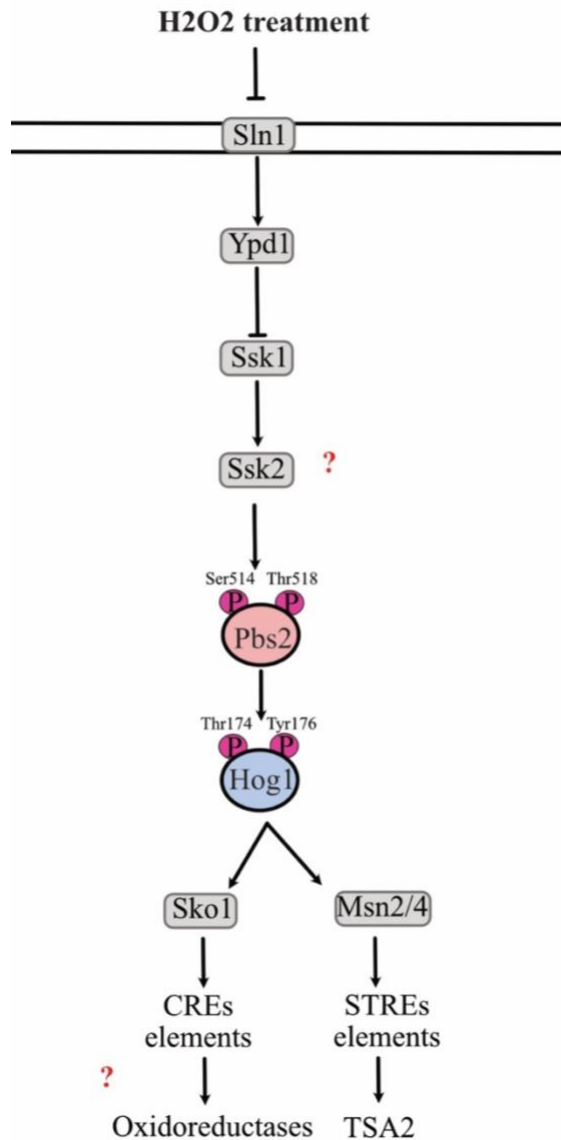


Figure 19: Hog1 signalling upon H₂O₂ treatment.

This scheme summarizes the signalling pathway of Hog1 in response to H₂O₂ treatment based on the cited and discussed papers. Upon oxidative stress, Hog1 appears to be activated through the Sln1-Ypd1-Ssk1-Ssk2-Pbs2 pathway, although the involvement of Sho1 is still under debate. The activation of Ssk2 by a parallel pathway is still unclear. Following activation, Hog1 predominantly remains in the cytoplasm but influences the activity of transcription factors Sko1 and Msn2/4. Sko1, acting through CREs elements, appears to control the basal expression of oxidoreductases that contribute to the defence against oxidative stress. However, the induction of these oxidoreductases upon oxidative stress is independent of Hog1. Msn2/4, on the other hand, acts through STREs elements and appears to induce the expression of genes such as *TSA2*. It is important to note that the contribution of other transcription factors in the oxidative stress response mediated by Hog1 remains to be investigated. Further research is necessary to determine the involvement of additional factors and their specific roles in the transcriptional response to oxidative stress dependently on Hog1.

4.3. Oxidative repair genes induced by Hog1

It appears that there is an overlap between the genes regulated under osmotic and oxidative stress conditions, suggesting a convergence of signal transduction pathways between these two types of stress. The interplay between osmotic and oxidative stress can be explained by several non-exclusive hypotheses. First, the decrease in cell volume and the subsequent increase in iron concentration after osmotic stress may lead to respiratory dysfunction, resulting in elevated levels of ROS. Second, stress-induced metabolic changes may promote enhanced oxidative metabolism for energy production, leading to an increase in ROS levels. Third, the production of glycerol during osmotic stress can impact the redox metabolism by reducing end-products, further affecting the cellular redox balance.

Five genes (*GRE2*, *AHP1*, *SFA1*, *GLR1*, and *YML131w*) encoding oxidoreductases involved in the repair of oxidative damage have been found to be induced in a Hog1-Sko1p dependent manner upon osmotic stress (Rep *et al*, 2001). However, in a *sko1* deletion mutant, these genes are highly expressed in a constitutive manner and show little to no induction during osmotic stress. Interestingly, during oxidative stress induced by H₂O₂ treatment, the induction of *GRE2* is not mediated by Hog1-Sko1p, but rather by the transcription factor Yap1 implicated in ROS detoxification. Nonetheless, the altered basal expression of these genes in *hog1 sko1* mutants upon H₂O₂ treatment suggests that they may partially contribute to the sensitivity of Hog1 mutants to oxidative stress. Hog1 appears to play a crucial role in regulating the Tsa2 antioxidant protein (Wong *et al*, 2003). *TSA2* is known to be induced in response to osmotic stress and H₂O₂-induced oxidative stress. The increase in *TSA2* expression following osmotic shock is entirely dependent on the presence of Hog1. During oxidative stress, Hog1 collaborates with other factors such as Snk7 and Yap1 to facilitate the upregulation of *TSA2*. The activation of *TSA2* by Hog1 is suggested to occur through the involvement of the Msn2/Msn4 transcription factors.

Hog1 may not be directly involved in transcriptional regulation upon oxidative stress (Dolz-Edo *et al*, 2013). A study showed that Hog1 and Sko1 regulates CREs elements exclusively after an osmotic stress and not oxidative stress. In additions the STREs elements regulation upon oxidative stress seems to be independent of Hog1. This was contradicted by another study suggesting that CREs and STREs can be regulated by Hog1 upon oxidative stress (Lee *et al*, 2017b). The different results of the two studies can be due to different H₂O₂ concentration used. Nevertheless, the involvement of the transcription factors Hot1 and Smp1 upon oxidative stress through Hog1 was not yet tested.

4.4. Hog1 affects mitochondrial respiration

A study conducted by Vendrell *et al.* aimed to elucidate the molecular mechanisms underlying cell death caused by sustained activation of Hog1 (Vendrell *et al*, 2011) (Figure 20). The researchers found that Hog1 can modulate mitochondrial respiration, leading to an increase in ROS levels. Sustained activation of Hog1, achieved through a constitutively active *PBS2^{DD}* mutant, induced programmed cell death (PCD), also known as apoptosis-like cell death, in yeast. This PCD was accompanied by an elevation in ROS levels. In anaerobic conditions, PCD was suppressed, indicating that ROS formation is necessary for this process.

Moreover, the study demonstrated that mitochondrial respiration is inhibited after osmotic stress in a Hog1-dependent manner. The *PBS2^{DD}* mutant exhibited Hog1-dependent inhibition of O₂ consumption, but this inhibition was not dependent on *GPD1*, involved in the glycogen metabolism. Interestingly, the study also revealed that mutation in the *SCF^{CDC4}* gene suppresses cell death caused by sustained activation of Hog1 and the subsequent increase in ROS levels, in a manner dependent on *Msn2/4*. In the absence of *SCF^{CDC4}*, the degradation of *Msn2/4* is slower, leading to increased *Msn2/Msn4*-dependent gene expression. *PNC1*, a target of *Msn2/4*, is involved in this suppression by controlling *Sir2*. Deletion of *MSN2/4*, *PNC1*, or *SIR2* in a *SCF^{CDC4}* background abolished the suppression of PCD, while overexpression of these factors prevented PCD. Therefore, Hog1 acts through the activation of *Msn2/4*, which regulates *PNC1* and subsequently *Sir2*, to prevent ROS accumulation and cell death. The precise function of *Sir2* in this pathway is still unknown, but it is suspected to act through its involvement in ribosomal DNA (Vendrell *et al*, 2011). These findings highlight the importance of tightly regulating MAPK activation for an appropriate stress response and demonstrate the dual role of Hog1 in inducing or suppressing ROS accumulation, depending on the context.

Multiple studies have further elucidated the role of Hog1 in altering mitochondrial function and increasing ROS levels under conditions of sustained activation. One such study focused on the interaction between Hog1 and *Isc1* (inositol phosphosphingolipid phospholipase C), which is involved in ceramide production, a key regulator of cell signalling (Barbosa *et al*, 2012). In the absence of *Isc1*, there is an increase in ceramide levels, leading to constitutive activation of Hog1. Deletion of *ISC1* renders cells hypersensitive to H₂O₂ and shortens their chronological lifespan. Interestingly, the double mutant *isc1Δ hog1Δ* exhibits higher viability compared to the single mutants. The premature aging observed in *Isc1* deletion cells is partially suppressed when Hog1 is also deleted, correlating with lower intracellular oxidation and protein carbonylation. Furthermore, mitochondrial dysfunction observed in the *isc1Δ* mutant is a consequence of Hog1 activation. The *isc1Δ* mutant, which is unable to grow on glycerol, can grow in the absence of Hog1, and the double mutant exhibits higher cytochrome c oxidase activity compared to the single mutants. Additionally, while the catalase activity decreases in the absence of Hog1, disruption of Hog1 in *isc1Δ* cells results in higher activity of the catalase *Cta1*. Interestingly, in the absence *Pgs1* involved in the cardiolipin synthesis, the replicative lifespan is shortened. This shortening is suppressed by the down regulation of the HOG1 pathway in *pgs1Δ* cells, which lack cardiolipin and exhibit mitochondrial dysfunction (Zhou *et al*, 2009). Taken

together, these studies highlight that uncontrolled and sustained activation of the Hog1 pathway can have negative effects on yeast cells, which differ from its transient activation during stress responses. It underscores the importance of tight regulation and proper modulation of the Hog1 pathway to ensure cellular homeostasis and survival.

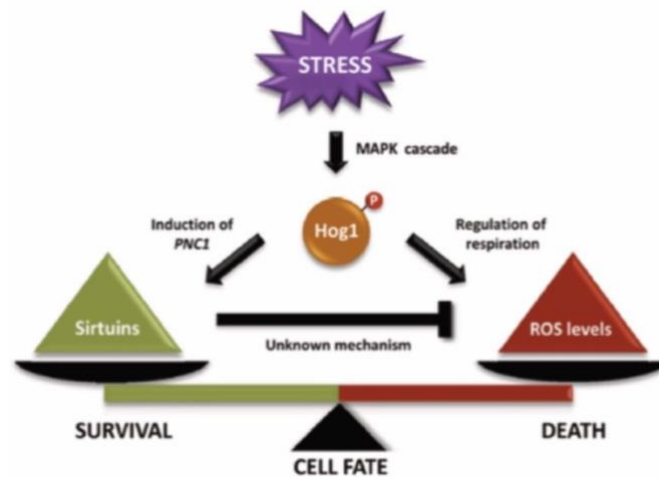


Figure 20: The dual function of Hog1 towards ROS.

Hog1 plays a dual function in response to ROS during stress. Upon activation, Hog1 inhibits mitochondrial respiration, which ultimately results in an increase in ROS levels within the cell. Interestingly, through its regulation of the transcription factors Msn2/4, Hog1 induces the expression of PNC1, which in turn activates the Sir2. The activation of Sir2 by PNC1 leads to a reduction in ROS levels, although the precise mechanism behind this effect is not yet fully understood. This regulatory mechanism mediated by Hog1 and Msn2/4 provides a counterbalance to the increase in ROS caused by Hog1 activation, potentially preventing excessive ROS accumulation and subsequent apoptosis-like cell death. Sustained activation of Hog1 and prolonged elevation of ROS levels, particularly from mitochondria, can lead to cell death. Retrieved from (Vendrell et al, 2011).

5. Hog1 regulates autophagy and mitophagy

Hog1 plays a significant role in mitophagy during starvation conditions and post-log cultures (Mao et al, 2011; Aoki et al, 2011) (Figure 21). Although Hog1 is activated during later stages of mitophagy, it remains in the cytoplasm, and its transcription factors are not required for this process. Hog1 does not directly affect the transcription of Atg32, a protein involved in mitophagy. Instead, Hog1 is indirectly related to the phosphorylation of Atg32. During mitophagy, the phosphorylation and activation of Hog1 rely on Pbs2 and Ssk1, while other upstream proteins in the canonical Hog1 pathway are not necessary. This indicates that the signalling mechanism of Hog1 during mitophagy differs from the one characterized in the

canonical pathway. Moreover, hyper-osmotic stress does not induce mitophagy, further supporting the distinct role of Hog1 in this process.

Under starvation conditions, Hog1 does not contribute to general autophagy suggesting that it regulates specifically mitophagy (Bicknell *et al*, 2010). However, the combination between starvation conditions and hypo- or hyper-osmotic conditions, shows that Hog1 stabilizes autophagy (Figure 21) (Prick *et al*, 2006). In response to rapamycin treatment, autophagy does not respond to hypo- or hyper-osmotic stress, and Hog1 is not involved in this context (Prick *et al*, 2006). However, Hog1 has been shown to play a role in autophagy induced during the late stage of endoplasmic reticulum (ER) stress, aiding in ER homeostasis and cell survival (Figure 21) (Bicknell *et al*, 2010). During persistent ER stress, Pbs2 phosphorylates Hog1, which translocates to the nucleus to regulate genes expression. Unlike its response to osmotic stress, Hog1 remains phosphorylated both in the nucleus and the cytoplasm during ER stress. From the cytoplasm, Hog1 influences the stability of Atg8. Notably, the deletion of Atg8 results in cells that are more resistant to persistent ER stress, highlighting the dual function of Hog1 in promoting both cell survival and cell death during ER stress. In addition, MMS stress that leads to genotoxic stress induces the phosphorylation of Hog1 and its activation independently of phosphatases (Huang *et al*, 2020). Sln1 branch is mainly responsible of this activation by impairing phosphorelay between Ypd1 and Ssk1. Upon its activation, Hog1 stays in the cytoplasm and does not translocate to nucleus to regulate genes expression. In the cytoplasm Hog1 induces autophagy in a manner dependent on its kinase function (Figure 21). Other studies also highlight the role of Hog1 in autophagic processes in different mutants and treatments (Shen *et al*, 2017; Babele *et al*, 2018; Bothammal *et al*, 2022; Teixeira *et al*, 2015).

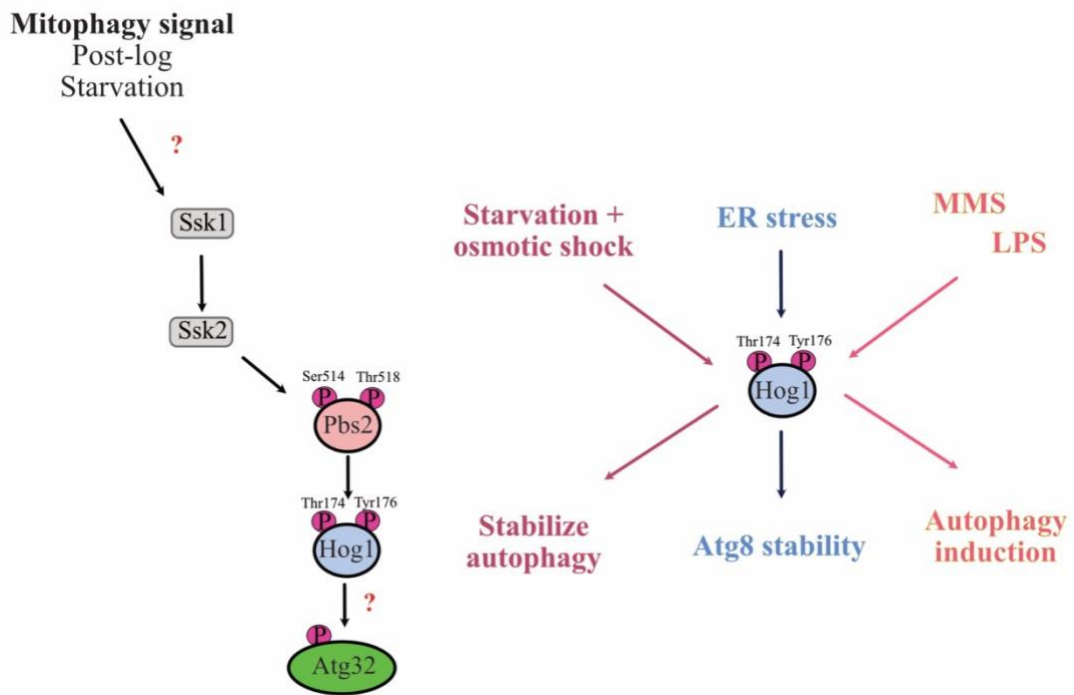


Figure 21: Hog1 modulates autophagy and mitophagy.

This figure summarizes the role of Hog1 in modulating autophagic responses based on the cited and discussed papers. Under post-log and starvation conditions, Hog1 is activated through the Ssk1-Pbs2 pathway, independent of the upstream components of the canonical pathway. This activation of Hog1 is indirectly linked to the phosphorylation of Atg32, a protein involved in selective autophagy. In the context of starvation combined with osmotic shock, Hog1 plays a role in stabilizing the autophagic response. Furthermore, Hog1 influences the stability of Atg8, another important protein involved in autophagy, in response to endoplasmic reticulum (ER) stress. Additionally, Hog1 has been found to induce autophagy in response to exposure to substances such as methyl methane sulfonate (MMS) and leptospiral lipopolysaccharide (LPS).

V- Thesis questions

The initial response to short telomeres, which triggers replicative senescence, and other essential telomeric functions and maintenance modes are evolutionarily conserved between budding yeast and mammalian cells. However, while mitochondrial alterations also appear to be conserved, there is currently a lack of data on oxidative stress levels and regulations in budding yeast during replicative senescence. Preliminary data from our laboratory indicated an increase in ROS levels during replicative senescence, along with the activation of Hog1. Furthermore, Hog1 seemed to impact the onset of senescence and affect ROS levels. Therefore, the objective of my project was to assess ROS levels during replicative senescence and investigate potential pathways linking telomerase inactivation with oxidative stress and the activation of Hog1.

The first goal was to measure ROS levels during replicative senescence using a reliable quantification method to assess any fluctuation. The second goal was to identify if Hog1 is activated during senescence due to its canonical pathway and if it plays a role regarding ROS levels. Since senescence is directly linked to telomere length, we wanted also to investigate any possible action of Hog1 directly on telomeres homeostasis which could impact senescence. Moving beyond the second goal, the subsequent objective encompassed examining whether Hog1's role during senescence is contingent upon checkpoint activation, or if it operates independently of Mec1. Lastly, we wanted to delve into the question of whether autophagic processes, in fermentative conditions, contribute to replicative senescence and if it's through the mediation of Hog1. These questions were addressed in the submitted manuscript in the first part of the results section.

In addition, we were interested in TERRA as a possible target of Hog1 during replicative senescence. The goal was to check if Hog1 affects TERRA levels during senescence. This was addressed in the unpublished section of the results.

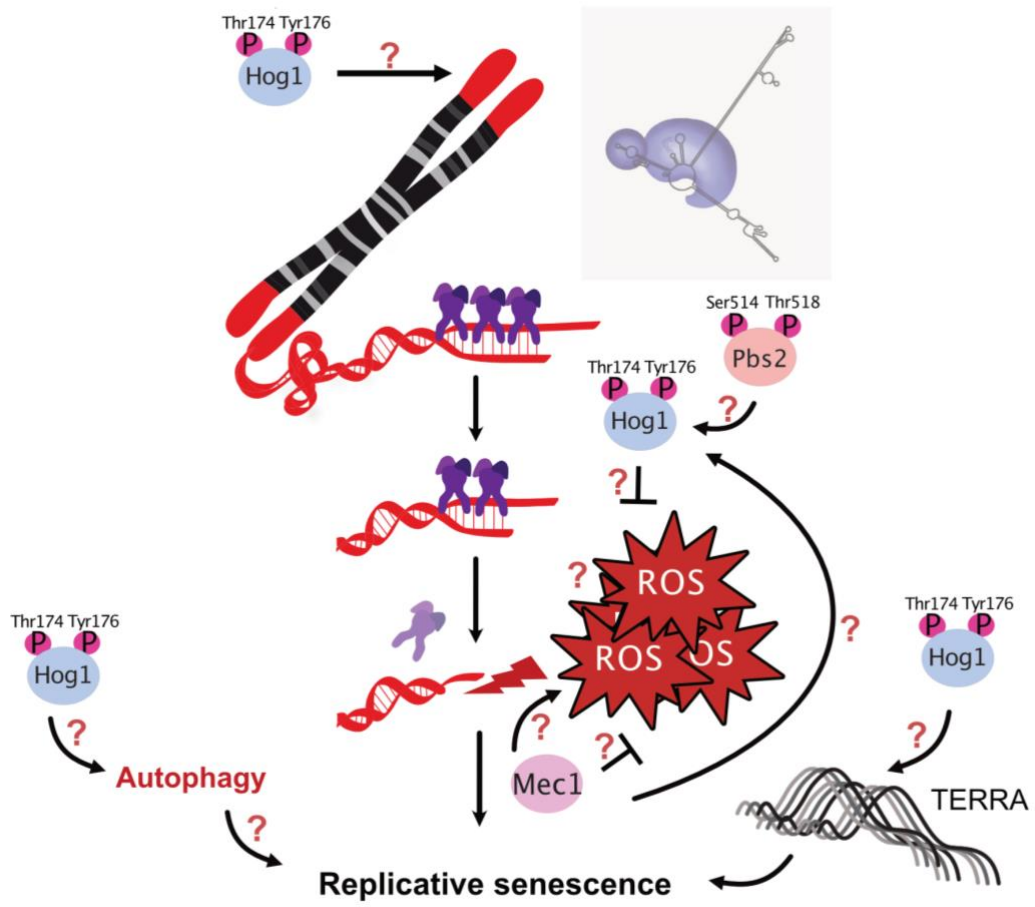


Figure 22 : Scheme recapitulating thesis questions

Results

Part 1: Submitted Manuscript

Article n°1: Hog1 acts in a Mec1-independent manner to counteract oxidative stress following telomerase inactivation

Bechara Zeinoun, Maria Teresa Teixeira*, Aurélia Barascu*

Title page

Hog1 acts in a Mec1-independent manner to counteract oxidative stress following telomerase inactivation

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Running title: Hog1's role in replicative senescence

Abstract

Replicative senescence is triggered when telomeres reach critically short length and activate permanent DNA damage checkpoint-dependent cell cycle arrest. Mitochondrial dysfunction and increase in oxidative stress are both features of replicative senescence in mammalian cells. Here, we show that reactive oxygen species (ROS) levels increase in the telomerase-negative cells of *Saccharomyces cerevisiae* during replicative senescence, and that this coincides with the activation of Hog1, a mammalian p38 mitogen-activated protein kinase (MAPK) ortholog. Hog1 activation is dependent on Pbs2, the MAPK kinase (MAPKK) in its canonical pathway, and counteracts increased ROS levels during replicative senescence. While Hog1 deletion accelerates replicative senescence, we found this could stem from decreased telomere length and reduced cell viability prior to telomerase inactivation. ROS levels also increase upon telomerase inactivation when Mec1, the yeast ortholog of ATR, is mutated, suggesting that oxidative stress is not simply a consequence of DNA damage checkpoint activation in budding yeast. We speculate that oxidative stress is a conserved hallmark of telomerase-negative eukaryote cells, and that its sources and consequences can be dissected in *S. cerevisiae*.

Keywords: Hog1 / Oxidative stress / Replicative senescence / *Saccharomyces cerevisiae* / Telomeres.

Introduction

Telomeres are essential structures found at the ends of linear eukaryotic chromosomes, consisting of DNA sequences, proteins, and long non-coding RNA (LncRNA) telomeric transcripts ([Shay & Wright, 2019](#)). Telomeres crucially safeguard chromosome integrity by protecting against degradation and fusion events ([Jain & Cooper, 2010](#)). However, due to the “DNA end-replication problem”, telomeres gradually shorten with each cell cycle. Telomerase, a specialized reverse transcriptase, counteracts telomere shortening by adding repetitive telomeric sequences to chromosome ends. In human somatic cells, the telomere-protective functions become compromised when the telomeres shorten with cell divisions due to telomerase inactivation coupled with the “DNA end-replication problem”. When telomere lengths become critically short, they activate an irreversible DNA damage checkpoint-dependent cell cycle arrest, known as replicative senescence ([Campisi & d'Adda, 2007](#); [d'Adda et al, 2003](#)). The unicellular eukaryote, *Saccharomyces cerevisiae*, relies on telomerase for its long-term viability ([Lundblad & Szostak, 1989](#)), but similar to human somatic cells, telomerase inactivation in budding yeast also leads to replicative senescence. When budding yeast cells divide in the absence of telomerase, they cease proliferation and enter a metabolically active state, arresting in the G2/M phase of the cell cycle ([Enomoto et al, 2002](#); [Ijima & Greider, 2003](#)). This cell cycle arrest in *S. cerevisiae*, which is akin to mammalian cells, relies on the activation of the DNA damage checkpoint kinases, Mec1 and Tel1 (the yeast orthologs of ATR and ATM, respectively), in addition to Rad53 phosphorylation ([Abdallah et al, 2009](#); [Khadaroo et al, 2009](#); [Teixeira, 2013](#)). Remarkably, not only is the triggering of replicative senescence in response to short telomeres evolutionarily conserved, but also many other essential telomeric functions and maintenance mechanisms ([Kupiec, 2014](#); [Wellinger & Zakian, 2012](#)).

Studying replicative senescence is challenging due to the inherent heterogeneity resulting from intracellular differences in telomere lengths and the immense intercellular variations ([Xu & Teixeira, 2019](#)). Intriguingly, data collected from various organisms indicate that mitochondrial defects, oxidative stress, and chronic inflammation can accelerate telomere shortening and dysfunction ([Ahmed & Lingner, 2018, 2020](#); [Fouquerel et al, 2019](#); [Opresko et al, 2002](#)). These factors are potential sources of cell-to-cell variation and contribute to genome instability during replicative senescence ([Passos & von Zglinicki, 2005](#)). Notably, senescent human fibroblasts exhibit modifications in mitochondrial structure and function, accompanied by elevated reactive oxygen species (ROS) levels and oxidative damage ([Hutter et al, 2004](#); [Mai et al, 2010](#); [Passos et al, 2010](#); [Passos et al, 2007](#); [Sitte et al, 2001](#); [Sitte et al, 2000](#)).

Similar metabolic alterations have also been observed in budding yeast during replicative senescence. A previous study revealed that the absence of telomerase resulted in increased mitochondrial mass, and a transcriptomic analysis indicated that energy production genes were up-regulated and stress response genes were induced ([Nautiyal et al, 2002](#)). However, data regarding ROS level alterations and their regulation during senescence in budding yeast is currently lacking.

P38, a member of the mitogen-activated protein kinase (MAPK) family, is critical for various cellular processes, including cellular senescence and oxidative stress responses ([Martínez-Limón et al, 2020](#)). In budding yeast, the MAPK Hog1, the ortholog of mammalian p38, is crucial for the defence against many stressors, including osmotic ([Brewster et al, 1993](#)) and oxidative stress ([Haghnazari & Heyer, 2004](#)). The canonical pathway of Hog1 activation involves two branches, the Sho1 and Sln1 branches, which converge to activate the MAPK kinase (MAPKK), Pbs2 ([O'Rourke et al, 2002](#)). Pbs2 then interacts with and phosphorylates Hog1 at the conserved residues, Thr¹⁷⁴ and Tyr¹⁷⁶, leading to its activation. Hog1 is a multifunctional protein with important functions in both the cytoplasm and nucleus, and it is vital for stress adaptation ([de Nadal & Posas, 2022](#)). Its roles encompass regulating gene expression by activating transcription factors, participating in gene initiation and elongation, regulating the cell cycle, and contributing to various steps in mRNA metabolism. Notably, Hog1 is activated in response to H₂O₂ stress ([Haghnazari & Heyer, 2004](#)) and regulates antioxidant genes by activating the transcription factors, Msn2/Msn4 ([Wong et al, 2003](#)) and Sko1 ([Rep et al, 2001](#)). In the absence of Hog1, cells become more sensitive to H₂O₂, which was previously shown to correlate with reduced expression of the *TSA2* gene ([Wong et al., 2003](#)). Conversely, sustained Hog1 activation can lead to cell death, which has been linked to alterations in mitochondrial respiration and increases in ROS levels ([Vendrell & Posas, 2011](#)). Hog1 counters this ROS increase by inducing *PNC1* and activating Sir2. Multiple studies have demonstrated that uncontrolled Hog1 activation disrupts mitochondrial function and elevates ROS levels, underscoring the critical importance of regulating Hog1 activation ([Barbosa et al, 2012](#); [Zhou et al, 2009](#)). Furthermore, Hog1 has been implicated in autophagic processes and is required for mitophagy, the selective process of mitochondria degradation ([Aoki et al, 2011](#); [Huang et al, 2020](#); [Mao et al, 2011](#)). Interestingly, Hog1 also positively regulates the localization of the Sir complex to telomeres following osmotic stress and the silencing of telomeric regions ([Mazor & Kupiec, 2009](#)).

Here, we report that ROS levels increase during replicative senescence in budding yeast. During this process, Hog1 is activated by Pbs2 and plays a role in ROS detoxification. This countering of ROS increase occurs independently from the actions of Mec1. We also find that autophagy does not participate in replicative senescence in budding yeast. However, Hog1

participates in maintaining telomere length homeostasis and affects cell viability. Our results thus suggest that Hog1 serves as a link between telomeres and ROS metabolism.

Results

ROS levels increase during replicative senescence in budding yeast

Human senescent cells exhibit increased ROS levels during replicative senescence, however, data relating to *S. cerevisiae* senescent cells is lacking ([Passos et al., 2010](#); [Passos et al., 2007](#)). Budding yeast telomerase is constitutively active but experimentally inactivating it triggers replicative senescence ([Teixeira, 2013](#)). We used a validated *TetO2-TLC1* system, where *TLC1*, which encodes the telomerase RNA template, is controlled by a repressible promoter by doxycycline, enabling the conditional shut-off of telomerase ([Bah et al., 2011](#); [Khadaroo et al., 2009](#); [Soudet et al., 2014](#)). To measure ROS levels, we used DCF, a molecule that can be directly oxidized by ROS and produce fluorescence in quantities reflecting ROS levels, which we can quantify by flow cytometry. Replicative senescence was detected from day three following culture with doxycycline as cell proliferation capacity decreased ([Figure 1A](#)). We also observed a simultaneous increase in ROS levels in the absence of telomerase ([Figure 1B](#)). These results were recapitulated in strains where *TLC1* was deleted; in these strains, we also found that ROS levels declined as cultures recovered their initial proliferation capacity following the emergence of post-senescence survivors ([Figure S1](#)). These data indicate that similar to mammalian models, budding yeast telomerase-negative cultures exhibit increased ROS levels.

Hog1 is activated during replicative senescence in a Pbs2-dependent manner and counteracts increase in ROS levels

We next investigated how ROS are regulated during replicative senescence. We focused on the multifunctional MAPK, Hog1, which is activated by oxidative stress ([de Nadal & Posas, 2022](#); [Haghnazari & Heyer, 2004](#)). To determine whether Hog1 activation occurred during replicative senescence, we prepared protein extracts from senescent cultures and used a specific antibody to detect phosphorylated forms of Hog1. We observed that Hog1 was phosphorylated during replicative senescence from day three, concomitant with an increase in ROS levels ([Figure 2 C and B, respectively and S2](#)). This phosphorylation was dependent on Pbs2, the MAPKK that precedes Hog1 in its canonical pathway ([O'Rourke et al., 2002](#)). We also observed that *HOG1* or *PBS2* deletion resulted in a similar premature loss of cell viability, when telomerase was inactivated ([Figure 2 A](#)). This suggests that the Hog1 pathway may play a role in senescent cells. While the HOG1 pathway activates transcription factors of antioxidant genes to reduce ROS levels ([Vendrell & Posas, 2011](#)), excessive Hog1 activity

can increase ROS levels by disrupting mitochondrial respiration ([Barbosa et al., 2012](#); [Vendrell & Posas, 2011](#)). To understand which of these Hog1 functions was involved in replicative senescence, we measured ROS levels in the absence or presence of Hog1 during senescence. Our results showed that the increases in ROS levels started earlier and reached higher levels in *hog1* Δ compared to *HOG1* strains (Figure 2B). This suggests that increases in ROS levels trigger an oxidative stress response that activates the Hog1 pathway, which is required for ROS detoxification in telomerase-inactivated cells.

HOG1 deletion affects telomere length homeostasis and cell viability

Given the direct relationship between replicative senescence and telomere shortening, we investigated the potential influence of Hog1 on telomere length homeostasis prior to telomerase inactivation, and the rate of telomere shortening in the absence of telomerase. We thus performed telomere-PCR on DNA samples from *HOG1* and *hog1* Δ strains to determine telomere length. Our results showed that *HOG1* deletion resulted in slightly shorter telomeres of ~30 bp prior to telomerase inactivation (Figure 3A-B, S3A). However, no significant differences in telomere shortening rates were observed in the absence of telomerase in either the *HOG1* or *hog1* Δ strains; shortening rates were measured to be approximately 2.5 bp/cell population doubling (Figure 3C-D, S3B), similar to previously published results ([Marcand et al., 1999](#); [Soudet et al., 2014](#)). We therefore conclude that Hog1 contributes to the maintenance of telomere length homeostasis. As telomere length homeostasis results from a balance between telomere lengthening by telomerase and telomere shortening due to the “DNA end-replication problem”, we suggest that Hog1 might interfere with telomerase recruitment or activity.

We reasoned that the accelerated senescence we observed in the *hog1* Δ strains when telomerase was inactivated could be due to shorter telomere lengths prior to telomerase inactivation, rather than because *HOG1* is required for cell growth in the absence of telomerase. To confirm this, we employed a microfluidics system, which allows consecutive cell cycles from individual cell lineages (herein referred to as lineages) to be tracked, thereby enabling more precise quantification of cell proliferation ([Xu et al., 2015](#)). In the presence of telomerase, *HOG1* cells grew indefinitely with a spontaneous mortality rate of ~0,38% (Figure 3E). In contrast, the absence of *HOG1* caused the mortality rate to increase to ~5,8% even in the presence of telomerase (Figure 3F). This has not previously been observed with cell population growth (liquid or solid). This data indicates that *HOG1* loss alone causes some cell death, which, when combined with telomerase inactivation, could have contributed to the accelerated senescence we observed.

Collectively, these findings suggest that the apparent acceleration of senescence we observed in the absence of Hog1 could be attributable to a marked increase in intrinsic mortality rates combined with the initial shorter telomeric lengths. However, this does not preclude a significant role for Hog1 in detoxifying ROS during replicative senescence, particularly as *hog1* Δ strains exhibit much higher ROS levels throughout replicative senescence compared to *HOG1* strains (Figure 2B).

Autophagic processes do not modify the onset of replicative senescence

Autophagy is a process that involves self-eating and bulk degradation, where organelles and their components are delivered to vacuoles to be degraded and recycled (Yin *et al.*, 2016). Autophagy can be selective, when specific cargo, such as damaged organelles, are degraded (Suzuki, 2013). The specific degradation of dysfunctional mitochondria is known as mitophagy. Hog1 plays a role in autophagy under certain conditions (Babele *et al.*, 2018; Bicknell *et al.*, 2010; Prick *et al.*, 2006) and is considered to be a mitophagy activator (Aoki *et al.*, 2011; Mao *et al.*, 2011; Shen *et al.*, 2017). We therefore investigated whether autophagic processes were essential during replicative senescence. We deleted the *ATG8* and *ATG32* genes, which encode two proteins essential for bulk autophagy and mitophagy in budding yeast, respectively (Suzuki, 2013). These mutants *atg8* Δ and *atg32* Δ displayed a blockage of bulk autophagy and mitophagy respectively, confirmed by an assay based on Rosella, a fluorescence-based pH biosensor (Rosado *et al.*, 2008) (Figure S4).

We then investigated whether blocking these processes in wild-type cells would affect senescence dynamics. Liquid senescence assays revealed that senescence remained unchanged in the absence of autophagy or mitophagy, suggesting that these processes were not essential for the viability of telomerase-negative budding yeast cells (Figure 4A). Similarly, following telomerase inactivation, the senescence profiles remained unchanged when either *ATG8* or *ATG32* were deleted in a *hog1* Δ background (Figure 4A). We concluded that Hog1 activity occurs independently from autophagic processes, which do not alter the onset of replicative senescence in budding yeast.

Hog1 acts in a Mec1-independent manner to regulate ROS levels during replicative senescence

We hypothesized that ROS level increases could result from cells being in a senescent state. Mec1 is a pivotal kinase in budding yeast, essential for the DNA damage checkpoint and the onset of replicative senescence (Hector *et al.*, 2012). Mec1 and the DNA damage checkpoint pathway are also known to protect cells against oxidative stress (Tsang *et al.*, 2014). Hog1 and

Mec1 are both necessary to combat the oxidative stress induced by hydrogen peroxide, but act independently ([Haghnazari & Heyer, 2004](#)). A hypomorph mutant of *MEC1*, *mec1-21*, contains a G to A substitution at position 2644, outside the kinase domain ([Ritchie et al, 1999](#)). The *mec1-21* mutant displays lower dNTP levels and shorter telomeres (~50 bp) compared to wild type strains ([Fasullo et al, 2009](#); [Ritchie et al., 1999](#)). The *mec1-21* mutant retains essential functions but is defective for the S phase checkpoint and Rad53 activation following UV and HU exposure ([Fasullo & Sun, 2008](#); [Sun & Fasullo, 2007](#)). We thus used the *mec1-21* mutant to investigate whether the actions of Hog1 against ROS were Mec1-dependent during replicative senescence. We inactivated telomerase and measured cell growth and proliferation capacity over time in the $P_{TetO2}\text{-}TLC1$, $P_{TetO2}\text{-}TLC1\ hog1\Delta$, $P_{TetO2}\text{-}TLC1\ mec1-21$, and triple mutant strains. When compared to *MEC1* cells, we observed that *mec1-21* accelerated the loss of viability under telomerase-negative conditions ([Figure 4B](#)), consistent with the initial shorter telomeres. We also verified that Rad53 phosphorylation was impaired in the *mec1-21* strains ([Figure 4C](#)). Yet, under these conditions, where the DNA damage checkpoint was disabled, telomerase inactivation resulted in more pronounced increases in ROS levels. This indicates that Mec1 also participates in ROS detoxification in the absence of telomerase ([Figure 4D](#)). In addition, while the triple mutant, $P_{TetO2}\text{-}TLC1\ hog1\Delta\ mec1-21$, displayed a much lower proliferation capacity, it showed an even more pronounced increase in ROS compared to the respective single mutants ([Figure 4D](#)). These results are consistent with a model where Hog1 and Mec1 are both involved in ROS detoxification during replicative senescence but act in independent pathways.

Discussion

Here, we have shown that increased ROS levels are a feature of replicative senescence in budding yeast. Hog1, one of the five MAPKs of *S. cerevisiae*, is activated by Pbs2 during replicative senescence and counteracts increases in ROS levels, likely independently of Mec1. In addition, Hog1 regulates telomere length homeostasis, and its deletion results in a marked increase in cell mortality rates. Our findings also indicate that autophagic processes are not essential in the context of replicative senescence in budding yeast.

Previous studies have shown that Hog1 is activated in response to exogenous acute stresses, such as H₂O₂ exposure, where it is essential for triggering antioxidant genes and maintaining cell viability ([Haghnazari & Heyer, 2004](#); [Wong et al., 2003](#)). Given our findings that ROS levels increase during replicative senescence, it is plausible that oxidative stress directly triggers Hog1 pathway activation. Replicative senescence is an endogenous process resulting from telomerase inhibition, that leads to numerous cellular modifications at both genomic and metabolic levels. Consequently, other modifications may also contribute to Hog1 activation. Notably, a previous study proposed that Hog1 activation in response to H₂O₂ stress primarily occurs through the Sln1-Ypd1-Ssk1-Ssk2-Pbs2 pathway, with Ssk2 acting as the MAPKKK that specifically activates Hog1 in response to oxidative stress, but not Sho1 branch ([Lee et al., 2017](#)). Therefore, determining the Hog1 pathway upstream of Pbs2 might help clarify the origin of Hog1 pathway activation in the absence of telomerase.

Microfluidics analysis, where cells grow individually, showed that *HOG1* deletion in the presence of telomerase increased cell mortality rates by ~15 fold. This increase may have gone undetected in other studies where cells were grown in populations as colonies or liquid cultures due to competition and selection of the fittest cells. Similar mortality rates have been described for other mutants considered “viable”, underscoring the high sensitivity of the microfluidics method ([Xu et al., 2015](#)). We speculate that the Hog1 pathway might be important in response to certain intrinsic stresses, and that it becomes essential in rare situations. Accordingly, a potential role for Hog1 under normal stress-free cellular conditions, unrelated to telomeres, has been described ([Reynolds et al., 1998](#)).

We have shown that Hog1 participates in telomere length homeostasis in budding yeast. This could be due to Hog1 acting to positively regulate telomere transcriptional silencing through the localization of the Sir complex following osmotic stress ([Mazor & Kupiec, 2009](#)). It could also be that the absence of Hog1 disrupts subtelomeric heterochromatin, which would alter telomere length homeostasis.

In conclusion, this study has shown that the metabolic alterations observed in human senescent cells are conserved in budding yeast. These alterations involve a conserved MAPK Hog1/p38 pathway, although the outcome might differ in different species. As most basic functions in telomere biology are conserved in eukaryotes, determining the mechanistic link between telomere shortening and increases in ROS levels in budding yeast will be essential to clarify how telomeres have evolved in the context of eukaryotic evolution.

Material and Methods

Yeast strains

All yeast strains used in this study were derived from a W303 background corrected for *RAD5* and *ADE2* (Table 1). Gene deletions were constructed as previously described (Longtine *et al.*, 1998). *Mec1-21* point mutation was constructed using Crispr-Cas9, as previously described (Lemos *et al.*, 2018). Strains expressing Rosella constructs from plasmids were constructed as previously described (Rosado *et al.*, 2008). Primers used are listed in Table 2.

Liquid senescence experiments

Strains were grown at 30°C in liquid-rich media (YPD). Cell suspensions were diluted to 0,001 OD_{600nm} with a final concentration of 30 µg/mL of doxycycline (*Sigma-Aldrich #D9891*), and the OD_{600nm} was measured after 24 hours. Cultures were similarly diluted for several days and daily samples were taken for analysis.

ROS detection

Yeast cultures with an OD_{600nm} of 0,4 were incubated at 30°C in darkness for one hour in 500 µL of sterile 1X PBS, containing DCF-DA (2',7'-Dichlorofluorescein diacetate) (*Sigma-Aldrich #D6883*) at a final concentration of 10 µM. Samples were washed and then resuspended in 500 µL of 1X PBS. Fluorescence was then analysed by flow cytometry using the settings, 488 (λ_{ex})/533 (λ_{em}) in Accuri C6 or MACSQuant Analyzer 10. The mean intensity values were then plotted. The mean values of *P_{TetO2}-TLC1* at day 0 without doxycycline were subtracted.

DNA extraction

Cells with an OD_{600nm} of 5 were centrifuged for four minutes at 2000 g and washed in 500 µL of sterile distilled water. After centrifugation, 200 µL of lysis buffer (Triton 100 X - 10% SDS sodium dodecyl sulfate - 5 M NaCl – 0,5 M EDTA ethylenediaminetetraacetic acid - 1 M Tris - H₂O), 200 µL of 0,45 µm acid-washed glass beads (*Sigma-Aldrich #G8772*), and 200 µL of phenol: chloroform: isoamyl alcohol solution (25:24:1, *Sigma-Aldrich #77617*) were added to the cell pellet. The tubes were vortexed for 15 minutes at 4°C, followed by the addition of 200 µL of TE (Tris/EDTA) at pH 8. After five minutes of centrifugation at maximum speed, the

aqueous phase was transferred to a new tube containing the same volume of isopropanol. After mixing by inversion, the samples were centrifuged for one minute at maximum speed. The resulting DNA pellet was washed in 500 μ L of ethanol. Finally, after centrifuging and drying in a speed vacuum (three minutes at 40°C), the DNA pellet was resuspended in 50 μ L of TE and 0,1 μ L of RNase A (100 mg/ml) and incubated for 30 minutes at 37°C. The quality and integrity of the DNA were checked by agarose gel electrophoresis (1% agarose, 0,5X TBE). The quantity was evaluated using Qubit2 (*Thermofisher*). Samples were stored at -20°C.

SDS-PAGE and Western blot

Cells with an OD_{600nm} of 5 were collected by centrifugation. The pellet was lysed in 0,2 M of sodium hydroxide (NaOH) for 10 minutes on ice. After adding trichloroacetic acid (TCA) at a final concentration of 0,5%, the samples were incubated again on ice for 10 minutes. After centrifuging for 10 minutes at maximum speed at 4°C, the pellet was resuspended in 50 μ L of Laemmli 4X buffer and denatured at 95°C for five minutes. Protein samples were electrophoresed on a 10% denaturing gel (or 7,5% for Rad53 detection) of 37,5:1 Acrylamide:Bis-acrylamide (*Sigma-Aldrich* #A3699). Proteins were then transferred to a nitrocellulose membrane (*Amersham Protran 0.45 NC, GE HealthCare*) and stained with Ponceau red. The following antibodies were used: *Cell Signalling*, #9211 to detect Phospho-Hog1, Santa Cruz, #sc-165978 to detect total Hog1, *Abcam*, #ab104252 to detect both unphosphorylated and phosphorylated forms of Rad53, and the horseradish peroxidase-coupled secondary antibody (HRP) (*Sigma*, #A9044 and #A9169). The signals were revealed using an electrochemiluminescence reagent (*ClarityWestern ECL, Biorad*) and recorded using the ChemiDoc Imaging System (*Biorad*).

Telomere-PCR

This method was adapted from ([Forstemann et al, 2000](#)). In brief, 40 ng of genomic DNA was denatured at 98°C for five minutes before the tailing reaction in 20 μ L of *New England Biolabs* Buffer 4 (1X), 100 μ M of dCTP, and 1U of terminal transferase (*New England Biolabs* #M0315L). The reaction was incubated at 37°C for five minutes, followed by five minutes at 94°C, and then maintained at 4°C. For the PCR reactions, 5 μ L of the polyC-tailed products were used with 1X Taq Mg-free Buffer (*New England Biolabs*), 500 nM of each primer ([Table 2](#)), 200 μ M of dNTPs, and 1 U of Taq Standard Polymerase (*New England Biolabs* #M0273) in a final volume of 30 μ L. The following PCR program was used: three minutes at 94°C, followed by 45 cycles of 20 seconds at 94°C, 15 seconds at 57°C, 20 seconds at 72°C, and

finally, five minutes at 72°C. The TeloPCR products were then loaded onto a large 2% agarose gel with 1X TBE buffer and 100 ng/ml of ethidium bromide (BET). A 50bp molecular weight marker was also loaded (*New England Biolabs #N3236*). Electrophoresis was then performed at 50V for 15 hours. Visualization and analysis were performed using the ImageLab® software (*Biorad*).

Microfluidics analysis

Microfluidics analyses were performed as previously described ([Xu et al., 2015](#)).

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Conflicts of interest

The authors declare no conflict of interest.

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Tables

Table 1: strains used in this study

Strain name	Figure	Genotype
yT787	1, 2, 3, 4, S2, S3, S4	<i>Mata ura3-1 trp1-1 leu2-3,112 his3-11,15 tlc1::HIS3MX6-P_{TetO2}-TLC1</i>
yT1473	2, 3, 4, S2, S3, S4	<i>yT787 hog1::TRP1</i>
yT1714	2, S2	<i>yT787 pbs2::TRP1</i>
yT1743	4	<i>yT787 mec1-21</i>
yT1744	4	<i>yT787 hog1::TRP1 mec1-21</i>
yT1701	4, S4	<i>yT787 atg8::NAT</i>
yT1702	4, S4	<i>yT787 hog1::TRP1 atg8::NAT</i>
yT1705	4, S4	<i>yT787 atg32::NAT</i>
yT1706	4, S4	<i>yT787 hog1::TRP1 atg32::NAT</i>
yT338	S1	<i>TLC1/ tlc1Δ</i>

Table 2: Primers used in this study

Name	Description/resulting strain	Sequence
oT568	deletion of <i>TLC1</i> (yT)	5'-GCA ATG GTG ACA TAT AGA TCT CAA GGT TCT CAA TTA AAA GAC CTT CTT TGT AGC TTT TAG TGT GAT TTT TCT GGT TTG AGC GGA TCC CCG GGT TAA TTA A-3'
oT1569	deletion of <i>TLC1</i> (yT)	5'-GAC AAT TAC TAG GAT GTT CTT CTA TTT TTT TAT TTT TAT TTG TAT ATT GTA TAT TCT AAA AAG AAG AAG CCA TTT GGT GGG AAT TCG AGC TCG TTT AAA C-3'
oT210	insertion of P _{TetO2} upstream of <i>TLC1</i> (yT787)	5'- AAT ACG ATT AAG CAA ACG CAA CAG CCA TTG ACA TTT TCA TAG GGT ACC TAT CTT CCT CTC ATA GGC CAC TAG TGG ATC TG-3'
oT543	insertion of P _{TetO2} upstream of <i>TLC1</i> (yT787)	5'-AAA AAA CTT CCT CTT TAG CAA TGG TGA CAT ATA GAT CTC AAG GTT CTC AAT TAA AAG ACC CGG ATC CCC GGG TTA ATT AA -3'
oT1550	deletion of <i>HOG1</i> (yT1473)	5'-GGT AAA TAC TAG ACT CGA AAA AAA GGA ACA AAG GGA AAA CAG GGA AAA CTA CAA CTA TCG TAT ATA ATA CGG ATC CCC GGG TTA ATT AA-3'
oT1551	deletion of <i>HOG1</i> (yT1473)	5'-CCA TAA AAA AAA GAA ACA TCA AAA AGA AGT AAG AAT GAG TGG TTA GGG ACA TTA AAA AAA CAC GTG AAT TCG AGC TCG TTT AAA C-3'
oT1252	deletion of <i>PBS2</i> (yT1714)	5'-ATT ATT ATA TTA AGC AGA TCG AGA CGT TAA TTT CTC AAA GCG GAT CCC CGG GTT AAT TAA-3'
oT1253	deletion of <i>PBS2</i> (yT1714)	5'-TAT ATT CAC GTG CCT GTT TGC TTT TAT TTG GAT ATT AAC GGA ATT CGA GCT CGT TTA AAC-3'
oT1415	<i>Mec1-21</i> point mutation (yT1743-yT1744)	5'-AAA CTA CAG GAT AAT ATC TTG TTT T-3'
oT1416	<i>Mec1-21</i> point mutation (yT1743-yT1744)	5'-AAG ATA TTA TCC TGT AGT TTG GAT CA-3'
370	deletion of <i>ATG8</i> (yT1701-yT1702) *	5'-GAT AAG AGA ATC TAA TAA TTG TAA AGT TGA GAA AAT CAT AAT AAA-3'
371	deletion of <i>ATG8</i> (yT1701-yT1702) *	5'-CGA TTT TAG ATG TTA ACG CTT CAT TTC TTT TCA TAT AAA AGA CTA-3'

333	deletion of <i>ATG32</i> (yT1705-yT1706) *	5'-GTC CTA ATC ACA AAA GCA AAA GCG TAC GCT GCA GGT CGA C-3'
369	deletion of <i>ATG32</i> (yT1705-yT1706) *	5'-AAG TGA GTA GGA ACG TGT ATG TTT GTG TAT ATT GGA AAA AGG TTA-3'
oT182	TeloPCR Y' Fwd	5'-CTG TAG GGC TAA AGA ACA GGG-3'
oT169M	TeloPCR Rev	5'-GCG GAT CCG GGG GGG GGG G-3'

*Obtained from N. Belgareh-Touzé and O. Ozturk

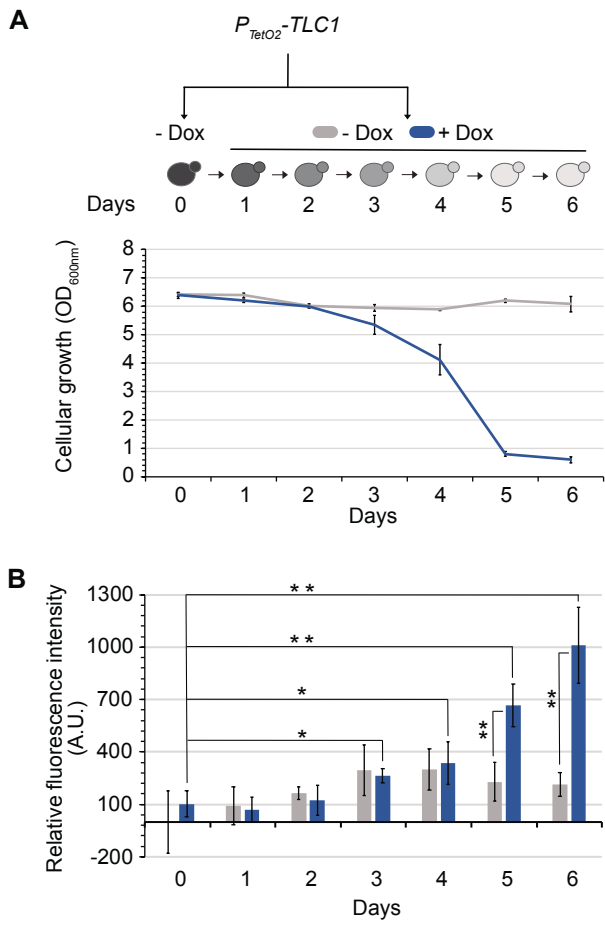


Figure 1: ROS levels increase during replicative senescence in budding yeast. Each consecutive day, cells with the genotype indicated were diluted in media either with or without doxycycline (Dox) to enable conditional shut-off of telomerase, and grown for 24 hours. Cell density at OD_{600nm} (**A**) and ROS levels (**B**) are plotted as mean \pm SD of three independent clones. P-values were calculated by two-tailed Student's t-test (* < 0,05; ** < 0,01) and only significant differences have been represented.

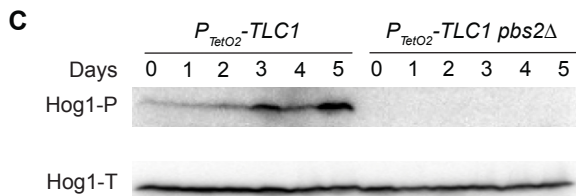
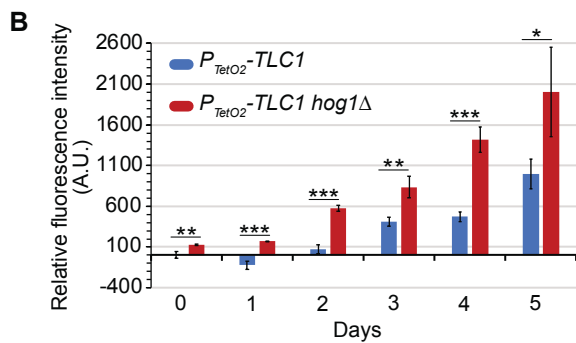
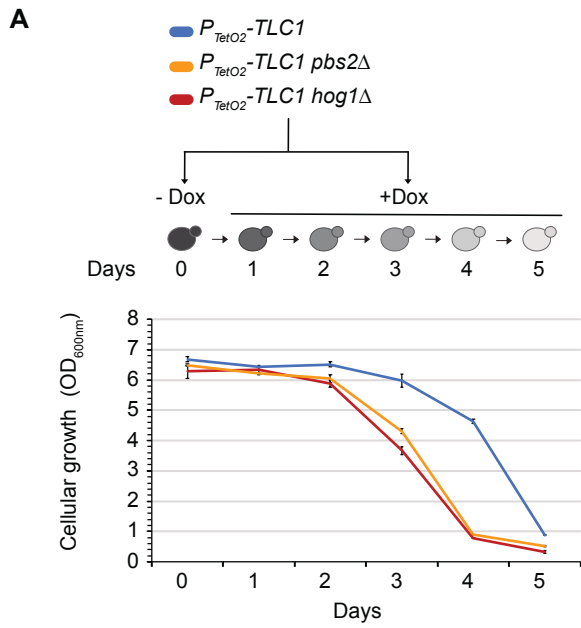


Figure 2: Hog1 is activated during replicative senescence in a Pbs2-dependent manner and counteracts increase in ROS levels. Cells with the genotypes indicated were treated as described in Figure 1. Cell density at OD_{600nm} (**A**) and ROS levels (**B**) are plotted as mean \pm SD of three independent clones. P-values were calculated by two-tailed Student's t-test (* < 0,05; ** < 0,01; *** < 0,001) and only significant differences between the two strains used have been represented. (**C**) Protein extracts analysed by Western blot using an antibody against phosphorylated forms of Hog1's human ortholog p38 (Hog1-P), or total Hog1 (Hog1-T).

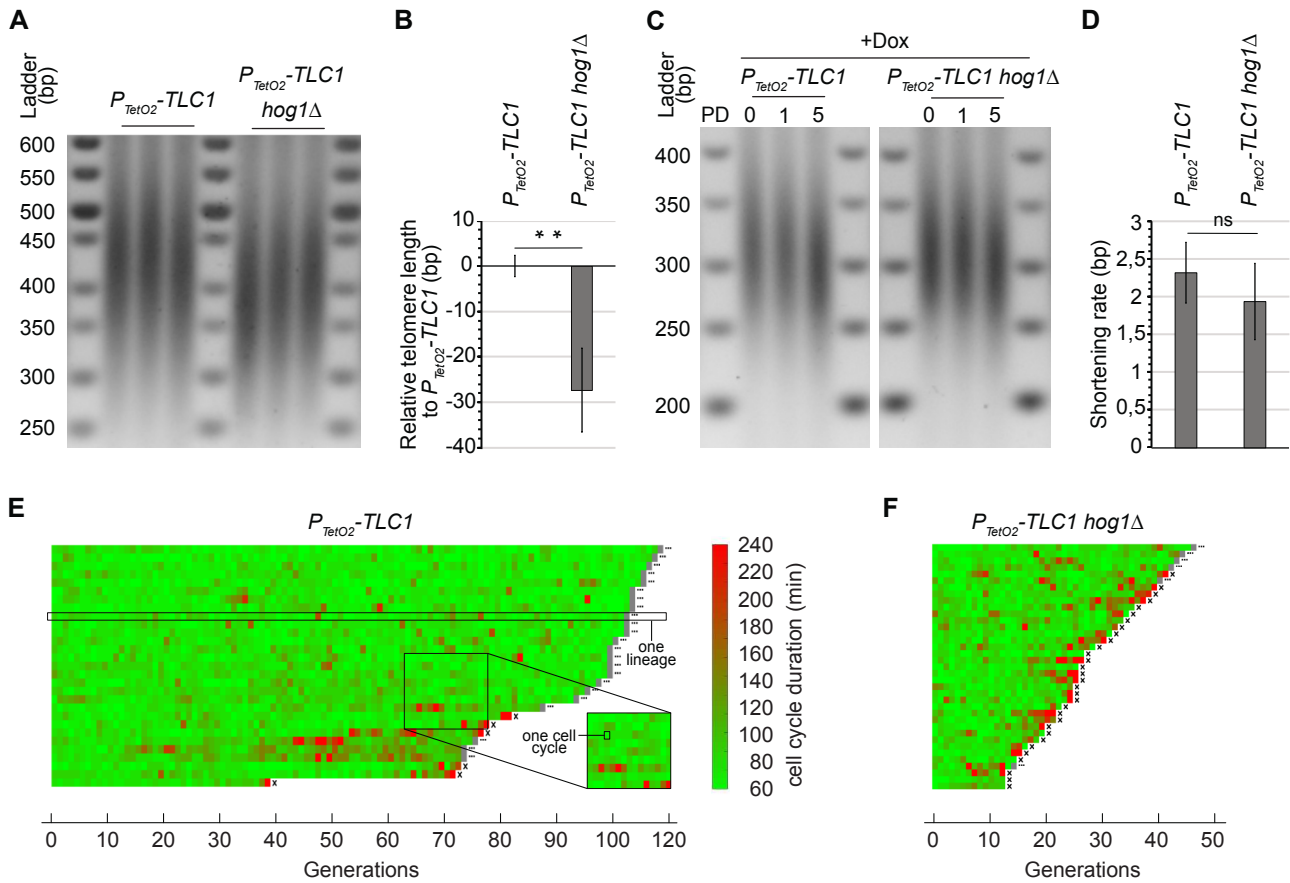


Figure 3: *HOG1* deletion affects telomere length homeostasis and cell viability. Representative telomere-PCR of Y' telomeres from the strains indicated (**A**) and their quantification, plotted as mean \pm SD (**B**). P-values were calculated by two-tailed Student's t-test (** $<$ 0,01). (**C**) Cells of the genotypes indicated were pre-cultured overnight in doxycycline-containing media to enable telomerase shut-off. Cells were then diluted and grown in the same media for the indicated population doublings (PD). Representative telomere-PCR of Y' telomeres from genomic DNA extracts are shown. (**D**) Quantification of telomere shortening was measured between PD 1 to 5 and plotted as mean \pm SD. (**E,F**) Microfluidics results of independent lineages with the genotypes indicated. Cells were introduced into the microfluidics microcavities and cultured in SD. Each horizontal line represents the consecutive cell cycles (generations) of a single lineage, and each segment corresponds to one cell cycle. An ellipsis (...) at the end of the lineage line indicates that the cell was living after the experiment, whereas an X indicates cell death. Cell cycle duration is indicated by the coloured bar.

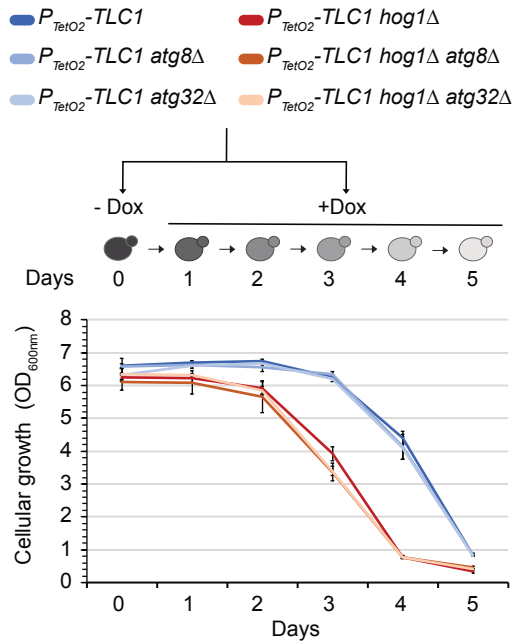
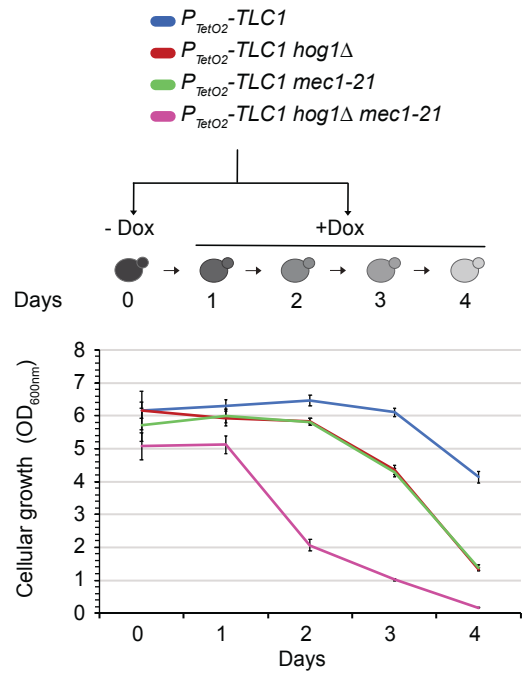
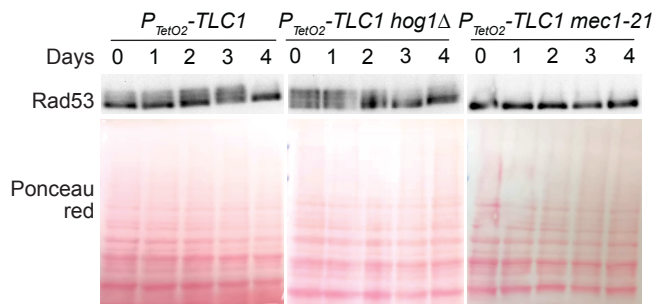
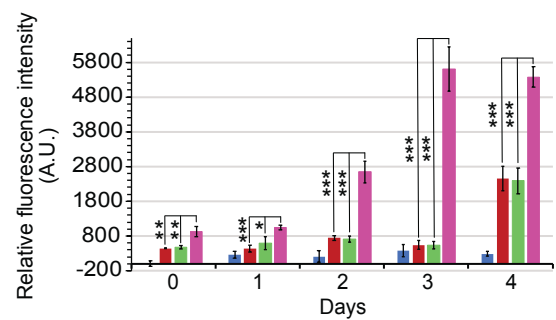
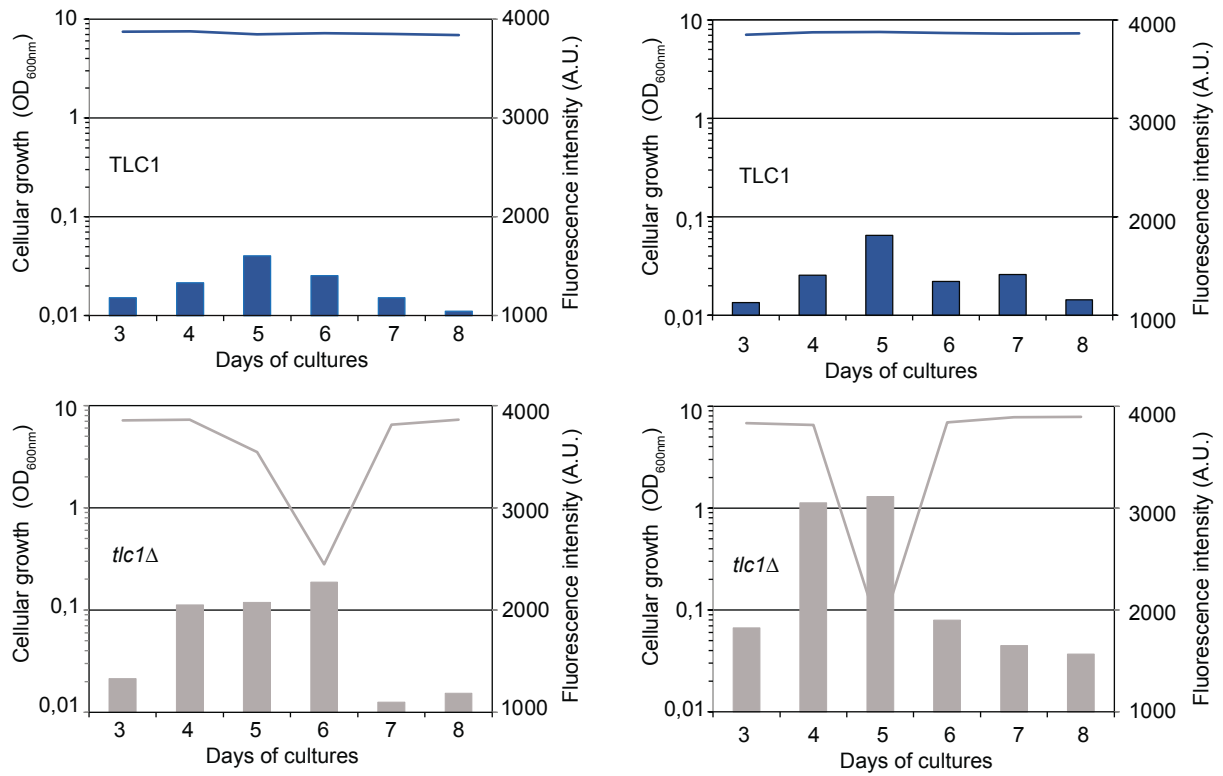
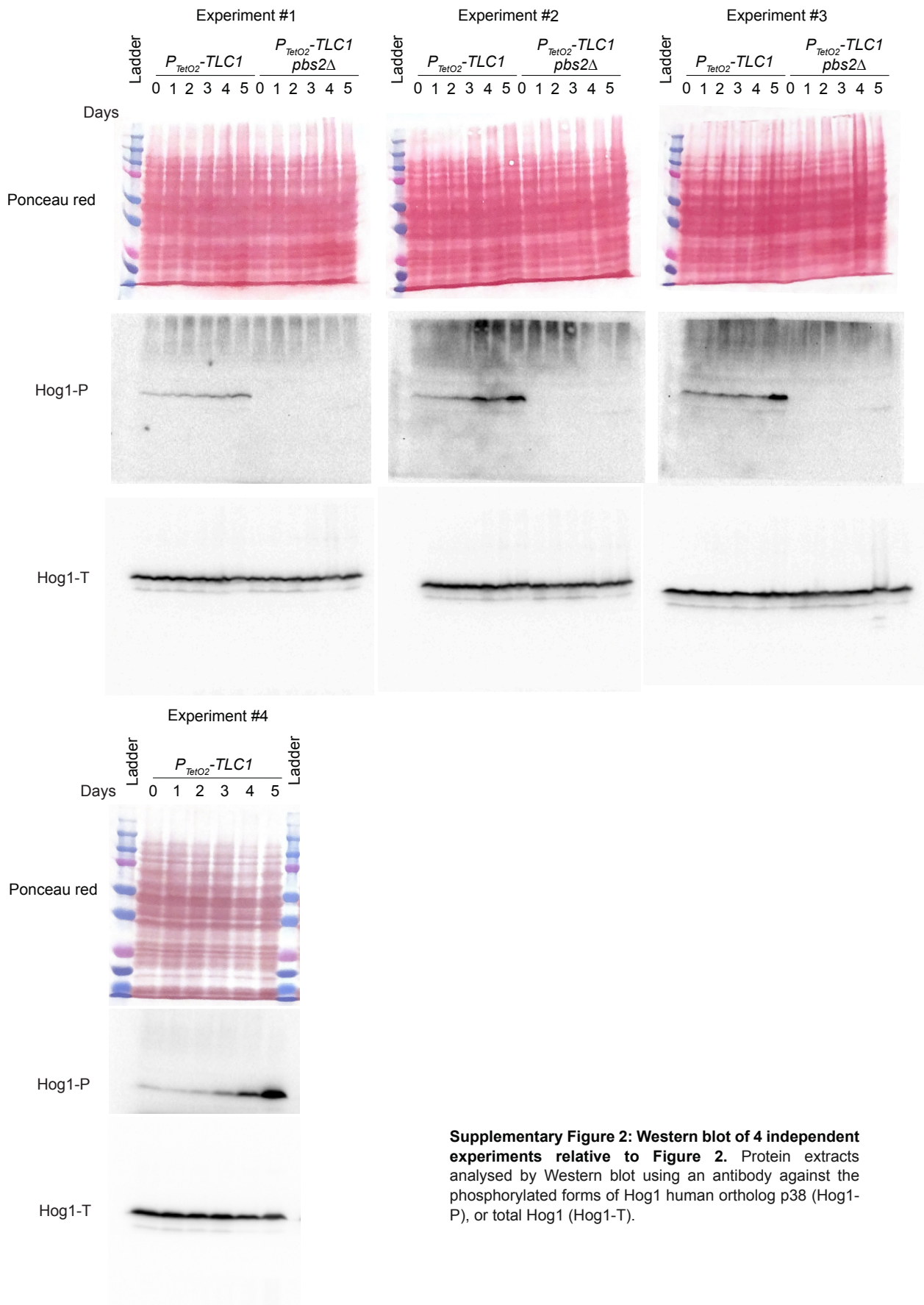
A**B****C****D**

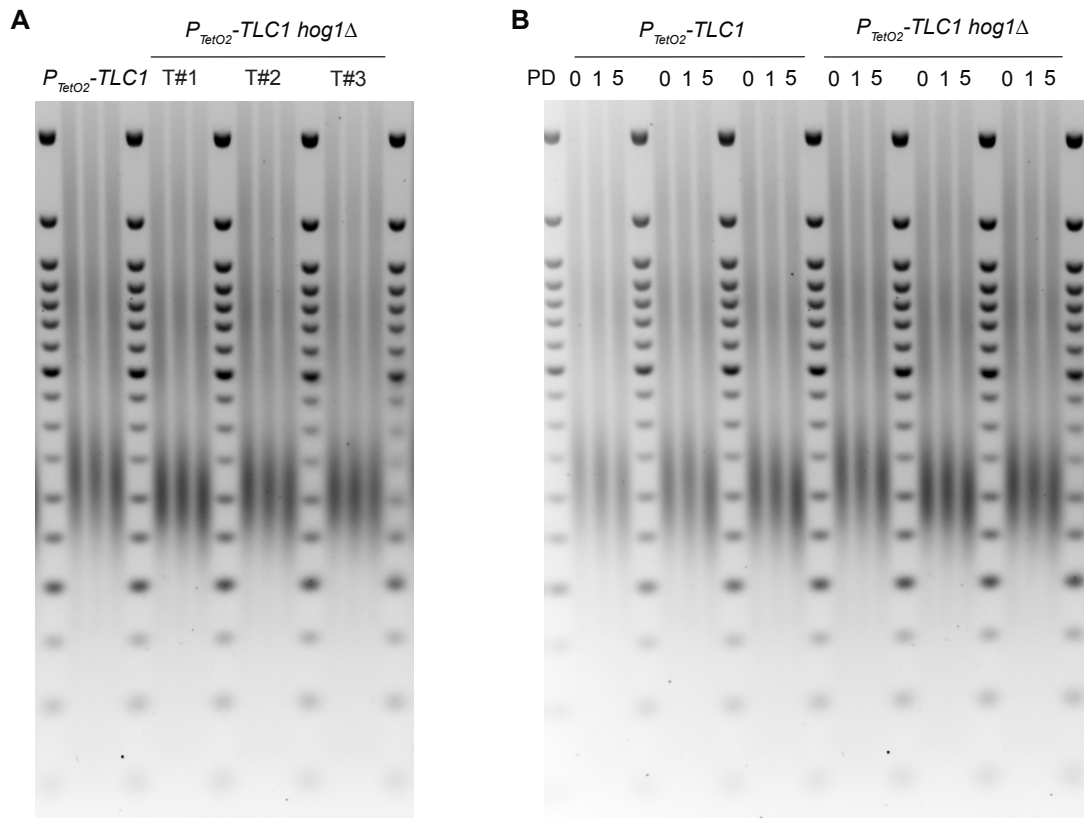
Figure 4: Hog1 interaction with multiple pathways. Cells with the genotypes indicated were treated as described in Figure 1. Cell density at OD_{600nm} (**A, B**) and ROS levels (**D**) are plotted as mean ± SD of three independent clones. P-values were calculated by two-tailed Student's t-test (* < 0,05; ** < 0,01; *** < 0,001) and only significant differences between the triple mutant and the double mutants have been represented. (**B**) Representative telomere-PCR of Y' telomeres from the strains indicated. (**C**) Protein extracts analysed by Western blot using an antibody against Rad53. The mobility shift of the band indicates Rad53 phosphorylation.



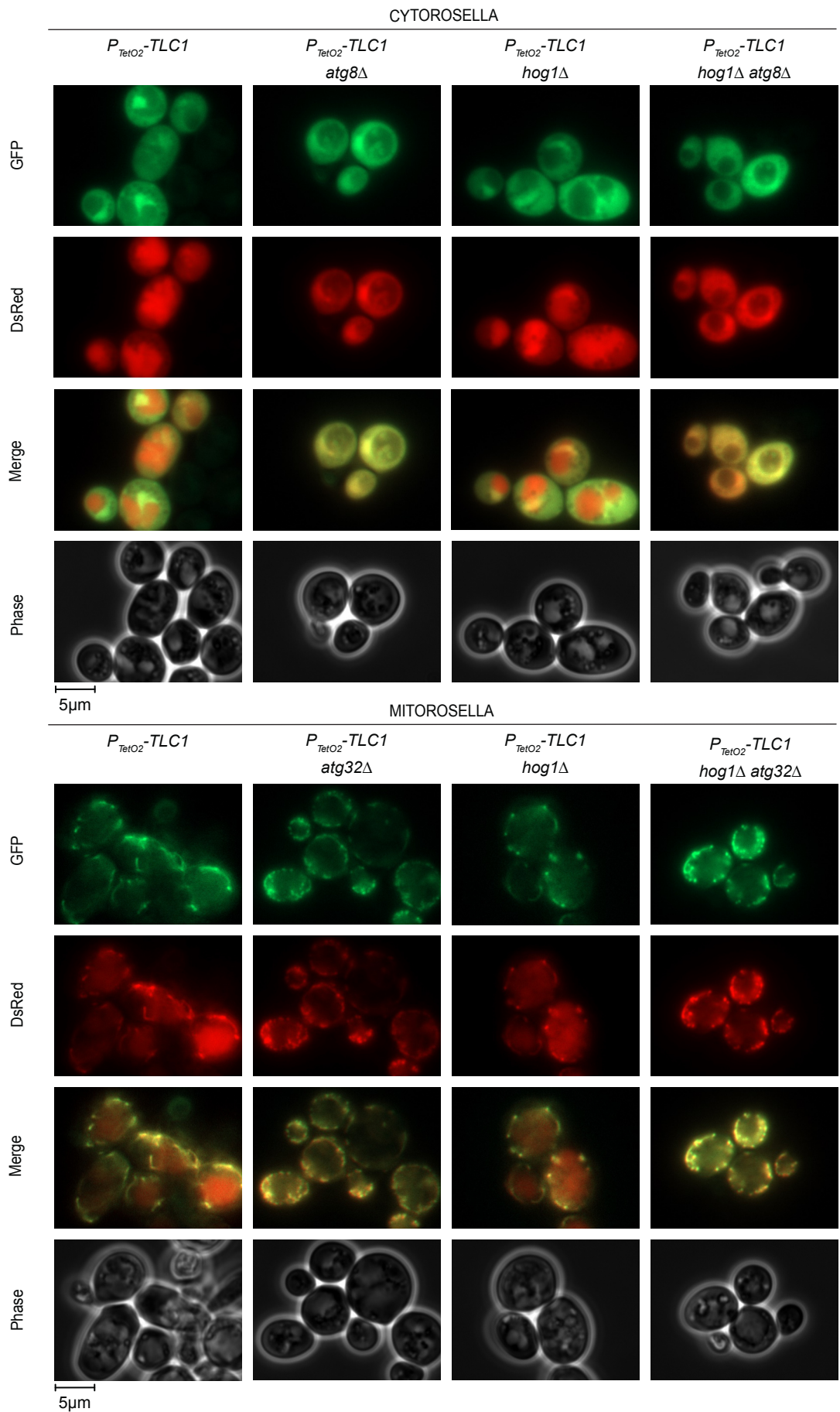
Supplementary Figure 1: ROS levels increase during replicative senescence in *tlc1Δ* strains. A diploid *TLC1/tlc1Δ* strain was dissected, and the four spore-derived colonies obtained after two days were pre-cultured. Each consecutive day, cultures were diluted, as described in Figure 1, and grown for 24h. Cell density at OD_{600nm} (curve-left axis) and ROS levels (histogram-right axis) are plotted.



Supplementary Figure 2: Western blot of 4 independent experiments relative to Figure 2. Protein extracts analysed by Western blot using an antibody against the phosphorylated forms of Hog1 human ortholog p38 (Hog1-P), or total Hog1 (Hog1-T).



Supplementary Figure 3: *HOG1* deletion affects telomere length homeostasis. (A) Technical triplicates of telomere-PCR of Y' telomeres from the independent transformants (T#1-3) and strains indicated. **(B)** Technical triplicates of the experiment described in Figure 3C. Population doublings (PD).



Supplementary Figure 4: Strains deleted for *ATG8* and *ATG32* show an autophagy- and mitophagy-impaired phenotype, respectively. Images of fluorescence microscopy following autophagy and mitophagy induction by nitrogen depletion for 24 hours. Cytorosella is a fusion between a cytoplasmic targeting signal, a DsRed, and a GFP sensitive to pH. Mitorosella is similar to cytorosella but with a mitochondrial targeting signal.

Part 2: Unpublished results

Hog1 seems to act independently of TERRA during replicative senescence

1. *Hog1 and TERRA transcription during replicative senescence*

As mentioned in the introduction, upon osmotic stress, Hog1 is essential for the transcription of several LncRNA (Nadal-Ribelles *et al*, 2014). Unpublished results from the lab of our collaborator Dr. Francesc Posas suggested that upon an osmotic stress, Hog1 binds subtelomeric regions to possibly induce the transcription of TERRA from several telomeric ends. In addition, TERRA is thought to play a role in the oxidative metabolism. In fact, TERRA is upregulated after ROS induction in mammalian cells (Galigniana *et al*, 2020) and during the diauxic shift in budding yeast, when ROS levels also increase (Perez-Romero *et al*, 2018). The increase in ROS levels, the activation of Hog1 and the upregulation of TERRA (Zeinoun *et al*, 2023) are all detected during replicative senescence. We thus speculated that during senescence, TERRA may also be a potential target of Hog1. We performed a RT-qPCR using primers from several telomeric ends to detect TERRA levels on samples from liquid senescence experiment in $P_{TetO2}\text{-TLC1}$ and $P_{TetO2}\text{-TLC1 } hog1\Delta$ strains. We recapitulated results from the literature showing that TERRA increases in the course of senescence (Figure 23). This increase was also detected in the absence of Hog1 but results were highly variable. Since TERRA levels are higher at the shortest telomeres, and in our cells, telomeres display various lengths, this leads to variability in different experiments. This heterogeneity makes it difficult to compare the two strains and draw a definitive conclusion if Hog1 influences TERRA levels during senescence. Yet, the fact that our preliminary results show that TERRA transcripts can be detected in senescence in $P_{TetO2}\text{-TLC1 } \Delta hog1$ cells suggest that TERRA transcription does not fully depend on Hog1 in this context.

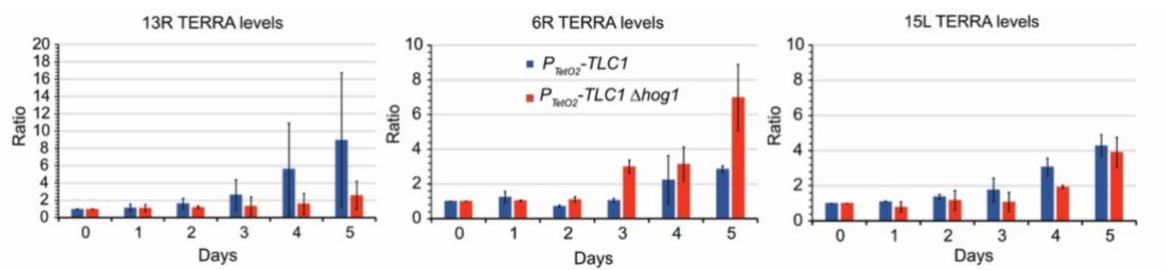


Figure 23: TERRA increase during replicative senescence appears to be independent from Hog1.

TERRA levels were measured by RT-qPCR on RNA samples from liquid senescence experiment at 13R, 6R and 15L telomeres in $P_{TetO2}\text{-TLC1}$ and $P_{TetO2}\text{-TLC1 } \Delta hog1$ strains. The ratio was calculated using the $\Delta\Delta CT$ method using *ACT1* as a reference gene and plotted as mean \pm SD of two independent clones.

2. *Hog1, TERRA and acceleration of senescence*

We thought to use a complementary genetic approach to test if Hog1 acts to regulate TERRA during senescence. Overexpression of RNaseH1 results in the degradation of TERRA in the context of telomeric R-loops, which also increase during replicative senescence (Balk *et al*, 2013). In addition, overexpression of RNaseH1 accelerates senescence, and this has been interpreted as evidence for TERRA contributing to the variability of telomerase-negative cells (Graf *et al*, 2017). We reasoned that if the acceleration of senescence of $P_{TetO2}\text{-TLC1 } hog1\Delta$ cells is caused by the absence of TERRA increase and its subsequent beneficial R-loops, the additional removal of RNA:DNA hybrids by RNaseH1 overexpression should not further accelerate senescence, but instead display an epistatic phenotype. We thus conducted a liquid senescence experiment in $P_{TetO2}\text{-TLC1}$ and $P_{TetO2}\text{-TLC1 } hog1\Delta$ strains where RNaseH1 is overexpressed. Our result confirms the literature showing that the overexpression of RNaseH1 accelerates senescence but the combination with the deletion of *HOG1* shows high variability. Thus, we could not draw a definitive conclusion on this subject (Figure 24).

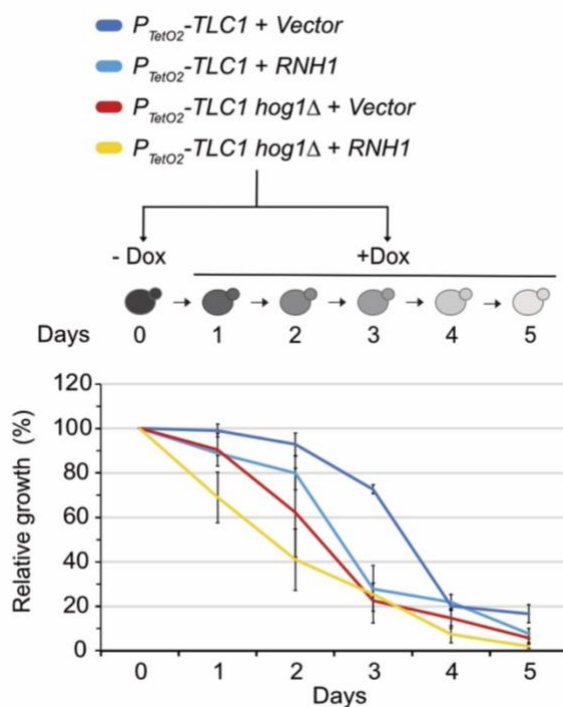


Figure 24: *Hog1, TERRA and acceleration of senescence.*

Liquid senescence experiment in *SD-URA* selective media using $P_{TetO2}\text{-TLC1}$ and $P_{TetO2}\text{-TLC1 } hog1\Delta$ strains containing either an empty vector or a *Rnh1*-OE plasmid (*RNH1*). The graph represents the relative growth of each strain plotted as mean \pm SD of three independent transformants.

3. FinalCut system to bypass replicative senescence heterogeneity

We wished to bypass the heterogeneity of telomeres length varying from cell to cell in order to control TERRA expression and draw a definitive conclusion on whether Hog1 influences TERRA during replicative senescence. We thus decided to take advantage of a new system developed in the laboratory. This system, called FinalCut, is based on the CRISPR-Cas9 technology to cut specifically the 6R telomere up to a desired defined length (*Figure 25A*). Thus, by synchronizing replicative senescence and specifying the defined short telomere, we wanted to assess ROS levels and TERRA levels with great precision at this telomere in the presence and absence of Hog1. This would cancel the limitation that can mask a possible effect of Hog1 on TERRA transcription and can give us more insights about the time of increase of ROS levels during senescence. For this, we used a control strain, where Cas9 is induced but cannot cut due to the absence of the PAM sequence, and 3 different strains that give us 3 different telomeric lengths after the cut: 100 bp, 40 bp and 20 bp. We induced the cut for 3, 6 and 9 hours and took samples for analysis (*Figure 25B*). Upon the induction of Cas9, the 6R telomere is cut to the desired length: 100, 40 or 20 bp (*Figure 25C*). The phosphorylation of Rad53 seen by a band shift on western blot reveals that cells start to activate the checkpoint and enter replicative senescence for the Tel6R::40bp at 6 and 9 hours and for the Tel6R::20bp at 3, 6 and 9 hours (*Figure 25D*). However, to our surprise Hog1 is activated in the culture conditions required for the use of the FinalCut system even before the cut which is a media containing raffinose as a carbon source (*Figure 25E*). We reasoned that Hog1 could thus be important for viability of cells in raffinose growth conditions, potentially precluding the use of *hog1* Δ strains with the Final Cut system. We thus decided to not further use this system to measure the dependency of TERRA transcription on *HOG1*. Importantly, we measured ROS levels and saw that they significantly increase in the Tel6R::20 bp at 9 hours (*Figure 25F*). These data suggest that the increase in ROS during replicative senescence is delayed and comes after the checkpoint activation. Our results are thus consistent with published data that show that in human and mice cells, mitochondrial dysfunctions and oxidative stress are subsequent and delayed to the checkpoint activation ([Passos et al, 2010](#)).

Prior to replicative senescence, events occurring early after telomerase inactivation, such as reversible DNA-damage checkpoints-dependent non-terminal arrests are observed in microfluidics experiments in which the consecutive single-cell divisions are monitored from

telomerase inactivation to senescence (Xu *et al*, 2015; Coutelier *et al*, 2018). These events have been proposed to be the ground for replicative senescence heterogeneity and genomic instability, but are expected to be infrequent and diluted out in the experimental setting we used here (Coutelier *et al*, 2018; Martin *et al*, 2021; Rat *et al*, 2023). Therefore, more investigations, in particular at single cell level, would be required to establish the chronology of events regarding ROS increase and checkpoint activation or cell death.

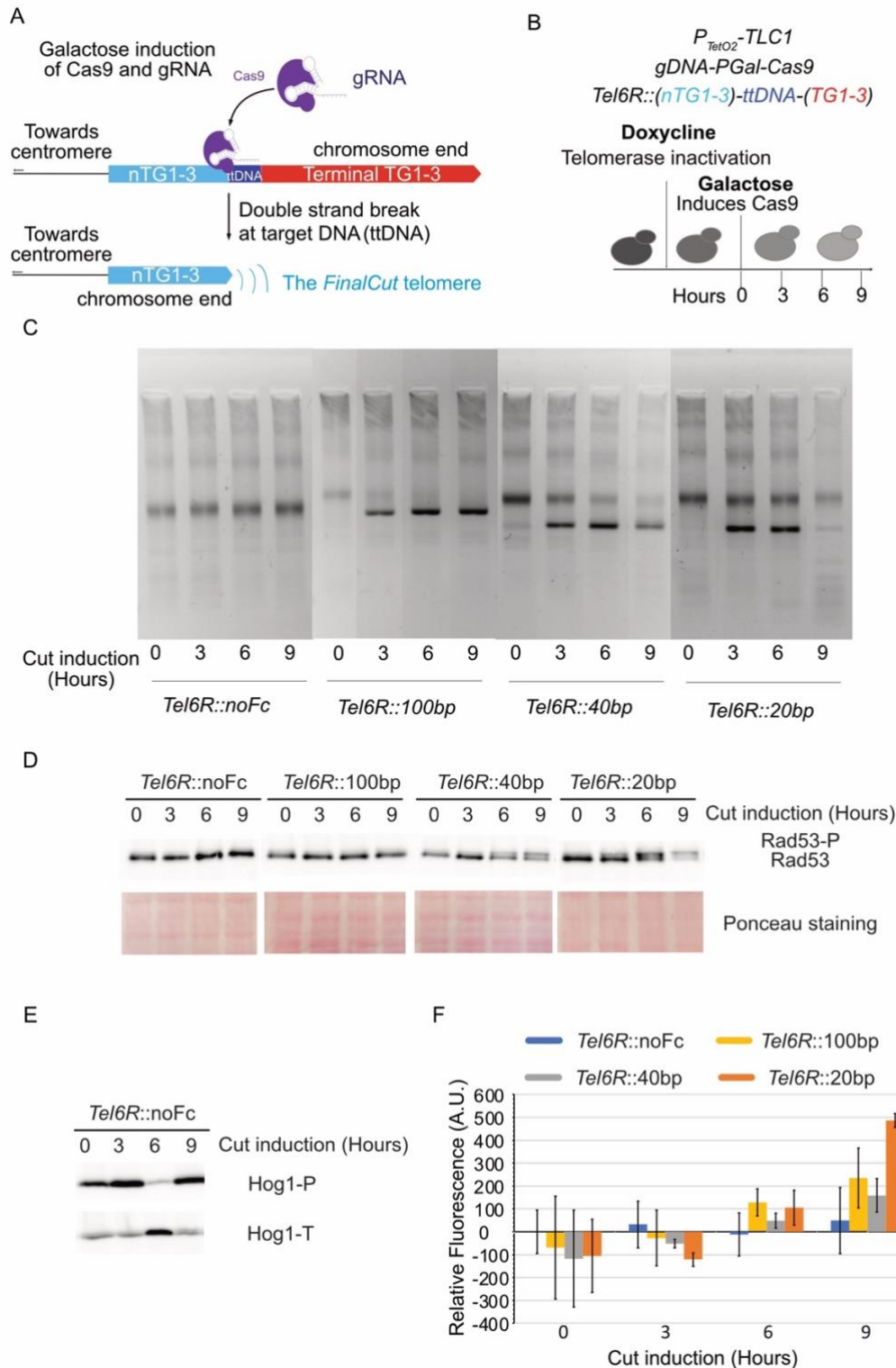


Figure 25: FinalCut system to bypass replicative senescence heterogeneity.

(A, B): Experimental design (C): TeloPCR of 6R telomeres of indicated strains. The smears and the precise bands represent respectively the heterogenous uncut telomeres and the precise length of cut telomeres. (D): Protein extracts analysed by Western blot using anti-Rad53 antibodies. (E): Protein extracts analysed by Western blot using an antibody against the phosphorylated form of Hog1's human ortholog p38 (Hog1-P) or total Hog1 (Hog1-T). (F): ROS levels are plotted as mean \pm SD of three independent transformants (normalized to 0 hours of the Tel6R::noFC).

Discussion

In the course of my thesis project, the primary focus was exploring the potential occurrence of oxidative stress during replicative senescence in *Saccharomyces cerevisiae*. Our results show that ROS levels increase at a population level in telomerase-negative cultures before the crisis of senescence and then decrease with the emergence of survivors. The secondary focus was to investigate if Hog1 is activated and play a role during replicative senescence. We show that Hog1 is activated during replicative senescence by its canonical MAPKK Pbs2 and the deletion *HOG1* results in a more pronounced increase in ROS levels. Thus, Hog1 may be a key player in mitigating ROS levels increase during replicative senescence in budding yeast. Notably, I uncovered that Hog1 acts independently of Mec1 that also seems to counteracts ROS levels. Another facet of our investigation centered on whether Hog1 influences telomere homeostasis. Our results demonstrate that Hog1 does indeed impact telomere length, as its absence shortens telomeres. We also unveiled Hog1's indispensability for cell survival even under normal, stress-free conditions. In order to understand how Hog1 acts, we also explored its potential involvement in autophagic processes during replicative senescence. Intriguingly, our results dismiss the notion of autophagy participating in budding yeast replicative senescence. In addition, we tested Hog1's potential involvement in TERRA expression. We found that Hog1 and TERRA likely operate independently during replicative senescence.

Throughout the upcoming section, I will engage in a comprehensive discussion of the obtained results, adding it to the existing data in budding yeast and drawing comparisons with existing literature in mammalian cells.

I. Oxidative stress is a feature of replicative senescence in budding yeast

1. ROS levels increase during replicative senescence

Oxidative stress has been documented during replicative senescence in many models, but there is a lack of corresponding data for budding yeast (Passos *et al*, 2007, 2010; Hutter *et al*, 2004; Allen *et al*, 1999; Lee *et al*, 2002; El Maï *et al*, 2020). Consequently, the primary objective was to quantify levels of ROS during replicative senescence in *S. cerevisiae*. Telomerase is constitutively active in *S. cerevisiae*, thereby maintaining telomere lengths

between 200 and 400 base pairs (Teixeira, 2013). To investigate replicative senescence, we employed two strategies to inhibit telomerase activity. First, we replaced the promoter of *TLC1*, the telomerase LncRNA, with a TetO2 promoter that can be repressed using doxycycline. Second, we deleted *TLC1* gene. The outcomes from both of these approaches consistently demonstrated an elevation in ROS levels upon telomerase inactivation in budding yeast. These findings are in accordance with existing data reported in other organisms. For subsequent experiments and further analysis, we used the P_{TetO2}-*TLC1* system because manipulations are easier. In *tlc1Δ* we also identified that survivors that evaded senescence exhibited a decline in ROS levels, bringing them to levels akin to actively proliferating cells. As a result, we propose that the underlying cause of the ROS increase can be attributed to specific metabolic alterations occurring exclusively within senescent cells with growth defects. We wanted to test whether ROS increase affect growth, which can accelerate senescence. However, our attempt to control ROS levels during senescence using antioxidants was unsuccessful. Thus, we could not draw a conclusion on this subject.

2. Mitochondrial dysfunction as a possible source of ROS increase during replicative senescence

The increase of ROS during replicative senescence in mammalian cells has been established to emanate from a mitochondrial origin. In these studies, MitoSOX, a fluorescent probe that can specifically detect superoxide anions within the mitochondrial matrix, was employed (Passos *et al*, 2007, 2010). Our employed DCF method lacks the capacity to elucidate the specific source of the ROS upsurge, but rather detects diverse ROS species. A previously published study has documented metabolic alterations and an increase in mitochondrial mass within senescent budding yeast cells as compared to young or survivor cells (Nautiyal *et al*, 2002). This observation is in accordance with our findings, which reveal elevated ROS levels within senescent cells in contrast to actively proliferating cells and survivors. From this, we favor the hypothesis that the production of ROS in budding yeast during replicative senescence might also stem from the mitochondria. Subsequently, the ROS levels decline in survivor cells would be due to post-senescent cells having restored functional mitochondria. So, either mitochondria dysfunction in senescent cells is reversible, or post-senescent survivors derive from cells preceding senescence. Thus, it would be interesting to monitor mitochondrial functions alterations in budding yeast senescent cells and survivors and use MitoSOX to detect

and quantify superoxide anions stemming from mitochondrial production during replicative senescence and in survivors.

3. ROS increase and checkpoint activation = what's the chronology of events?

Another important question was whether ROS increase during replicative senescence comes before or after the activation of the checkpoint. Studies in human and mice suggested that ROS and mitochondrial dysfunctions occur after checkpoint activation and that ROS contribute to the stabilization of the DDR response induced by dysfunctional telomeres (Passos *et al*, 2010). Our results, employing the FinalCut system, have shed light on this subject and support the idea that ROS levels increase follows checkpoint activation. Specifically, while the checkpoint was activated at intervals of 3, 6, and 9 hours subsequent to the 6R telomere cut to a 20 bp length, the increase in ROS levels was only discernible after the 9-hour timepoint. Consequently, we concluded that mitochondrial dysfunction and ROS increase in budding yeast could potentially result from the activation of the checkpoint mechanism. Furthermore, it's plausible that this ROS increase might be instrumental in establishing and fortifying the DDR response, much akin to the mechanisms observed in mammalian models. However, since telomeres are cut to a short length, senescence is accelerated when employing the FinalCut system and type B cells from which could stem most of the heterogeneity are reduced. Consequently, this could mask any potential rise in ROS levels occurring prior to checkpoint activation, as there may not be sufficient time for metabolic changes to occur within this timeframe. In addition, our results show that even if Mec1 is mutated, ROS levels increase upon telomerase inactivation. This suggests that oxidative stress is not a simple consequence of the DNA damage checkpoint activation in budding yeast. In order to establish the chronology of events between mitochondrial dysfunctions, ROS increase and the DNA damage checkpoint activation in a time resolved manner, we plan to take advantage of our custom microfluidic device. This would allow us to monitor the consecutive divisions of single cells until senescence over several days with high temporal resolution. Our first preliminary results validate the use of Yap1-GFP, the major ROS detoxifying protein in budding yeast to monitor ROS levels and show that ROS levels increase after the non-terminal arrests in type B senescent cells where the checkpoint is activated. Also, we plan on using Tom70 and pre-Cox4, fused to fluorescent proteins, to monitor respectively the structure and the dysfunctions of mitochondria. We will soon be able to couple these signals to the validated Yap1-GFP system to establish the detailed chronology

of events during replicative senescence between ROS increase mitochondrial alterations and the checkpoint activation.

II. Hog1 is activated during replicative senescence by its canonical MAPKK Pbs2

After we established that oxidative stress is a feature of replicative senescence in budding yeast, we were interested in delineating potential pathways that participate in ROS detoxification. The fact that mammalian MAPK p38 is activated and participates in replicative senescence (Iwasa *et al*, 2003), led us to ask if its orthologue in budding yeast, Hog1, plays a role in this process. In addition, Hog1 has been shown to be activated after a H₂O₂ stress (Haghnazari & Heyer, 2004). Our result shows that Hog1 is phosphorylated and thus activated during replicative senescence correlating with the observed ROS increase. Importantly, Hog1 is known to be activated by two different branches, the Sln1 and the Sho1 branch that converge in the MAPKK Pbs2 preceding Hog1 phosphorylation (de Nadal & Posas, 2022). Our results show that *PBS2* deletion results in the non-activation of Hog1. This led us to speculate that Hog1 is activated by its canonical pathway during senescence. Of note, the activation of p38 in senescent human fibroblasts, is reduced when its canonical MAPKK6 is mutated (Iwasa *et al*, 2003). The remaining question is whether ROS are the direct cause of Hog1 activation. Interestingly, after an oxidative stress, a study showed that Hog1 is mainly activated through the Sln1-Ypd1-Ssk1-Ssk2-Pbs2 pathway and that Ssk2 served as the MAPKKK that activates Hog1 (Lee *et al*, 2017b). Nevertheless, the signal of Hog1 activation during replicative senescence is internal and not external like an H₂O₂ stress thus it would be not surprising if the canonical sensors Sln1 and Sho1 are not required for its activation during this process. Thus, testing mutants that works upstream Pbs2 in the two branches may give us more insights about the mechanistic and the cause of Hog1 activation during senescence.

III. Hog1 regulates telomeres length homeostasis and affects cell viability

Our results show that the deletion of *HOG1* accelerates replicative senescence, contrasting with the inhibition of p38, which extends the lifespan and delays the onset of senescence (Iwasa *et al*, 2003). This suggests that Hog1 potentially plays an important role during senescence and is essential for cells viability in budding yeast telomerase-negative cells. However, our microfluidics results where cells grow individually show that even in the presence

of telomerase, the deletion of *HOG1* causes ~5,8% of cell mortality compared to ~0,38% in *P_{TetO2}-TLC1* strain. This increase mortality has gone undetected typically in our classical liquid senescence experiments most probably because cultures reach saturation the first day after telomerase inactivation, precluding the detection of small growth defects at the level of a population where competition and selection of the fittest cells operate. While a slight growth defect has been described (Piao *et al*, 2012), some studies have suggested a basal role of Hog1 (Mazor & Kupiec, 2009; Reynolds *et al*, 1998). We also tested if Hog1 affect telomeres length homeostasis that can directly impact the dynamics of senescence. Our results show that telomeres are initially shorter when Hog1 is deleted but the shortening rate in the absence of telomerase is similar to the *P_{TetO2}-TLC1* strain. This indicates that Hog1 is essential for telomeres length homeostasis but does not accelerate telomeres shortening. Importantly, multiple studies have demonstrated the interaction of p38 with telomeric proteins, suggesting a potential impact on telomere length homeostasis. Upon exposure to varying doses of doxorubicin, an anticancer drug, p38 is activated and downregulates the expression of Trf2, leading to the induction of senescence or apoptosis (Paolo *et al*, 2009). Following acute exercise in mice skeletal muscle, strong activation of p38 inhibits the expression of Trf1 (Ludlow *et al*, 2012). Furthermore, p38 has been shown to interact with Trf2 and positively regulate its expression in head and neck squamous cell carcinoma (Roy *et al*, 2018). In a mouse model, the proinflammatory cytokine TNF-alpha activates p38, leading to the phosphorylation of Atf7. This phosphorylation event releases Atf7/telomerase from telomeres, resulting in telomere shortening (Maekawa *et al*, 2018). Additionally, upon TNF-alpha exposure p38 modulates TERRA levels resulting in shorter telomeres in male offspring (Liu *et al*, 2019). Interestingly, Hog1 has been shown to positively regulate the localization of the SIR complex at telomeres after an osmotic stress (Mazor & Kupiec, 2009). Thus, we speculate that the shorter telomeres in the absence of Hog1 could be due to an alteration of the telosome. This can be tested by assessing the quantity of telomeric proteins in the presence and absence of Hog1. In addition, it is also possible that Hog1 interfere with the recruitment of telomerase which would impact telomeres length. Taken all these results, the marked mortality and the initial short telomere in the absence of Hog1 could in part explain the accelerated senescence. Thus, we cannot conclude on Hog1 activation being essential for replicative senescent cells. However, this does not exclude the fact that Hog1 could play relevant roles. Importantly, the increase in ROS in the absence of Hog1 could affect some telomeres and produce damages to telomeric sequences and for proteins. This would not be detectable when assessing average telomeres length, but would

have significant impact on senescence since only one dysfunctional telomere in budding yeast is sufficient to activate the checkpoint and results in the onset of replicative senescence.

IV. Does Hog1 regulate ROS levels during replicative senescence?

One major result we obtained is that ROS increase is higher when either Hog1 or Mec1 are absent, and even higher when cells lack both proteins.

A first hypothesis posits that Hog1 and Mec1 may not directly influence ROS levels during replicative senescence. Instead, ROS levels would be a consequence of replicative senescence with the observed increase in ROS levels in the absence of Hog1 potentially resulting from a premature onset of replicative senescence caused by shorter telomere length and diminished cellular viability. This notion is further supported by the parallel increase in ROS levels in the *mec1-21* mutant, which may similarly signify premature entry into replicative senescence. This is substantiated by comparable senescent curves and ROS increase in *P_{TetO2}-TLC1 hog1Δ* and *P_{TetO2}-TLC1 mec1-21*, while the *P_{TetO2}-TLC1 hog1Δ mec1-21* exhibits even greater impairment of cell growth in the absence of telomerase, coupled with a more pronounced rise in ROS levels than the double mutants. Evidence from studies in mice indicates a direct association between dysfunctional telomeres and mitochondrial dysfunction, leading to elevated ROS levels (Sahin *et al*, 2011). Consequently, it is plausible that the observed indirect increase in ROS levels in Hog1 and Mec1 mutants stems from shorter telomeres in their absence. However, the basal activity of Hog1 appears to regulate telomere homeostasis, but the activation of Hog1 during replicative senescence implies a potential role in this process, whether in counteracting ROS levels or otherwise. In contrast to Hog1 in budding yeast, p38 in mammals was shown to be essential for maintaining high levels of ROS after DNA damage response upon dysfunctional telomeres (Passos *et al*, 2010). This implies that p38 and Hog1 activation during replicative senescence may have opposite outcomes, and the mechanisms linking telomeres to Hog1 and ROS are potentially different between yeast and mammalian cells.

Notably, Hog1 is an important player in ROS levels regulation. On one hand, Hog1 can activate the transcription of antioxidant genes to limit ROS levels and is essential for cell survival after an oxidative stress (Rep *et al*, 2001; Wong *et al*, 2003). On the other hand, in the case of a sustained activation, Hog1 has been shown to interfere with the mitochondria and increase the levels of ROS (Vendrell *et al*, 2011). This let us wonder if Hog1 participates in the detoxification or generation of ROS during replicative senescence. Our results that in the

absence of Hog1, ROS levels increase earlier and are higher than in *P_{TelO2}-TLC1* cells could thus be in accordance with the hypothesis that Hog1 would participate in ROS detoxification during replicative senescence. In addition, our results suggest that Mec1, also likely participate in ROS detoxification during senescence but independently from Hog1. This is also in accordance with a study that showed that upon an H₂O₂ stress Hog1 and Mec1 are both necessary but act independently (Haghnazari & Heyer, 2004). Therefore, to explain our results, we favor the hypothesis is that in response to telomere shortening-dependent ROS increase, Hog1 activates transcription factors as Msn2/4 and Sko1 to induce the transcription of antioxidant genes. To test this hypothesis, it would be interesting to measure if the antioxidants genes expression or activity increase during replicative senescence and if the absence of Hog1 results in less expression or activity. Testing defective mutants for the transcription factors that work downstream of Hog1 and checking if STREs and CREs elements are induced during replicative senescence in a Hog1-dependent manner would also give more insight about Hog1's role. These experiments would be crucial to directly link Hog1 to ROS detoxification during replicative senescence.

V. Autophagic processes do not participate in replicative senescence

Published data in mammalian models show conflicting results on the role of autophagic processes during senescence. High flux of recycled amino acids and metabolites generated by autophagy has been shown to promote senescence and facilitate the SASP phenotype (Narita *et al*, 2011). In contrast, inhibition of autophagy during senescence has been shown to contribute to senescence and the SASP phenotype (Kang *et al*, 2015) and the knockdown to autophagic components results in a premature senescence and an increase in ROS levels (Kang *et al*, 2011). Moreover, studies have shown that replicative senescence inducing pathways can decrease mitophagy. Indeed, p53 has been shown to impair mitophagy through its interaction with Parkin, the protein responsible for recognizing damaged mitochondria. This leads to the accumulation of damaged mitochondria in cigarette smoke induced senescence and promotes cardiac dysfunction during ageing (Ahmad *et al*, 2015; Hoshino *et al*, 2013). Furthermore, a study suggested that ROS produced in senescent cells can result in the accumulation of p53 in the cytosol which would impair mitophagy (Manzella *et al*, 2018). In addition, fission processes highly correlate with mitophagy, in contrast to fusion processes (Youle & van der Bliek, 2012). However, mitochondria have been shown to be hyperfused during senescence (Mai *et al*, 2010).

In accordance with this, the expression of S-nitrosoglutathione reductase (GSNOR) that regulates S-nitrosylation decreases during aging and senescence which decreases fission processes and impairs mitophagy (Rizza *et al*, 2018). Importantly, autophagy increases and is responsible for cell death during replicative crisis which follows replicative senescence (Nassour *et al*, 2019). Our results in budding yeast show that blocking autophagy or mitophagy does not grossly impact the dynamics of replicative senescence. We also show that the acceleration of senescence in the absence of Hog1 is independent from bulk autophagy and mitophagy since blocking these processes do not further impact senescence. Yet, all these results do not exclude that autophagic processes may happen during senescence in budding yeast. We tried to measure autophagy by tagging proteins involved in these processes with GFP then by following with Western blot their degradation, however, this method was not sensitive enough to use with our liquid senescence experiments. In addition, the rosella system showed high level of mortality when cells express this system, precluding this system to be used in senescence. Tagging a more abundant cytoplasmic protein like Pgk1 then following its degradation would be a good alternative in order to draw a conclusion on if autophagic processes occurs in fermentative conditions during replicative senescence.

VI. Hog1 acts independently of TERRA during replicative senescence

Upon an osmotic stress, Hog1 has been shown to modulate the transcription of LncRNAs (Nadal-Ribelles *et al*, 2014). TERRA, the telomeric LncRNAs, is crucial for telomeres homeostasis and has been proposed to be involved in the recruitment of telomerase (Cusanelli *et al*, 2013) and the maintenance of telomeres length during replicative senescence (Balk *et al*, 2013). Preliminary unpublished results from the lab of our collaborator (F. Posas, IRB Barcelona) show that upon an osmotic stress Hog1 binds subtelomeric regions and induces the transcription of TERRA. Therefore, we wanted to investigate if Hog1 action during replicative senescence is through the modulation of TERRA levels. Our first results show that TERRA increase during replicative senescence in the presence or the absence of Hog1 suggesting that Hog1 may not be involved in modulation TERRA levels during this process. Our attempt to overexpress RNaseH1 in order to test if Hog1 acts to regulate TERRA R-loops during replicative senescence shows highly variable results. This could be explained by the high mortality rate observed when overexpressing RNaseH1. The activation of Hog1 in FinalCut strains cultures conditions limited us from using this system to measure TERRA levels in the absence and presence of Hog1 from a precise cut telomere. All of these suggest that Hog1 likely

acts independently of TERRA during replicative senescence. In order to draw a final conclusion on this, it would be interesting to check if Hog1 binds at subtelomeric regions during replicative senescence by performing chromatin immunoprecipitation experiments. An alternative hypothesis may be that Hog1 indirectly influence TERRA transcription through its downstream transcription factors or chromatin remodelers. A similar mechanism has been observed in mammalian cells involving p38. In this case, p38 phosphorylates its transcription factor, Atf7, upon exposure to TNF-alpha, resulting in the release of Atf7 from subtelomeric promoters of TERRA. This process induces an increase in TERRA levels through heterochromatin disruption (Liu *et al*, 2019). Notably, no studies to date have connected p38 and TERRA during replicative senescence in mammalian cells. Therefore, it is also plausible that Hog1 may modulate TERRA in specific stress conditions, even if it does not affect TERRA during replicative senescence.

Conclusion

In conclusion, data obtained during my thesis highlight that oxidative stress is a feature of replicative senescence in budding yeast and that Hog1 regulates telomeres homeostasis and may contribute to the link between telomeres and ROS metabolism. Based on our results and data from the literature, we propose a model (Figure 26). The basal activity of Hog1 contributes to the regulation of telomeres length and cellular viability in the presence of telomerase. In the absence of telomerase, telomeres shorten after each cell cycle until one of them reaches a critical length and activates the DNA damage checkpoint. This leads to replicative senescence and the persistent arrest in the G₂/M phase of the cell cycle where deep changes in gene expression and metabolic alterations would impact mitochondrial structure and function and would result in the increase of ROS levels. Hog1 is activated during replicative senescence by its MAPKK Pbs2 and is likely to counteract ROS increase independently of the Mec1 which also likely contribute to ROS detoxification. Hog1's action is independent from autophagic processes and from TERRA. Further experiments are required to unveil precisely the mechanistic behind Hog1's activation, if ROS increase stems from the mitochondria and if Hog1 and Mec1 regulate directly ROS levels during replicative senescence.

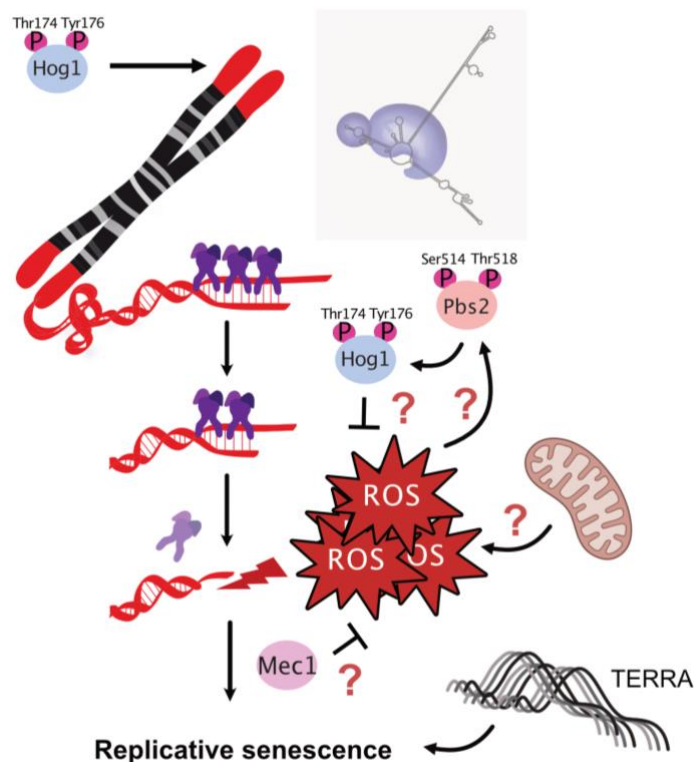


Figure 26 : Model of Hog1 implication during replicative senescence.

Annexes

Article n°2: TERRA and telomeres maintenance in the yeast
Saccharomyces cerevisiae

Bechara Zeinoun, Maria Teresa Teixeira and Aurélia Barascu

Genes 2023, 14, 618

Review

TERRA and Telomere Maintenance in the Yeast *Saccharomyces cerevisiae*

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Abstract: Telomeres are structures made of DNA, proteins and RNA found at the ends of eukaryotic linear chromosomes. These dynamic nucleoprotein structures protect chromosomal tips from end-to-end fusions, degradation, activation of damage checkpoints and erroneous DNA repair events. Telomeres were thought to be transcriptionally silent regions because of their constitutive heterochromatin signature until telomeric long non-coding RNAs (LncRNAs) were discovered. One of them, TERRA (TELomeric Repeat-containing RNA), starts in the subtelomeric regions towards the chromosome ends from different telomeres and has been extensively studied in many evolutionarily distant eukaryotes. Changes in TERRA's expression can lead to telomeric dysfunction, interfere with the replicative machinery and impact telomere length. TERRA also co-localizes in vivo with telomerase, and can form RNA:DNA hybrid structures called R-loops, which have been implicated in the onset of senescence and the alternative lengthening of telomere (ALT) pathway. Yet, the molecular mechanisms involving TERRA, as well as its function, remain elusive. Here, we review the current knowledge of TERRA transcription, structure, expression, regulation and its multiple telomeric and extra-telomeric functions in the budding yeast *Saccharomyces cerevisiae*.

Keywords: TERRA; R-loops; senescence; telomere maintenance; survivors; *Saccharomyces cerevisiae*



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1. Introduction

Chromosomes are subjected daily to constant genotoxic stresses, which can be of endogenous (replicative stress, oxidative stress, etc.) or of exogenous origin (irradiation, exposure to chemical agents, etc.). Among the different types of DNA damage, double stranded breaks are the most toxic because they can lead to the loss of large pieces of chromosomes. DNA damage repair mechanisms ensure genome stability through the orchestration of a set of enzymatic activities, which specifically identify these accidental double-stranded ends and repair them, thus limiting the loss or alteration of genetic information for future generations. However, since eukaryotic chromosomes are linear, they have natural ends that resemble double-strand breaks called telomeres. The main function of these nucleoprotein structures is to maintain the integrity of chromosomes by protecting them from the DNA repair machinery. Thus, functional telomeres are not recognized as accidental double-strand breaks and do not become fused or degraded by repair processes [1].

The nucleotide sequence of telomeres is highly conserved in eukaryotes, formed by regular or irregular tandem repeats of a guanine-rich motif, which terminates in most organisms studied with a 3' tail. This composition is due to the activity of telomerase, the main enzyme ensuring telomere length maintenance. In budding yeast, telomeres are approximately 300 +/− 75 b and telomeric DNA is histone-free, formed by an irregular repeated sequence T(G)_{1–3}/(C)_{1–2}A and terminates with a 5 to 10 nt 3' single-strand overhang [2–4]. The length of the telomeric single-strand region is also dynamic and heterogeneous in nature, increasing transiently in the late S phase (about 40–100 nt) [2,5–7].

The protection and stability of the telomere structure is ensured by a specialized protein complex referred to as “shelterin” in mammalian cells. In *S. cerevisiae*, Rap1 was first described as a transcription regulator of many essential genes across the yeast genome, but also binds double-stranded telomeric DNA to protect chromosome ends from fusions. At telomeres, Rap1 recruits Rif1/2 proteins, which contribute to telomere protection functions and to regulating telomerase. In addition, Rap1 recruits the Sir3/4 (silent information regulator) proteins, which interact with Sir2, a NAD⁺-dependent histone deacetylase, to repress transcription. The CST complex, found at the 3′ overhang, comprises Cdc13, Stn1 and Ten1 to protect the C-strand from 5′ to 3′ resection, and regulates telomerase [8].

To better understand the essential functions of telomeres, we must decipher the dynamics and changes in telomere length, protein composition and structure along the cell cycle and over consecutive cell divisions. In fact, DNA replication is one of the mechanisms that inevitably affects telomeres. The enzymatic features of DNA polymerases do not support the complete replication of telomeres, generating the so-called end replication problem. This causes a progressive shortening of telomeres with each cell cycle. Telomerase is a reverse transcriptase that serves to counteract this end-replication problem in most eukaryotes studied to date. In budding yeast, telomerase is composed of a catalytic reverse transcriptase subunit Est2, two accessory subunits, Est1 and Est3, and a long non-coding RNA, TLC1, that serves as a template for the iterative synthesis of telomeric repeats. Telomerase is constitutively expressed in yeast cells to maintain telomeres at an average size that results from a balance between permanent shortening and lengthening and to enable unlimited yeast proliferation [9]. However, in many multicellular organisms such as humans, only few cells including stem and germ cells express telomerase. Somatic cells repress telomerase, so telomeres shorten with each cell division until they reach a critical size that leads to a permanent arrest of proliferation called replicative senescence. At the molecular level, this arrest is caused by the activation of the DNA damage response at telomeres. In *S. cerevisiae*, the senescence signal in telomerase-negative cells is thought to come from the first telomere reaching a critical reduced size [10,11], an idea which was consolidated by mathematical simulations and formal genetics [12]. Consistent with this hypothesis of senescence being driven by the shortest telomeres, five dysfunctional telomeres were detected in human cells in culture at the onset of senescence [13]. Thus, telomeres play a central role in controlling cell viability, and their proper functioning ensures genome stability.

Subtelomeres are genomic regions found upstream of the telomeres and are made up of long, heterogeneous and variable repeated elements, subject to rapid evolution. Subtelomeric regions do not contain genes essential for cell viability under rich growth conditions, but the extreme plasticity of these regions ensures rapid adaptation of yeast under various stress conditions [14–17]. This is because subtelomeres include multigene families coding for functions related to interaction with the environment. Unlike telomeres, the subtelomeric regions of *S. cerevisiae* are covered by nucleosomes [18,19]. The Sir proteins play a key role in the compaction of these regions and the telomere position effect (TPE) that results in the silencing of genes near telomeres [20,21]. In *S. cerevisiae*, all subtelomeric regions have an X element, followed by one to four repeats of a 5–7 kb-long Y′ element in nearly half of the telomeres [22–25]. However, despite the heterochromatin marks found in these regions, the subtelomeric sequences and even telomeres are transcribed into different non-coding RNAs [26–28]. Telomeric transcription was described in *S. cerevisiae*, *Schizosaccharomyces pombe* and several other mammals, birds, fish, plants and protozoa species, suggesting that telomeric transcription spans the whole eukaryote evolution [27,29–39]. Among the non-coding RNAs transcribed from the regions closer to the tip are ARRET, α ARRET, subTERRA and TERRA. Here, we focus on the most recent findings on TERRA in *S. cerevisiae*.

2. TERRA Transcription and Primary Structure

TERRA (TElomeric Repeat-containing RNA) was first discovered in mammalian cells as a long non-coding RNA which contains UUUAGG repeats and varies in length from 100 b to more than 9 kb, colocalizes with telomeres, and is considered part of telomeric heterochromatin [26,40]. TERRA transcription at telomeres is an apparently conserved feature in eukaryotes, and its length and quantities vary within different cell lines, species, tissues and developmental stages. In budding yeast, Northern blot analysis showed that TERRA is heterogeneous, ranging from 100 to 1200 b, with an average of 380 b. In *S. cerevisiae*, TERRA levels are kept low mainly by the 5′-3′ exonuclease Rat1, a fraction of which is found at telomeres and degrades TERRA [36,41]. Xrn1, the cytoplasmic paralog of Rat1, has no effects on TERRA levels, supporting the idea that yeast TERRA is mostly nuclear.

TERRA LncRNA is transcribed by RNA Polymerase II, using the telomeric C-rich strand as a template (Figure 1). ChIP experiments revealed that RNA pol II associates with telomeres, and inactivating this polymerase by an *rpb3-2* temperature-sensitive allele blocks TERRA accumulation [36]. TERRA transcription starts at subtelomeres, toward the end of the chromosomes from both Y′ and X-only telomeres. Thus, TERRA has the same sequence as the G-rich sequence and harbors specific subtelomeric sequences at the 5′ end and telomeric repeats at the 3′ end [36,41]. Most of TERRA heterogeneity stems from its 3′ ends, yet its promoter remains ill-defined. Using 5′RACE and RLM-5′RACE, some transcription start sites (TSSs) of TERRA have been identified on several telomeric ends from Y′ and X-only telomeres [42–44]. This revealed that Y′ TERRA starts closer to telomeric repeats than X-only TERRA and that the +1 nucleosome for TERRA transcription is stabilized by Sir2. In mammalian cells, promoters have been found at numerous human chromosome ends in CpG-islands residing in subtelomeric regions, and TERRA was similarly shown to be affected by the chromatin state [45,46]. TERRA accumulates at the G1/S transition prior to the replication of telomeres and then decreases in the late S phase. Thus, in budding yeast, similarly to mammalian cells, TERRA is finely regulated through the cell cycle [47–49].

The canonical poly(A) polymerase Pap1 polyadenylates the majority of yeast TERRA, bestowing more stability to the molecule [36]. In a *pap1-1* temperature-sensitive allele, TERRA is destabilized at a restrictive temperature. CF1A, the endo-nucleolytic cleavage factor that acts prior to Pap1, seems to also be important for TERRA biogenesis [36] (Figure 1). In contrast, the deletion of *TRF4*, encoding an alternative poly(A) polymerase of the TRAMP complex (whose action targets RNAs for degradation by the exosome) increases TERRA levels in a background where TERRA already accumulates, indicating that TERRA can be polyadenylated by TRAMP and thus subjected to nuclear exosome degradation, but to a minor extent [36,50] (Figure 1). Consequently, TERRA would harbor a poly(A) tail on its 3′ end. Yet, only 7% of yeast TERRA is polyadenylated [50]. In human cells TERRA, the absence or presence of the poly(A) tail determines the association or not to chromatin [26,36,38,40,41,49]. In humans and fission yeast, TERRA harbors at its 5′ end a 7-methylguanosine cap structure not yet identified in budding yeast [49,51].

The low levels detected in wild-type yeast cells makes it difficult to quantify endogenous TERRA by Northern blot and RT-qPCR [36]. Thus, to further study TERRA biogenesis and functions, TERRA levels have been artificially raised using artificial regulatable promoters inserted before the telomeric tract or in subtelomeric regions [36,42,52,53]; but, these methods have shown that it interferes with Ku70/80 function and creates an Exo1-dependent resection at the chromosome ends (see below). Another approach was through in vivo detection of endogenous TERRA molecules. Several 21 nt-long stem-loop MS2 phage sequences were inserted in tandem within the TERRA locus of a single telomere. Upon transcription, the resulting TERRA molecules can then be detected by live cell imaging through the high-affinity binding of the MS2 phage coat protein fused to a fluorescent protein expressed in cells. Using this approach, it was concluded that TERRA is detected only in a small population of cells (10%) from each given telomere, and the transcription product forms a perinuclear focus during the interphase [54]. In other words, for

each given chromosome end, TERRA appears to be stabilized at telomeres approximately one time every ten cell divisions. Thus, in each cell, ~10% of telomeres would have their TERRA stabilized.

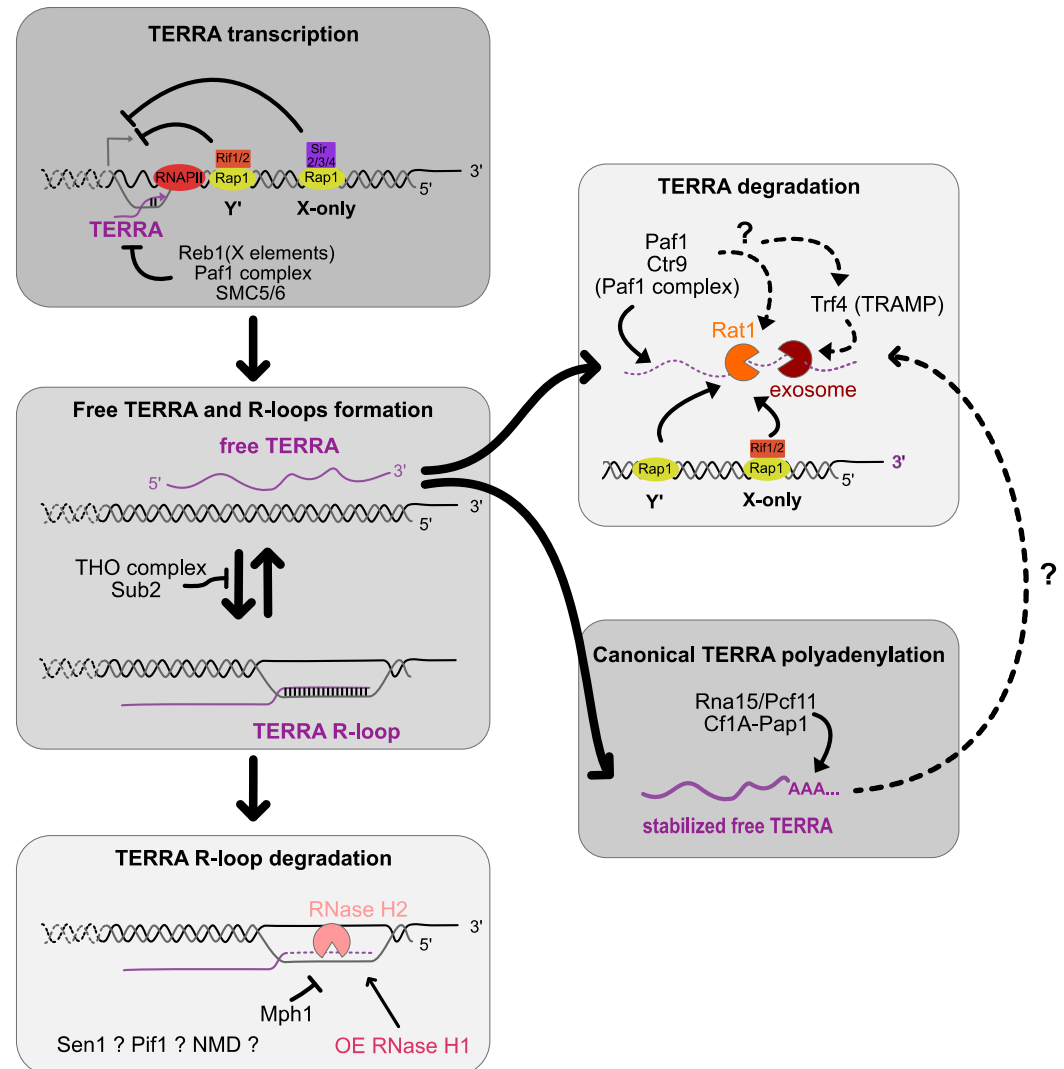


Figure 1. TERRA transcription and regulation in budding yeast. RNA polymerase II (RNAPII) transcribes TERRA from the 5' C rich telomeric strand starting in subtelomeres towards telomeric ends from both X-only and Y'-telomeres. Rap1 represses TERRA transcription by recruiting Sir2/3/4 on X-only telomeres and Rif1/2 on Y' telomeres. Reb1 binds on its binding sites residing in the X elements repressing TERRA probably by road-blocking transcription. TERRA transcription is also regulated by the Paf1 complex and SMC5/6 complex. Free TERRA is mainly degraded by the exonuclease Rat1 in manner dependent on Rap1. Rif1/2 is recruited by Rap1 and participates to the degradation of TERRA partially by recruiting Rat1 on X-only telomeres. The canonical poly(A) polymerase Pap1 stabilizes TERRA by polyadenylating its 3' end. TRF4 is also suspected to polyadenylate TERRA which may play a role in its degradation by targeting it to the exosome. The Paf1 complex may reduce TERRA levels by acting on Rat1 or/and Trf4. TERRA is also found under an RNA:DNA configuration forming R-loops with telomeric ends. The THO complex plays a role in TERRA processing and reduces TERRA RNA:DNA hybrids. RNase H2 is found at telomeric level and degrades TERRA RNA:DNA hybrids participating in their cell cycle regulations. RNaseH1 is not detected at telomeres in telomerase positive cells but its overexpression reduces TERRA R-loops. The helicase Mph1 plays a role in limiting telomeric RNA:DNA R-loops. The involvement and local regulation of these R-loops by Sen1, Pif1 helicases and the NMD pathway has yet to be tested.

3. Transcriptional, Co-Transcriptional and Post-Transcriptional Regulations

Subtelomeric elements control transcription in many ways, and this was first discovered through the study of the silencing of polymerase II-dependent genes inserted near telomeres: the so-called “telomere position effect” (TPE). TERRA transcription itself is subjected to some known silencing mechanisms, such as the ones regulated by the Sir complex. Another way subtelomeres limit TERRA transcripts’ level is by regulating distinct Rap1-mediated pathways (Figure 1) [41]. This was discovered by detecting an increase in TERRA levels at both X-only and Y’ telomeres in a *rap1-17* mutant in which the C-terminus of Rap1 was truncated, thus preventing the recruitment of both Rif1/2 and Sir2/3/4 complexes to telomeres. When dissecting which protein complex was responsible for this effect, it was found that at the X-only telomeres, Rap1 recruits both the Sir2/3/4 complex, which presumably represses TERRA transcription, and the Rif1/2 complex, which negatively regulates TERRA in part via Rat1-dependent degradation. In contrast, at the Y’ telomeres, the Sir2/3/4 complex has no effect on TERRA levels and the Rif1 effect is independent of Rat1. Subtelomeres also contain binding sequences for Reb1, an essential transcription factor. In *reb1^{ts}*, TERRA levels increase 75-fold compared with WT, suggesting an effect of Reb1 in the transcription of TERRA (Figure 1) [43]. Since most subtelomeric binding sites of Reb1 reside downstream of the TSS of TERRA in the X element, it appears that Reb1 represses TERRA levels by a roadblock transcriptional termination mechanism, independently of Sir-complex-dependent chromatin modifications. The PAF1 complex is a transcription elongation factor that affects RNA levels through transcriptional and post-transcriptional modifications and via its binding to RNAPol II. In budding yeast, this complex is recruited at telomeres and limits TERRA levels through its subunits Paf1 and Ctr9 [50]. While the exact mechanism, possibly co-transcriptional, is still elusive, it appears that it is mostly independent from Rat1, the Sir complex and the exosome (Figure 1). The SMC5/6 complex, one of the structural maintenance of chromosome protein complexes and which plays an organizational and structural role in telomeres, is also involved in limiting TERRA levels [55]. While this complex is involved in the recruitment and sumoylation of the Sir complex, the effects of SMC5/6 complex on TERRA levels are also partially independent from Sir4 (Figure 1).

Overall, at all telomeres, the Sir complex and Rat1 are the key to limiting the global levels of TERRA, but many other factors are involved in fine tuning TERRA quantities in cells. Still, the additional level of regulation of TERRA within the S phase was found to depend mainly on Rat1 [47].

4. TERRA RNA:DNA Hybrid Regulation

TERRA was found to form three-stranded RNA:DNA telomeric hybrid structures, called R-loops, whose regulation is linked to telomere size and homeostasis [36,47,56–58]. R-loops are RNA:DNA structures in a three-stranded configuration where the RNA hybridizes with the DNA template, leaving a single-stranded DNA loop. TERRA telomeric R-loops are preferentially formed due to the G-rich nature of TERRA that binds stably to the C-rich DNA template. In addition, R-loops are naturally stable since the interaction of RNA:DNA is conformationally stronger than DNA:DNA. Depending on the levels and the location of R-loops, they could be beneficial or deleterious to the genome [59]. R-loops can be a source of replication stress, interfering with the DNA replication machinery and leading to replication fork collapses and collisions. This was shown to generate DSBs triggering recombination events [60]. In contrast to TERRA, which is hard to detect in unchallenged cells, RNA:DNA hybrids are readily detected at telomeres by chromatin immunoprecipitation using an antibody (S9.6) that recognizes the RNA:DNA hybrids, known as the DRIP technique (DNA-RNA ImmunoPrecipitation) [47,56–58]. Using this technique, it was found that RNA:DNA hybrids accumulate in the early S-phase, before telomere replication, which is known to happen later in the S-phase, suggesting that TERRA and R-loops are regulated throughout the cell cycle in a coordinated way with replication such that the potential harmful outcomes of the TERRA R-loops are avoided [47]. Recently, R-ChIP experiments

using a catalytic dead form of RNase H1, Rnh1-cd, yields more sensitivity in detecting hybrids at telomeres and could be used to detect more layers of TERRA regulation [61].

RNase H1 and RNase H2 enzymes contribute to the removal of RNA:DNA hybrids by endonucleolytic cleavage of the hybridized RNA [62,63]. While RNase H1 is composed of the gene product of *RNH1*, the RNase H2 holoenzyme is made of three subunits: Rnh201 (the catalytic one), Rnh202 and Rnh203. Additionally, while RNase H1 functions independently of the cell cycle and mostly responds to R-loop stress, RnaseH2 may only be effective in certain cell cycle phases. The deletion of RNase H enzymes and the OE of RNase H1 are mostly used to control R-loop levels in the cell. The first evidence that TERRA forms telomeric R-loops in budding yeast comes from the overexpression of Rnh201, which was found to specifically reduce TERRA in a *rat1-1* mutant in which TERRA accumulates [36]. Conversely, in an *rnh1 rnh201* double mutant, R-loop levels increase [53,56,57], suggesting that telomeric R-loops are controlled by endogenous RNase H enzymes (Figure 1). Nevertheless, most of TERRA in a wild-type cell appears not to be in an R-loop configuration, since the over-expression of RNase H1 only reduces TERRA at some telomeres. Most importantly, RNase H2 participates in the regulation of TERRA R-loops during the S phase [47]. The telomeric protein Rif2 contributes to RNase H2 recruitment to degrade telomeric R-loops, and accordingly the deletion of Rif2 causes the accumulation of hybrids, showing its importance in preventing R-loop formation. In contrast, RNase H1 could not be detected at telomeres in telomerase-positive cells, suggesting that TERRA R-loops are mainly removed by RNase H2 in unchallenged conditions.

The THO complex plays a role in transcription elongation and mRNA export. In budding yeast, four proteins constitute this complex: Tho2, Hrp1, Mtf1 and Thp2 [64]. A fraction of this complex was found at the telomeres and plays a role in telomere stability [57]. Indeed, THO promotes the telomere maintenance through the control of TERRA biogenesis. Similar to an *rnh1 rnh201* double mutant, a *thp2* mutant has no effect on the global pool of TERRA, but shows an increase in telomeric RNA:DNA hybrids. In contrast, the deletion of *THO2* and *HRP1* downregulates the levels of TERRA at both *Y'* and X-only element telomeres and increases TERRA hybrids at telomeres [53,57,65]. THO complex deregulation can thus lead to an inappropriate assembly of TERRA into native ribonucleotides, thereby impairing its localization. Therefore, this complex plays a role in TERRA processing and suppresses R-loops at telomeric levels (Figure 1).

The SMC5/6 complex not only limits the level of TERRA (as discussed above) but can also act on R-loops through the negative regulation of Mph1, the helicase yeast homolog of mammalian FANCM. Mph1 was found to accumulate at RNA:DNA hybrids, which increase in RNase H mutants during replication to prevent the accumulation of DNA damage [58]. In addition, Mph1 accumulates at short telomeres in an R-loop-dependent manner. The model would show that Mph1 is recruited when R-loops accumulate to participate in recombinogenic activities triggered by R-loops. The SMC5/6 complex would thus be essential for the regulation of Mph1 to limit its pro-recombinogenic activity at RNA:DNA hybrids.

Other factors may also be implicated in the fine regulation of TERRA telomeric R-loops including the Sen1 and Pif1 RNA/DNA helicases that may locally unwind these hybrids. The nonsense-mediated mRNA decay pathway (NMD) involved in the RNA quality control surveillance mechanism may also regulate TERRA. NMD mutants have shorter telomeres because of an increased level of telomere-capping factors Stn1 and Ten1 [66,67]. High-throughput analysis shows that *Y'* element transcripts accumulate in an NMD mutant (*upf1Δ*) [68], but Northern blot analysis reveals that TERRA transcripts do not increase in NMD mutants (*upf1Δ*, *upf2Δ*, *upf3Δ*, *xrn1Δ*) [36]. This does not exclude that the NMD pathway could regulate TERRA association to telomeres locally without impacting the total amounts, as observed in mammalian cells [26].

5. TERRA and Telomeres Length

It has become clear that the TERRA pool and R-loop levels were found to increase when telomeres are shorter and increase even more when telomeres become critically short. This *cis* regulation, dependent on the amounts of Rap1 and Rif2 in *cis*, is dependent on telomere length, as detailed in the above sections [41,47,69]. Rat1 and RNase H2 are involved in this specific regulation, and the loss of telomeric proteins with the shortening of telomeres impairs their recruitment, resulting in more TERRA [47,69]. Thus, TERRA is upregulated at short telomeres.

Yet, in a telomerase-positive context, telomere length and TERRA levels were found to not always correlate inversely in mutants affected by the homeostasis of telomere length. *rat1-1*, *paf1Δ*, *nse3-1* and *thp2Δ* mutants have shorter telomeres and increase the TERRA pool or accumulate R-loops [36,50,57]. On the contrary, some mutants show a positive correlation between TERRA levels and telomere length and others are neutral. For example, *rap1-17* and *Rif1/2Δ* mutants have longer telomeres and increase the TERRA pool, but *Sir2/3* have milder changes in telomere length [41]. Mutants in the THO complexes *tho2Δ* and *hpr1Δ* have longer telomeres and show an increase in R-loops [57]. Over-elongated telomeres by tethering telomerase to telomeres only show a slight decrease in TERRA levels [41]. If we take a specific case, *sir2Δ* and *reb1^{ts}* have longer telomeres and more TERRA, but the double mutants have even more TERRA and shorter telomeres compared with single mutants. Therefore, one cannot rule out that some proteins involved in TERRA regulation can have other functions, which could impact telomeres independently of regulating TERRA.

6. TERRA Interacts with Telomerase and Regulates Its Function

TERRA expression increases with the shortening of telomeres in a *cis* regulation (as discussed above) and telomerase is recruited preferentially to short telomeres [70]. This is due to the increased occupancy of Rap1 molecules at longer telomeres inhibiting telomerase [71]. Since TERRA is transcribed from the C-rich strand and it contains telomeric repeats, one can speculate an interaction between TERRA and the telomerase template RNA, the *TLC1* LncRNA, which is complementary to TERRA. In budding yeast, telomerase impaired action was first suspected to be the cause of short telomeres observed in a *rat1-1* mutant where TERRA levels increase [36]. Indeed, telomere length in the *rat1-1 est2Δ* double mutant is epistatic to an *est2Δ* single mutant, indicating that telomerase-dependent lengthening of telomeres is impaired in *rat1-1* cells. The overexpression of RNaseH2 in the *rat1-1* context leads to less TERRA and suppresses the phenotype observed, which could suggest that TERRA:DNA hybrids may inhibit telomerase. However, TERRA telomeric hybrids were not detected specifically; rather, TERRA global levels were measured by Northern blot, and since RNase H2 works in a trimeric complex, we do not know if its over-expression actually reduces R-loops and, if so, to what extent. Further studies are needed to bring a clear conclusion on this matter.

Using live-cell imaging, the laboratory of P. Chartrand showed that TERRA expressed from a given telomere forms a perinuclear focus, which becomes multiple foci when telomeres shorten in the absence of telomerase [54,72]. TERRA foci colocalize and interact with *TLC1* RNA independently of DNA during the G1/early S phase preceding the clustering of telomerase during the late S phase, preferentially at the same telomere that transcribed TERRA. This suggests that TERRA would be the signal generated by these short telomeres, playing a role in the nucleation of *TLC1* RNA and the clustering of telomerase required for telomere elongation in *cis* [73]. Thus, TERRA could be assigned the role of a scaffold molecule. Evidence that TERRA interacts physically with telomerase and promotes its recruitment and activity in telomeres has been also reported in fission yeast [74].

In mammalian cells, TERRA interacts with telomerase and its template LncRNA in vivo [75]. Short TERRA-like oligonucleotides can base pair with telomerase template RNA, interact with telomerase and reduce the activity, but not the processivity, of telomerase in vitro [40,75,76]. In contrast, in vivo studies do not support that notion, showing that telomerase elongates telomeres efficiently independently of TERRA and telomere tran-

scription [46]. This does not exclude the fact that, *in vivo*, factors may exist participating in the switching of telomerase from TERRA to telomeric ends, repressing its inhibition depending on the cell-cycle phase [76,77].

All these studies agree that TERRA interacts with telomerase, but further investigation is required to draw a clear conclusion on how this interaction plays a role in telomere length maintenance and to unveil the detailed mechanisms behind it (Figure 2).

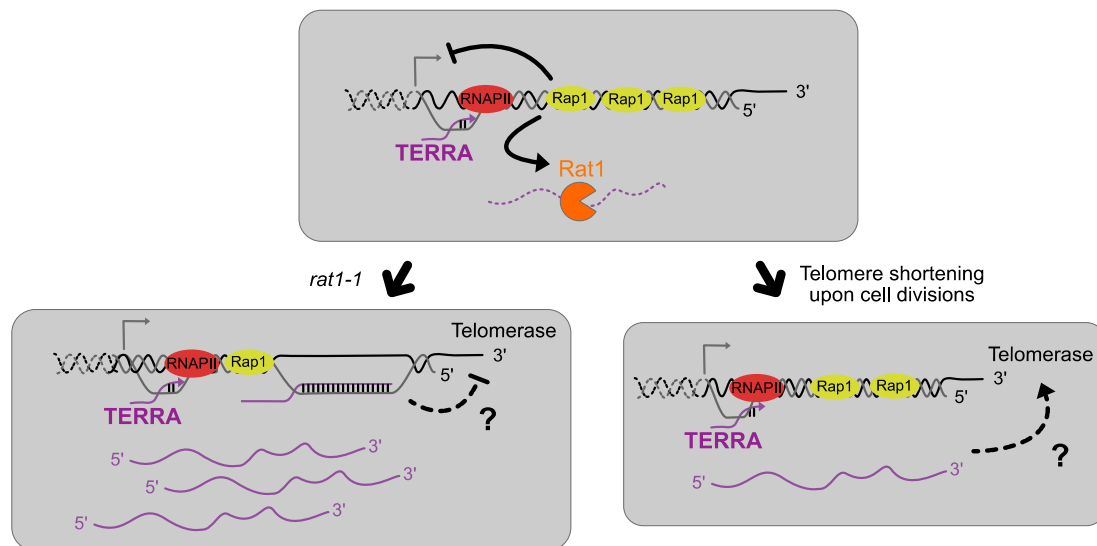


Figure 2. TERRA-telomerase possible interactions. In the absence of Rat1, the main exonuclease responsible for TERRA degradation, TERRA levels increase and are believed to form R-loops with telomeric ends suspected to impair the action of telomerase in elongating short telomeres. On the contrary, when telomeres shorten in a wild-type upon cell divisions, TERRA levels increase because of its *cis* regulation inhibition but TERRA is still regulated throughout the cell cycle. TERRA would then act as a scaffold molecule by interacting with telomerase to promote the elongation of the shorter telomeres during late S phase.

7. TERRA and Telomere Replication

Telomeres are replicated through semiconservative DNA replication and, if present, telomerase contributes to the maintenance of their length. Replication is tricky and when it comes to telomeres, considering their heterochromatin and repetitive structure, it is even more complicated, as slippage events may occur. Special helicases and telomere binding proteins are required for a complete replication [78–82]. In case replication forks stall and collapse, rapid loss of entire telomeric tracts can happen. Yet, telomeres display an intrinsic propensity for controlled degradations at the time of replication. Upon the passage of the replication fork, a physiologically limited 5′-3′ resection occurs at the tip of the leading strand to reform the 3′-G-overhang, which is essential for telomere functions at every passage of the replication fork [2,6]. The MRX-Sae2 complex plays a role in this end processing, followed by the action of Sgs1-Dna2 and Exo1 [83,84]. However, Exo1 can also contribute to unscheduled telomere end resection when telomeres lose their protection function in a mutant of the telomeric proteins Cdc13 or Ku70/80 [85–89]. Thus, TERRA and TERRA R-loops need to be finely regulated to avoid being a source of telomeric double-strand breaks or DNA degradations and loss of telomeric tracts. The cell cycle regulation of TERRA and TERRA R-loops, which accumulate early in the S-phase and decrease afterwards, necessarily contribute to preventing such deleterious events at the time of the passage of the replication fork [47]. Yet, many factors contribute to keeping TERRA in check to prevent telomere replication-related problems.

The notion that TERRA interferes with replication came from two sets of experiments in which TERRA was artificially induced. The first one used a galactose inducible promoter,

upstream activation sequence (UAS), just in front of telomeric repeats on telomere 7L (7L-Gal tiTEL), resulting in telomeric transcripts harboring only telomeric repeats [90]. The second one used a doxycycline regulatable promoter, upstream TERRA transcription start site on chromosome 1L (TetO7-1L tiTEL), thus resulting in the expression of full-length TERRA [42]. The result in both cases was a transcription-induced telomere shortening in cis, specific to the telomere from which the transcription is enhanced. Furthermore, R-loop levels did not change upon the forced induction of TERRA. These elements suggest that TERRA per se does not have a trans effect at other telomeres, but rather high levels of transcription within telomeric repeats could cause the shortening. The shortening observed was found to be independent of telomerase inhibition or the binding of telomeric proteins to telomeres (Ku70/80 and Rap1) [42]. In contrast, the shortening was found to be dependent on the S phase of the cell cycle, and thus possibly linked to the passage of the replication fork [90]. Accordingly, deletion of the nuclease *EXO1*, involved in resecting telomeres 5'-3', fully suppresses the phenotype observed [42,53]. It appears that high levels of TERRA interact with the Ku70/80 complex, resulting in an impairment of the Ku complex function in protecting chromosome ends from excessive degradation by the exonuclease Exo1. These observations, made in a context where TERRA is set experimentally high, underscore that TERRA interacts with telomeric proteins and can modulate nuclease processing activity also without forming R-loops configuration (Figure 3). Accordingly, in telomerase-positive cells, *EXO1* deletion, but not an overexpression of RNase H1, rescues the short telomere phenotype observed in a *thp2Δ* mutant [57]. In addition, R-loops have also been shown to cause sudden telomere loss events promoting telomeres shortening only when homology-directed repair (HDR) is impaired and RNase H mutants display higher levels of single-stranded telomeric DNA, suggesting a defect in replication-associated telomere processing [53,56].

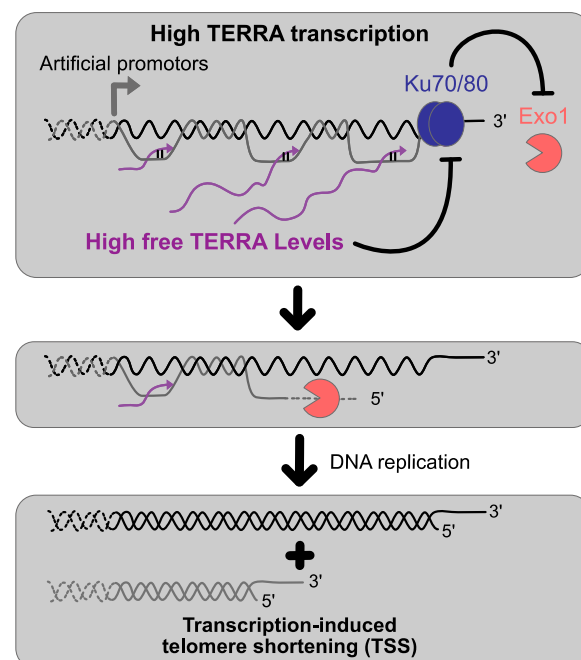


Figure 3. Effects of high TERRA transcription on telomere length homeostasis. The induction of TERRA transcription by artificial promoters results in high TERRA levels which promote Exo1-dependent 5'-3' resection at telomeres by impairing the Ku complex functions resulting in transcription-induced telomere shortening in cis.

In summary, TERRA and TERRA R-loops interfere with the replication machinery and disturb the passage of the replication fork and telomere processing, likely through several molecular mechanisms. Therefore, it is very possible that a large part of the replication

stress detected at telomeres is mediated by TERRA transcription, TERRA abundance or TERRA R-loops.

8. TERRA and R-Loop Functions during Senescence and Post-Senescence Survival

Replicative senescence is obtained in budding yeast after the experimental inactivation of telomerase, which is otherwise constitutively expressed and active. The cell proliferation of cultures of telomerase-negative cells is first almost indistinguishable from telomerase-positive ones, but consecutive daily dilutions and re-inoculations of these cultures display a progressive decline in proliferation capability. When cultures reach about 60–80 population doublings, corresponding to 4–5 days, telomerase-negative cells start displaying a severe growth defect. Yet, if allowed to grow with fresh media for one or two more days, cultures resume normal growth. Cells in these late cultures are called post-senescent survivors. In these cells, telomere length is maintained by HDR mechanisms, also called ALT for alternative lengthening of telomeres [91–95]. In budding yeast, it is a rare event estimated at a frequency of 1:10,000 [92], and two types of survivors can emerge from senescent cultures: type I and type II. Type I survivors gain multiple tandem Y' elements (even on X-only telomeres) by replication-dependent recombination and have short TG₁₋₃ terminal repeats, whereas type II survivors display very heterogeneous, and sometimes extremely elevated amounts of, TG₁₋₃ repeats at their chromosome ends. While the extension of these TG₁₋₃ telomeric repeats by rolling circle replication of telomeric circles was detected in survivor cells, it recently became obvious that the vast majority of telomere extensions in these cells are due to break-induced replication (BIR) and that type I and type II stem from a unified BIR mechanism [92,96,97]. Rad52, a central recombination factor in budding yeast, plays an essential role in the establishment and maintenance of ALT. Most cancers re-express telomerase to elongate telomeres and maintain cell proliferation, but a substantial number of tumors are telomerase-negative. In these, telomeres are also maintained by ALT through BIR [98]. ALT mechanisms are also responsible for relapses of cancers treated with anti-telomerase drugs [99]. Therefore, understanding senescence mechanisms, and more recently, the trigger of ALT, has been the focus of much attention. As a new player in telomere biology, TERRA was found to be at the heart of ALT mechanisms in both yeast and mammalian cells.

To start with, in *S. cerevisiae*, as mentioned above, all the studies so far agree that TERRA levels and R-loops, stemming from Y' and from X-only telomeres, increase with the shortening of telomeres in a telomerase mutant's background [41,47,54,56,58,100,101]. Then, similarly to human cells, TERRA and telomeric hybrids were found to even be present in post-senescent survivors of telomerase-negative cultures [102,103]. Because R-loops are expected to interfere with replication and thus with telomere shortening and stability, the question is how TERRA and/or R-loops impact senescence and ALT.

In order to study how TERRA affects senescence and survivor emergence, experimental conditions affecting TERRA and telomeric R-loop levels were combined with telomerase inactivation. The first trials were the forced induction of transcription at a single telomere, which resulted in a rapid loss of viability [42,53,90]. As mentioned, this induction of TERRA transcription caused an Exo1-dependent telomere shortening in cis, and the deletion of *EXO1* fully suppressed the acceleration of senescence. Consistently, reverting the shortening of the single telomere suppressed the phenotype. This would be in accordance with a single short telomere, more so unprotected, being sufficient to trigger replicative senescence, so that cells carrying at least a very short telomere display accelerated senescence [10,12].

An independent study aiming at studying the impact of TERRA levels on senescence showed that a 262 nt C₁₋₃A antisense RNA molecule is able to interact with TERRA in vivo and delay senescence [100]. This delay is independent of *EXO1* expression or the presence of HDR factors, since anti-TERRA RNA delays senescence in *exo1* or *rad52* contexts. However, this delay of senescence is epistatic with the deletion of *DOT1*, encoding an epigenetic regulator that methylates the nucleosomal histone H3Lys79. The authors concluded that

Dot1 interacts with TERRA to promote senescence through its N-terminal domain, which plays a role in telomere release from nuclear periphery. Thus, TERRA could constitute a signal from the short telomeres to trigger cellular senescence through its cooperation with Dot1 [100].

To increase the levels of R-loops, mutations in the THO complex were also introduced. While *HPR1* and *THO2* deletion mutants lead to slightly longer telomeres in the presence of telomerase, when combined with telomerase inactivation, the strains displayed an accelerated senescence and a faster selection of post-senescence survivors [56,65]. This was, in principle, in accordance with the idea that R-loops would be deleterious to telomere replication and would cause telomere losses, as detected in a *THP2* deletion mutant in a telomerase-positive background [57]. Accordingly, RNase H1 overexpression partially suppressed the accelerated senescence of the *tho2* and *hpr1* mutants [65]. However, this interpretation was questioned by the rapid appearance of post-senescent survivors. Importantly, overexpression of RNase H2 or an RNA complementary to TERRA with the objective of decreasing the levels of TERRA R-loops impaired the appearance of post-senescence survivors, suggesting an involvement of telomeric R-loops in starting ALT [65].

In another increased R-loop context, achieved via the deletion of both RNases H, senescence and consecutive survivor emergence were found to be delayed [56]. Conversely, the over-expression of RNase H1 in telomerase-negative cells reduced RNA:DNA hybrids and concomitantly accelerated senescence [47,56]. These results pointed to a model whereby R-loops, as stabilized in the absence of RNases H, prevent senescence. Furthermore, while Rad51 accumulates at the shortest telomeres in cells, together with the R-loops, the overexpression of RNase H1 abolishes both the R-loops and the Rad51 enrichment, suggesting a link between telomeric R-loops and HDR at the shortest telomeres [47]. Accordingly, the delay of senescence found in RNase H deletion is suppressed by the deletion of *RAD52* [56]. Additionally, in agreement with these results, Npl3, an RNA binding protein involved in many RNA processes, was shown to bind TERRA and to stabilize R-loops at short telomeres, which in turn promoted HDR to prevent premature replicative senescence [101]. This is attested by the fact that the deletion of *NPL3* accelerated senescence in an epistatic manner with *RAD52* deletion, and the phenotype was suppressed when RNaseH2 was deleted. Therefore, modulating TERRA and telomeric R-loops by different means would lead to different molecular structures involving TERRA with apparent distinct consequences. Notwithstanding, RNase H2 has been shown to be specifically recruited at telomeres, and specifically absent from short telomeres (Figure 4) [47]. Therefore, RNase H2 depletion may better reflect the actual molecular steps at telomeres when shortening, triggering senescence and subsequently enabling ALT, probably through the stimulation of HDR, and more specifically, BIR at telomeres (Figure 4). In accordance with this model, another independent study suggests that TERRA could play a role in the recombination and amplification of *Y'* elements in Type I survivors. Indeed, *paf1Δ* and *ctr9Δ* mutants, where TERRA levels increase, stimulate the induction of subtelomere *Y'*-element amplification in a Sir4 deficient background [104].

The next question is which mechanism can lead to such R-loop-dependent re-elongation of telomeres, in a context where R-loops are at the same time a trigger for replication stress? Rad53 is phosphorylated before the emergence of survivors, and this phosphorylation is delayed when RNase H1 is overexpressed, showing that the DNA damage checkpoint can be activated by RNA:DNA hybrids [65]. Therefore, similar to what was established in mammalian cells, TERRA transcription and R-loops may cause replication fork pauses when traversing telomeric repeats, generating appropriate substrates for BIR. In this context, the fact that RNase H mutants display high levels of ssDNA at telomeres in an *est2 rad52* background may attest for the accumulation of such a substrate when R-loops accumulate [53]. The displaced 3'-ssDNA overhang may be extended by R-loops and coated by Rad51, resulting in a strand invasion in a replication-independent manner. This notion was further tested in mammalian cells, where TERRA R-loops were recently shown to promote

ALT through an R- to D-loop switch, enabling the invasion of another telomere for BIR (Figure 4) [105].

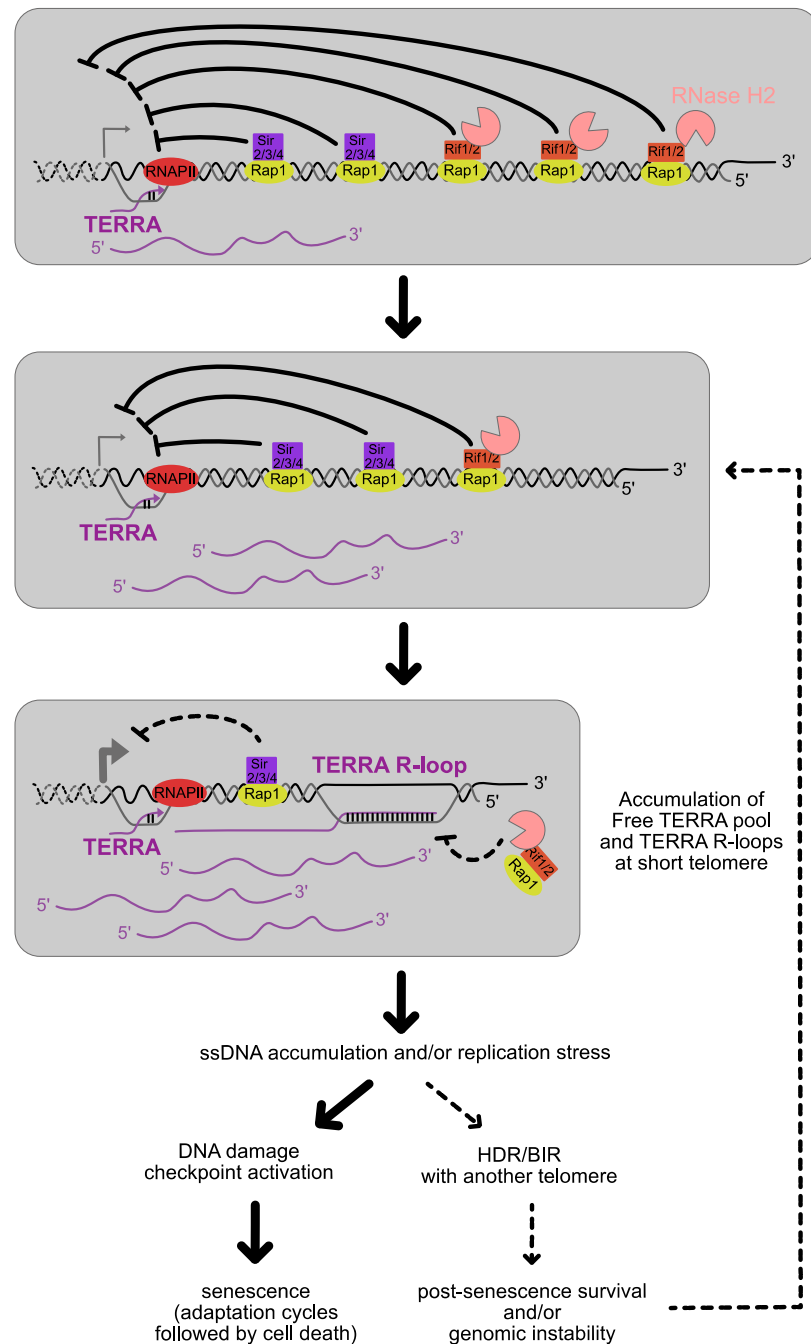


Figure 4. In the absence of telomerase, telomeres shorten gradually at each cell division. Free TERRA levels and TERRA R-loops increase at short telomeres due to the loss of the regulation in *cis* described in Figure 1. This generates structures that are prone to replication stress and/or recognized as DNA damage. A possible scenario is that at this point most cells undergo senescence, but in a tiny fraction the short telomeres may undergo a successful re-elongation via HDR with another longer telomere. Because these cells outgrow very fast the others, promoting or perturbing this process by introducing mutations, is expected to affect the rate of senescence. This process restarts again for each telomere reaching a critical short length.

In another vein, using cells displaying dysfunctional telomeres caused by the inactivation of the telomeric Cdc13 protein, and prone to Exo1-mediated degradation, Brian

Luke's laboratory found that the stabilization of RNA:DNA hybrids, through the loss of RNase H activity, prevents Exo1-dependent telomere-end resection at short dysfunctional telomeres [106]. Thus, TERRA hybrids at telomeres could also serve to limit the extent of 5'-3' degradations which are to increase as telomeres shorten and cells enter replicative senescence [107].

Taken together, telomeric R-loops trigger molecular mechanisms that endanger telomere semi-conservative DNA replication, enable telomere re-elongation and prevent degradation at the same time. ALT cells survive in the long term thanks to the fine-tuning of this duality [108]. In accordance with this notion, established yeast post-senescence survivors displayed growth defects. This was evidenced by careful analyses of individual sub-clones of established survivors, instead of mixed populations of cells [69]. It appeared that individual clones often experience phases of normal growth followed by severe proliferation defects. In parallel, the authors also follow the dynamics of single telomeres with unique lengths from the exact same cultures. They observe that individual telomeres undergo progressive telomere shortening up to a critical short length before being subjected to re-elongations of various lengths all of a sudden. They also correlate the extreme shortening of a given telomere with the accumulation of TERRA R-loops stemming from the short telomere in the exact same cultures. They show that the overexpression of RNase H1 causes a more pronounced loss of viability, in accordance with the model where R-loops help in sustaining ALT and proliferation. RNase H2 inactivation caused an inverse phenotype [69]. These results suggest that in ALT cells, the R-loop-related mechanism that triggers BIR at the shortest telomeres could well be the exact same than the one taking place in senescent cells when establishing ALT, meaning a very infrequent and perhaps deleterious event in most cases (Figure 4). These analyses also best illustrate the role of strong competition and powerful selection, masking the actual high mortality of ALT cells and unveiling clues for their fight in cancer patients.

9. Extratelomeric Functions of TERRA

Like other lncRNAs, TERRA seems to also play a central role in the regulation of other aspects of cell biology. In fact, the expression of many lncRNAs can change in response to stress adaptation [109]. More specifically, in mammalian cells, TERRA has been found in the extracellular inflammatory exosomes outside of the nucleus, playing a role in stimulating immune signaling, and was proposed as a marker revealing telomeric dysfunction during diseases [110,111]. Recent data also show that TERRA is induced in response to oxidative stress [112]. In budding yeast, live-cell imaging and RNA-FISH similarly show that TERRA undergoes transient induction and relocalization to the cytoplasm when yeast cells undergo diauxic shift [113]. These observations were not due to telomere shortening nor an increase in TERRA stability. These data thus suggest that a link between TERRA and an increase in oxidative stress also exist in budding yeast. While the biological significance of these observations remains to be established, these findings illustrate the complexity of TERRA biology and pave the way for new lines of research.

10. Conclusions

In budding yeast and other organisms, the impact of TERRA and TERRA-derived higher-order structures on telomere biology has progressed immensely since its discovery 15 years ago. Yet, much is left to understand regarding the regulation of TERRA expression, the formation of R-loops and perhaps other structures, and their biological consequences. In this review of TERRA, we focused on the unicellular simple model organism *Saccharomyces cerevisiae*, and yet, we face the complexity of TERRA regulation in time and space and the tremendous heterogeneity underlying its biology. The better understanding of TERRA is challenging, but the development of new tools to specifically modulate the expression and the structure of TERRA at individual telomeres, as well as to bypass the cell-to-cell variability in senescence phenotypes coupled to single-clone/single-cell analysis, will be

indispensable to crack the heterogeneity and the apparent stochasticity of TERRA behavior. These are approaches realistic to apply in the budding yeast model.

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Material and Methods

The following section describes the material and methods of the unpublished results part.

Yeast strains

All yeast strains used in this study are derived from W303 background corrected for *RAD5*, *ADE2* (*rad5-535* and *ade2-1* in the original strain) and have the promotor of telomerase under the control of a repressible promotor by doxycycline *P_{TetO2}-TLC1* (Soudet *et al*, 2014). Gene deletions and promotor insertions were constructed as described (Longtine *et al*, 1998). The strains that overexpress RNaseH1 were transformed by a replicative plasmid where the RNaseH1 sequence is under the control of the strong constitutive GPD promoter (Balk *et al*, 2013). The FinalCut strains were obtained from Prisca Berardi, a PhD student in the laboratory.

Table 1: Yeast strains used in the unpublished results part

Strain name	Figure	Genotype
yT787	22	<i>Mat α ura3-1 trp1-1 leu2-3, 112 his3-11,15 ADE2 LYS2 RAD5 tlc1::HIS3MX6- PrTetO2-TLC1</i>
yT1473	22	yT787 <i>hog1::TRP1</i>
yT1564	23	yT787 <i>pT-URA3</i>
yT1565	23	yT787 <i>P_{GPD}-RNH1</i>
yT1567 yT1568 yT1569	23	yT787 <i>hog1::TRP1 pT-URA3</i>
yT1556 yT1557 yT1558	23	yT787 <i>hog1::TRP1 P_{GPD}-RNH1</i>
yT1487	24	<i>Mat α ura3-1 trp1-1 leu2-3, 112 his3-11,15 ADE2 LYS2 RAD5 tlc1::HIS3MX6- PrTetO2-TLC1 trp1-1::AFB2-TRP1</i>
yT1503	24	yT1487 <i>telo6R::FC20bp</i>
yT1505	24	yT1487 <i>telo6R::FC40bp</i>
yT1596	24	yT1487 <i>telo6R::FC100bp</i>

Media

Table 2: Media used in the unpublished results part

Name	Composition (% w/v)
DO	1g Adenine (AD) 2g Isoleucine (I) 2g Uracil (UR) 2g Alanine (A) 2g Lysine (K) 2g Arginine (R) 2g Methionine (M) 2g Aspartate (D) 2g Phenylalanine (F) 2g Asparagine (N) 2g Proline (P) 2g Cysteine (C) 2g Serine (S) 2g Glutamate (E) 2g Threonine (T) 2g Glutamine (Q) 2g Tryptophan (W) 2g Glycine (G) 2g Tyrosine (Y) 2g Histidine (H) 2g Valine (V) 2g Leucine (Leu)
YPD liquid	1% peptone 1% yeast extract 2% glucose
SD-Ura liquid	0,67% nitrogenated yeast base without aa 2% glucose 0,2% DO-Ura
SD-Leu liquid	0,67% nitrogenated yeast base without aa 2% glucose 0,2% DO-Leu
SR-Leu liquid	0,67% nitrogenated yeast base without aa 2% raffinose 0,2% DO-Leu
SRG-Leu liquid	0,67% nitrogenated yeast base without aa 2% raffinose 3% galactose 0,2% DO-Leu

Transformation with replicative plasmid

Cells grown on solid media were resuspended in one-step buffer (40%, 4000 Polyethylene glycol (PEG); 0.2M lithium acetate (LiAc); 0.1M dithiothreitol (DTT)), 5 µg of DNA carrier (*Takara #630440*), and 1 µg of the appropriate replicative plasmid. Cells were then incubated for 30 min at 45°C, washed with 600 µL H₂O MilliQ, resuspended in 100 µ H₂O MilliQ, plated on appropriate selective media, and incubated for two days at 30°C.

Transformation with cassettes

10^8 exponential-phase growing cells were centrifuged for 4 minutes at 3000 rpm, washed in H₂O, and then washed and resuspended in 0.2 ml of 0.1M LiAc, 10mM Tris pH 8.0, and 1mM Ethylenediaminetetraacetic acid (EDTA). For each transformation, 38 μ L of this suspension was mixed with 2 μ L of denatured DNA carrier (10 mg/ml) (*Takara #630440*), 7 μ L of transforming DNA (versus water for the negative control condition), and 160 μ L of PEG 50% in LiAc-TE. After a 2 hours incubation at 30°C, 13 μ L of Dimethyl sulfoxide (DMSO) was added, and the incubation was extended for 10 minutes at 42°C. To dilute the PEG, 600 μ L of 0.15M sodium chloride (NaCl) were added to each tube. After centrifugation at 3000 rpm for 3 minutes, the pellets were resuspended in 90 μ L of 0.15M NaCl, spread using beads on an appropriate selective medium, and the plates were incubated for 48 hours at 30°C.

Liquid senescence experiments

The different strains used were cultivated at 30°C under 220 rpm rotation in liquid rich media (YPD) or in minimal SD-URA media. Cellular suspensions were diluted to 0,001 OD_{600nm} with a final concentration of 30 μ g/mL of doxycycline (*Sigma #D9891*) and the OD_{600nm} was measured after 24 hours of culture at 30°C under 220 rpm rotation. This was repeated for several days and samples were taken each day for analysis.

Galactose induction

FinalCut strains were inoculated in liquid SR-Leu and grown at 30°C under 220 rpm rotation from 6 to 9 hours. Cells from over-day pre-cultures were then diluted to 0,02 OD_{600nm} in SR-Leu + doxycycline (30 μ g/mL) (*Sigma #D9891*). After the overnight incubation (30°C 220 rpm) cell density was measured (expected OD_{600nm} between 0.4 and 1.0, corresponding to the log-phase) and corresponds to time zero (t=0h). To induce the expression of Cas9, time zero cells were diluted to 0,2 OD_{600nm} in SRG-Leu + doxycycline (30 μ g/ml) and incubated 3h at 30°C. This step was repeated twice to obtain time point 6 and 9 hours. For each time point (t = 0h, 3h, 6h, 9h) cells were collected and processed for TeloPCR, Western Blot and ROS analysis.

ROS detection

0,4 OD_{600nm} of yeast was incubated under 220 rpm rotation at 30°C in the dark for one hour in 500 µL of sterile phosphate buffered saline (PBS) 1X containing 2',7'-Dichlorofluorescein diacetate (DCF-DA) (*Sigma #D6883*) at a final concentration of 10 µM. Samples were washed and resuspended with 500 µL of PBS1X and then fluorescence was analyzed by flowing cytometry using the settings 488 (λ_{ex})/533 (λ_{em}) in Accuri C6. Mean intensity values are plotted.

Total RNA extraction

5 OD_{600nm} of yeast were resuspended in 500 µL of AE buffer (0.5M EDTA, 3M sodium acetate (NaAc)), 50 µL of sodium dodecyl sulfate (SDS) 10%, and 500 µL of phenol equilibrated with AE buffer. The sample was then vortexed in at 4°C twice for 3 minutes with a 2-minute pause in between. After two incubations of 5 minutes at 65°C, the sample was vortexed and placed on ice for 1 minute. After centrifugation at room temperature for 20 minutes at 13000 rpm, 400 µL of the upper phase were transferred to a new tube containing 500 µL of phenol chloroform. After a quick vortex agitation, the sample was centrifuged at room temperature for 10 minutes at 13000 rpm. The 360 µL of the upper phase were transferred to a new tube containing 1 ml of cold 100% ethanol and 40 µL of 3M NaAc at pH 5.3. After agitation, the tubes were placed at -20°C overnight. The next day, after centrifugation at 4°C at 13000 rpm for 30 minutes, the sample was washed in 1 ml of cold 70% ethanol. After centrifugation, the pellet was finally resuspended in 50 µL of RNase-free water. The quality and integrity of the RNA were checked by migration on a 1% agarose 0.5X TBE gel. The quantity was measured using a nanodrop (*Thermofisher*). Samples were stored at -80°C.

Reverse transcription and qPCR

After the total RNA extraction following the protocol described above, the samples were brought to a concentration of 10µg/50µL. Next, two consecutive DNA digestions were carried out at 37°C for 30 minutes using 0.5 µL of Turbo DNase (*Thermofisher #AM2238*). The DNase was then inactivated by incubating under agitation for 5 minutes with 5 µL of inhibitor (DNase inactive reagent) (*Thermofisher #AM2238*). After a 1.5 minutes centrifugation at 10,000 g, the supernatant was collected. In each PCR tube, the following components were mixed: 1 µL of 10mM dNTPs, 1.3 µL of 10mM TERRA-specific primer, 0.86 µL of 10mM *ACT1* primer, and

10 μ L of 2 μ g RNA. After a 90°C incubation for 1 minute, 7 μ L of the following mixture was added to each tube: 1 μ L of DTT (dithiothreitol), 4 μ L of Supercut buffer 5X, 1 μ L of SuperScript III, and 1 μ L of RNaseout (*ThermoFisher #18080093*). cDNA synthesis was achieved by incubating the samples at 55°C for 1 hour, followed by enzymatic inactivation at 70°C for 15 seconds. After dilution, quantitative PCR was performed using SYBR Green intercalating dye (*Biorad #1725114*) in combination with a real-time PCR system (*Applied Biosystems 7900HT*). Two independent biological replicates were performed for each qPCR sample. For each primer pairs, control samples without template (NT) were included to detect potential primer dimer formation. In addition, a -RT control was done where RNA samples were treated as described above for reverse transcription but without adding the SuperScript III. The master mix for each reaction was prepared on ice as follows: 2 μ L of cDNA, 0.25 μ L of 30 μ M forward primer, 0.25 μ L of 30 μ M reverse primer, 10 μ L of SYBR Green PCR Master Mix, and 7.5 μ L of H₂O, making a final volume of 20 μ L. The plate was sealed with an optical adhesive film (*Applied Biosystems*), briefly centrifuged at 2000 g, and placed in the quantitative PCR system. The PCR program used was as follows: 50°C for 2 minutes, followed by 10 minutes at 95°C, then 40 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. To determine the relative expression change of TERRA between samples, the relative quantification method based on Δ Ct calculations was used for both TERRA (target gene) and *ACT1* (reference gene). After confirming 100% primer amplification efficiency, the ratio $2^{(\Delta$ Ct TERRA)/2^{(\DeltaCt Reference)} was calculated. A ratio value less than 1 indicates decreased expression, while a value greater than 1 indicates increased expression. Additionally, analysis of the melting curves revealed the presence of a single peak for each primer pair used, confirming the primer specificity for the amplified sequences (*Primers used are listed in Table 3*).

Telomere PCR

40 ng of genomic DNA was denatured by heating it to 98°C for 5 minutes, after which it was placed on ice. The tailing reaction was carried out in a final volume of 20 μ L, which included 12 μ L of genomic DNA (40 ng), 2 μ L of NEB Buffer 4 (10X), 0.2 μ L of 10mM dCTP, 0.1 μ L of terminal transferase 20U/ μ L (*NEB #M0315L*), and 5.7 μ L of H₂O. The reaction was incubated at 37°C for 5 minutes, followed by 5 minutes at 94°C and finally kept at 4°C. PCR reactions were conducted using Taq Mg-free Buffer 1X, 500nM of each primer (*See*

supplemental Table 3), 200 μ M of dNTPs, 5 μ L of polyC-tailed product (after terminal transferase added a polyC tail to DNA), and 1U of Taq Standard Polymerase (*NEB #M0273*) in a final volume of 30 μ L. The amplification followed this program: 3 minutes at 94°C, followed by 45 cycles consisting of 20 seconds at 94°C, 15 seconds at the specific primer-subtelomeric temperature, then 20 seconds at 72°C, and finally 5 minutes at 72°C. The TeloPCR products were then combined with 6X loading buffer and loaded (20 μ L) onto a large 2% agarose gel 0.5X TBE, and 100 ng/ml of ethidium bromide (BET) and electrophoresed at 50V for 15 hours. A 50 bp molecular weight marker (*NEB #B7025*) was also included. Visualization and analysis were carried out using the ImageLab® software (*Biorad*).

SDS-PAGE and Western Blot

5 OD_{600nm} of cells were lysed in 0.2M sodium hydroxide (NaOH) for 10 minutes on ice. Following the addition of 0.5% final concentration of trichloroacetic acid (TCA), the samples were incubated again on ice for 10 minutes. After 10 minutes of centrifugation at maximum speed at 4°C, the pellet was resuspended in 50 μ L of Laemmli 4X buffer (0.250M Tris, 8% SDS, 40% glycerol, 20% 2-mercapto-ethanol and bromophenol blue) and denatured at 95°C for 5 minutes. Proteins samples were electrophoresed on a denaturing 10% polyacrylamide gel (37:5:1 Acrylamide/Bis) after concentration in a 5% stacking gel in Tris-Glycine-SDS 1X buffer (*Biorad #1610772*). Proteins were transferred to a nitrocellulose membrane (*Amersham Protran 0.45 NC, GE Health-care*) at 4°C in Tris-Glycine 1X buffer (*Biorad #1610734*). After verifying the transfer quality using Ponceau red staining (0.1% in 5% acetic acid), nonspecific binding sites on the membrane were blocked by incubating in 1X PBS-Tween 0.1% buffer containing 5% milk (Régilait) at room temperature for 45 minutes. The membrane was then incubated overnight with the primary antibody of interest Phospho-p38 MAPK (*cell signalling #9211*), T-Hog1 (*santa cruz #sc-165978*) or anti-Rad53 (*#ab166859*) in 1X PBS-Tween 0.1% buffer containing 5% milk at 4°C. After 3 washes of 3 minutes each in 1X PBS-Tween 0.1%, the membrane was incubated for 45 minutes with the appropriate secondary antibody coupled to horseradish peroxidase (HRP) (*sigma #A9044 #A9169*) in 5% milk at room temperature. After 3 washes of 3 minutes each with 1X PBS-Tween 0.1%, the signal was finally revealed using an electrochemiluminescence reagent (*ECL, Biorad*) and recorded using the ChemiDoc Imaging System (*Biorad*).

Microfluidics and mortality rate analysis

To manufacture the circuits, polydimethylsiloxane (PDMS) and a curing agent are mixed in a ratio of 11:1. The mixture is then degassed with a vacuum pump for 30 minutes and poured into the mold. After baking at 70°C for 5 hours, the circuit is carefully removed from the mold. A biopsy punch (1.5 mm, Harris Unicore) is used to create holes for the feeding and elimination within the circuit at an average flow rate. To seal the circuit, the surfaces of both PDMS and a glass coverslip (24 × 50 mm) are surface-activated by plasma generation (Plasma cleaner, Diener Electronic, Germany), allowing the two elements to covalently bond. The medium is introduced into the device via a tube and the force of a peristaltic pump (IPCN, Ismatec). Cells from an exponentially growing culture are then injected into the device using a 1 ml syringe. A constant average flow rate (28 µL/min) is maintained by the pump throughout the experiment. To monitor cell division, each chamber of the microfluidic device is photographed using a x100 immersion objective on a fully motorized Axio Observer Z1 inverted microscope (Zeiss), with constant focus maintained by a focus stabilizer (Definite Focuss, Zeiss). The temperature is also maintained at 30°C in the incubation chamber, which includes the entire microscope base, circuit, and objectives. Images are captured in a time-lapse every 10 minutes for several days. The use of a MATLAB program allows the determination of the time for each cell division in the chamber. This program also creates a graphical summary of the experiment results for all monitored chambers. MATLAB analysis produces a binary code that functions as a matrix for a script in R software. This script generates a survival curve for each strain utilized. Subsequently, the survival curve is plotted using another script, fitting with a decreasing exponential function $y = ae^{-bx}$, with a and b fitting parameters. b defines the mortality rate per division.

Oligonucleotides

Table 3: Primers used in the unpublished results part

Name	Sequence
oT210 insertion of P _{TetO2} upstream of <i>TLC1</i> (yT787)	5'- AAT ACG ATT AAG CAA ACG CAA CAG CCA TTG ACA TTT TCA TAG GGT ACC TAT CTT CCT CTC ATA GGC CAC TAG TGG ATC TG-3'
oT543 insertion of P _{TetO2} upstream of <i>TLC1</i> (yT787)	5'-AAA AAA CTT CCT CTT TAG CAA TGG TGA CAT ATA GAT CTC AAG GTT CTC AAT TAA AAG ACC CGG ATC CCC GGG TTA ATT AA -3'
oT1550 deletion of <i>HOG1</i> (yT1473)	5'-GGT AAA TAC TAG ACT CGA AAA AAA GGA ACA AAG GGA AAA CAG GGA AAA CTA CAA CTA TCG TAT ATA ATA CGG ATC CCC GGG TTA ATT AA-3'
oT1551 deletion of <i>HOG1</i> (yT1473)	5'-CCA TAA AAA AAA GAA ACA TCA AAA AGA AGT AAG AAT GAG TGG TTA GGG ACA TTA AAA AAA CAC GTG AAT TCG AGC TCG TTT AAA C-3'
oT529 qPCR ACT1 Fwd	5'- TGG ATT CCG GTG ATG GTG TT -3'
oT530 qPCR ATC1 Rev	5'- TTG TTC GAA GTC CAA GGC GA-3'
oT1999 qPCR 6R Fwd	5'-AGG GTA AAA ACC AGT GAG GCC-3'
oT2000 qPCR 6R Rev	5'- TAC TTC ACT CCA TTG CGC CC -3'
oT2011 qPCR 15L Fwd	5'- CAG GGT AAG TGG CAG TGG AG -3'
oT2012 qPCR 15L Rev	5'- TAC CAC AGC GAA CCA CGA TC -3'
oT1873 qPCR 13R Fwd	5'- ACG GTT ATG GTG CAC GAT GGG-3'
oT1874 qPCR 13R Rev	5'- TTA CCC TCC ATT ACG CTA CCT CC-3'
oT883 TeloPCR 6R Fwd	5'- ACG TTT AGC TGA GTT TAA CGG TG -3'
169M TeloPCR Poly G Rev	5'- GCG GAT CCG GGG GGG GGG G -3'

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Le stress oxydant associé à la sénescence répliquative et l'activation de Hog1 dans les cellules de *Saccharomyces cerevisiae* négatives pour la télomérase

La sénescence répliquative, le processus par lequel les cellules cessent de se diviser après un certain nombre de divisions, est déclenchée par une réponse aux dommages de l'ADN, qui résulte du raccourcissement des télomères au cours de chaque division cellulaire. Les données obtenues chez différents organismes montrent que les dysfonctionnements mitochondriaux et le stress oxydant sont des caractéristiques de la sénescence répliquative. Cependant, la production et la régulation des niveaux d'espèces réactives de l'oxygène (ROS) au cours de la sénescence répliquative chez la levure restent énigmatiques. L'objectif de ma thèse a été de quantifier les niveaux de ROS pendant la sénescence répliquative et de délimiter les voies reliant l'inactivation de la télomérase aux niveaux de ROS en utilisant *Saccharomyces cerevisiae* comme organisme modèle. J'ai démontré que le stress oxydant augmente pendant la sénescence répliquative chez *Saccharomyces cerevisiae*. L'orthologue de la MAPK p38, Hog1, s'active au cours de la sénescence répliquative par le biais de sa MAPKK canonique, Pbs2 et contribue probablement à la détoxification des ROS. Mes résultats suggèrent que l'action de Hog1 est indépendante de Mec1, qui probablement participe aussi à la détoxification des ROS. Hog1 semble aussi fonctionner indépendamment du long ARN non codant TERRA. De plus, j'ai établi un rôle indispensable de Hog1 dans le maintien de l'homéostasie de la taille des télomères et montré que la délétion de Hog1 entraîne une mortalité cellulaire marquée même en conditions de croissances normales. Mes recherches ont aussi révélé que les processus autophagiques n'ont pas d'impact sur l'établissement de la sénescence répliquative chez *Saccharomyces cerevisiae*. Dans l'ensemble, les données obtenues au cours de ma thèse mettent en évidence que le stress oxydant est une caractéristique de la sénescence répliquative chez *Saccharomyces cerevisiae* et que Hog1 régule l'homéostasie des télomères contribuant au lien entre les télomères et le métabolisme des ROS.

Mots clés : Sénescence répliquative, Stress oxydant, Télomères, Hog1, *Saccharomyces cerevisiae*

Replicative senescence-associated oxidative stress and Hog1 activation in telomerase negative *Saccharomyces cerevisiae* cells

Replicative senescence, the process by which cells cease to divide after a certain number of divisions, is triggered by a DNA damage response, resulting from telomeres shortening during each cellular division. Data obtained in different organisms show that mitochondrial dysfunctions and oxidative stress are features of replicative senescence. Yet, the production and regulation of reactive oxygen species (ROS) levels during replicative senescence in budding yeast are still elusive. The aim of my PhD was to quantify ROS levels during replicative senescence and delineate the pathways linking telomerase inactivation with changes in oxidative stress levels using *Saccharomyces cerevisiae* as a model organism. I demonstrated that oxidative stress increases during replicative senescence in budding yeast. The ortholog of the MAPK p38, Hog1, becomes activated during replicative senescence through its canonical MAPKK Pbs2, and likely contributes to ROS counteraction. My results suggest that Hog1's action is independent of Mec1, the major DNA damage checkpoint protein in budding yeast, which also likely counteracts the increase in ROS. Hog1 also likely functions independently of the telomeric long non-coding RNA TERRA. Moreover, I established the role of Hog1 in maintaining telomere length homeostasis and showed that the absence of Hog1 results in a marked cell mortality even in unchallenged conditions. Furthermore, my research revealed that autophagic processes do not impact replicative senescence onset in budding yeast. Collectively, data obtained during my thesis highlight that oxidative stress is a hallmark of replicative senescence in budding yeast and that Hog1 regulates telomeres homeostasis contributing to the link between telomeres and the ROS metabolism.

Keywords: Replicative senescence, Oxidative stress, Telomeres, Hog1, *Saccharomyces cerevisiae*