

**ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ**



**CNRS UMR 7104 – Inserm U 964**

**Thèse présentée par Théo ASPERT**  
**Soutenue le 17 décembre 2021**

Pour obtenir le grade de : **Docteur de l'université de Strasbourg**  
Discipline/ Spécialité : **Sciences de la vie/ Biophysique**

**Deciphering the complexity of aging dynamics  
using microfluidics and deep-learning**

**Dr Gilles CHARVIN**

DIRECTEUR DE THESE

**Pr Liesbeth VEENHOFF**  
**Dr Damien COUDREUSE**

RAPPORTRICE EXTERNE  
RAPPORTEUR EXTERNE

**Pr Michael RYCKELYNCK**  
**Dr Sahand RAHI**

EXAMINATEUR INTERNE  
EXAMINATEUR INVITE

*To life*

&

*A mon père*

# Remerciements

First of all, I would like to thank the jury members, Liesbeth Veenhoff, Damien Coudreuse, Mickael Ryckelynck and Sahand Rahi for accepting to evaluate my work, and especially to Liesbeth and Damien for reviewing the manuscript.

Aussi et biensûr, j'aimerais remercier Gilles Charvin pour m'avoir accueilli dans son labo. Presque 5 ans et un terrain de jeu quasiment illimité : microscopes, microfluidique et gadgets à volonté, le rêve ! C'était aussi un plaisir de plonger dans le monde du deep-learning, de développer et d'interagir ensemble quotidiennement. On a pas mal fait chauffer les serveurs de Teams et RocketChat !

Merci aussi au reste de l'équipe, Audrey, Sandrine et Sophie pour la bonne humeur, les chit-chats et l'interaction scientifique.

Merci à l'équipe Mendoza pour les discussions scientifiques, et notamment à Mercè : it was « *super cool!* » to crush yeast cells with you late in the lab!

Merci à Erwan, Didier et Serge pour leur aide technique en microscopie et en mécanique.

Merci aux voisins d'en face et squatteurs de machine à café : Hélène, Sara et Elena, et le voisin plus éloigné, Simon.

Basile, sans toi cette thèse aurait eu une saveur bien différente, alors merci pour les interactions et collaborations scientifiques et humaines. *“Je sais que je suis venu officiellement pour tenter de retirer l'épée, mais j'ai trouvé ici quelque chose de beaucoup plus précieux, j'ai trouvé la sérénité affective.” ;)*

And thanks to you for reading this thesis and taking an interest in my work! I hope you're not just here for the acknowledgement page :)

# Table of content

PREAMBLE .....	1
CHAPTER I: DECIPHERING THE COMPLEX DYNAMICS OF AGING USING MICROFLUIDICS AND DEEP-LEARNING .....	2
INTRODUCTION I: THE BIOLOGY OF AGING AND THE SPECIFIC CASE OF <i>SACCHAROMYCES CEREVISIAE</i> .....	2
1. GENERAL AGING CONCEPTS .....	2
1.1. Definition of aging .....	4
1.2. Evolutionary theories of aging .....	7
1.3. Molecular models of aging .....	10
1.4. Universality of aging.....	15
1.5. Laboratory models .....	16
2. AGING IN BUDDING YEAST: DIFFERENT MODELS.....	25
INTRODUCTION II: METHODS TO STUDY REPLICATIVE AGING IN YEAST .....	62
1. MICRODISSECTION.....	62
2. POPULATIONAL ENRICHMENT OF OLD CELLS .....	64
2.1. Density gradient and Elutriation .....	64
2.2. Mother Enrichment Program.....	65
2.3. Cell wall labeling .....	66
2.4. Conclusions on traditional assays .....	67
3. CAPTURING SINGLE-CELL DYNAMICS.....	68
SUMMARY OF THE INTRODUCTION AND OBJECTIVES.....	82
4. OBJECTIVES.....	88
RESULTS.....	89
5. RESULTS I: SETTING UP A PLATFORM FOR AUTOMATED AND HIGH-THROUGHPUT TRACKING OF REPLICATIVE LIFESPAN .....	89
1.1. Introduction .....	89
1.2. Results .....	89
1.3. Perspectives .....	128
6. RESULTS II: MEASURING THE PROBABILITY OF ERC EXCISION WITH AGE.....	130
1.1. Introduction .....	130
1.2. Results .....	130
1.3. Perspectives .....	136

GENERAL DISCUSSION.....	140
1. The ERCs pathway of aging: A countdown to death started by a stochastic trigger.....	141
2. Relevance of the ERC/rDNA pathway in other organisms.....	145
3. Other pathways of aging .....	145
4. A general framework to classify hallmarks of aging into pathways .....	147
5. Observing the daughters to understand aging factors .....	150
REFERENCES CHAPTER I .....	153
CHAPTER 2: MONITORING SINGLE-CELL DYNAMICS OF ENTRY INTO QUIESCENCE DURING AN UNPERTURBED LIFE-CYCLE .....	181
INTRODUCTION .....	181
1. GENERAL CONCEPTS OF QUIESCENCE .....	181
2. THE MOLECULAR PATHWAYS OF ENTRY INTO QUIESCENCE.....	181
3. STRUCTURAL REARRANGEMENTS IN QUIESCENCE .....	182
3.1. Cytoskeleton .....	183
3.2. Mitochondrial network and respiration.....	184
3.3. Proteasome .....	184
3.4. Phase transition of the cytoplasm .....	184
4. QUIESCENCE AND LIFE-CYCLE OF BUDDING YEAST .....	186
4.1. The life-cycle of budding yeast.....	186
4.2. Quiescence: one unique cellular state? .....	186
4.3. Assessing quiescence.....	188
SUMMARY AND MOTIVATIONS.....	189
RESULTS.....	190
DISCUSSION .....	215
REFERENCES CHAPTER II .....	217
APPENDIXES .....	221
APPENDIX 1: MICROFLUIDIC ENRICHMENT OF OLD CELLS AND SORTING ACCORDING TO THEIR SENESCENT STATE .....	221
APPENDIX 2: VERSATILE HEATING SYSTEM FOR LONG-TERM MICROFLUIDIC CULTURE....	227
APPENDIX 3: (COLLABORATION) SPECIALIZATION OF CHROMATIN-BOUND NUCLEAR PORE COMPLEXES PROMOTES YEAST AGING .....	230
APPENDIX 4: DISTINCT MECHANISMS UNDERLIE H <sub>2</sub> O <sub>2</sub> SENSING IN C. ELEGANS HEAD AND TAIL.....	257

APPENDIX 5: (COLLABORATION) SELF-LEARNING MICROFLUIDIC PLATFORM FOR SINGLE-CELL IMAGING AND CLASSIFICATION IN FLOW .....	302
PUBLICATIONS .....	324
DECHIFFRER LES DYNAMIQUES DU VIEILLISSEMENT A L'AIDE DE LA MICROFLUIDIQUE ET DE L'APPRENTISSAGE PROFOND .....	325
ABSTRACT .....	325
RESUME .....	325

---

# Abbreviations

ANN: Artificial Neural Network

ARS: Autonomously Replicative Sequence

DNA: Deoxyribonucleic acid

DSB: Double Strand Break

*E. coli*: *Escherichia coli*

ERC: Extrachromosomal rDNA Circle

GFP: Green Fluorescent Protein

ISC: Iron-Sulfur Cluster

LSTM: Long Short Term Memory network

NPC: Nuclear Pore Complex

PDMS: Polydimethylsiloxane

R- cells: Cells unable to respire, as opposed to R+ cells.

rDNA: ribosomal Deoxyribonucleic Acid

RLS: Replicative Lifespan

RNN: Recurrent Neural Network

ROS: Reactive oxygen species

rRNA: ribosomal Ribonucleic Acid

*S. cerevisiae*: *Saccharomyces cerevisiae*

*S. pombe*: *Schizosaccharomyces pombe*

SEP: Senescence Entry Point

TOR: Target Of Rapamycin

USCR: Unequal Sister Chromatid Recombination

WT: Wild type

---

## Preamble

If I had two words to summarize my thesis, I would use *single-cell* and *homeostasis*.

During the 4 years of my Ph.D., I focused on understanding how cells maintain and lose their homeostasis, mainly in two different contexts: replicative aging and quiescence. Since these phenomena are highly heterogeneous in a population and very dynamic at the cellular level, longitudinal single-cell approaches are required to accurately capture them. For that, I developed and used several tools based on microfluidics, timelapse microscopy and image analysis. Thus, this Ph.D. thesis has a strong methodological content but also aims at addressing biological questions.

This manuscript is split into two independent chapters. Chapter I is relative to replicative aging and is the core of the thesis. Chapter II is a side-project about quiescence, which has been done in close collaboration with Basile Jacquel, another Ph.D. student from the lab.

The appendix part contains a preliminary side-project, the development of a small tool and three published collaborations.

# Chapter I: Deciphering the complex dynamics of aging using microfluidics and deep-learning

A substantial part of my thesis consisted in developing methodological tools to address questions relative to aging. Hence, I decided to introduce the biology and the methodology in two different introductions, I and II.

Then come a short summary of the two introductions and the enunciation of the objectives of this part of the Ph.D.

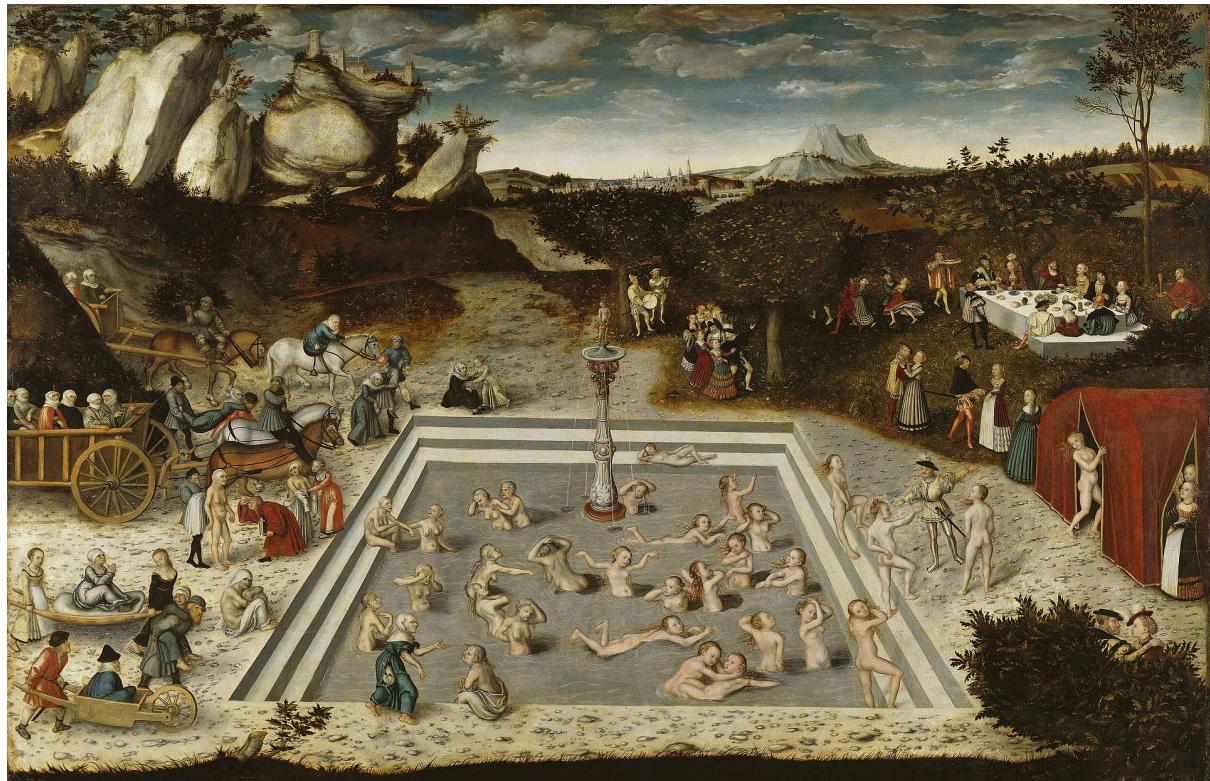
The Results section is also split into two parts relative to the methodology and to the biological question, with their own Perspectives paragraphs.

The chapter ends with a General discussion of the project on its globality.

## Introduction I: The biology of aging and the specific case of *Saccharomyces cerevisiae*

### 1. General aging concepts

Aging is one of the most familiar biological concepts, and anyone can observe its effects on older individuals and on themselves. At first sight, we could analogize it as the wearing of a machine due to its daily use. Nevertheless, understanding which gears are failing is challenging due to the complexity of the living machines. Indeed, most of the biological processes affect or are affected by aging. Consequently, a plethora of studies has been describing potential cellular or molecular causes over the years, which Medvedev tried to rationalize in the late 20th century [1]. Even though modern approaches and technologies now allow a more systematic and comprehensive analysis of this phenomenon, it remains a complex and poorly understood phenomenon.



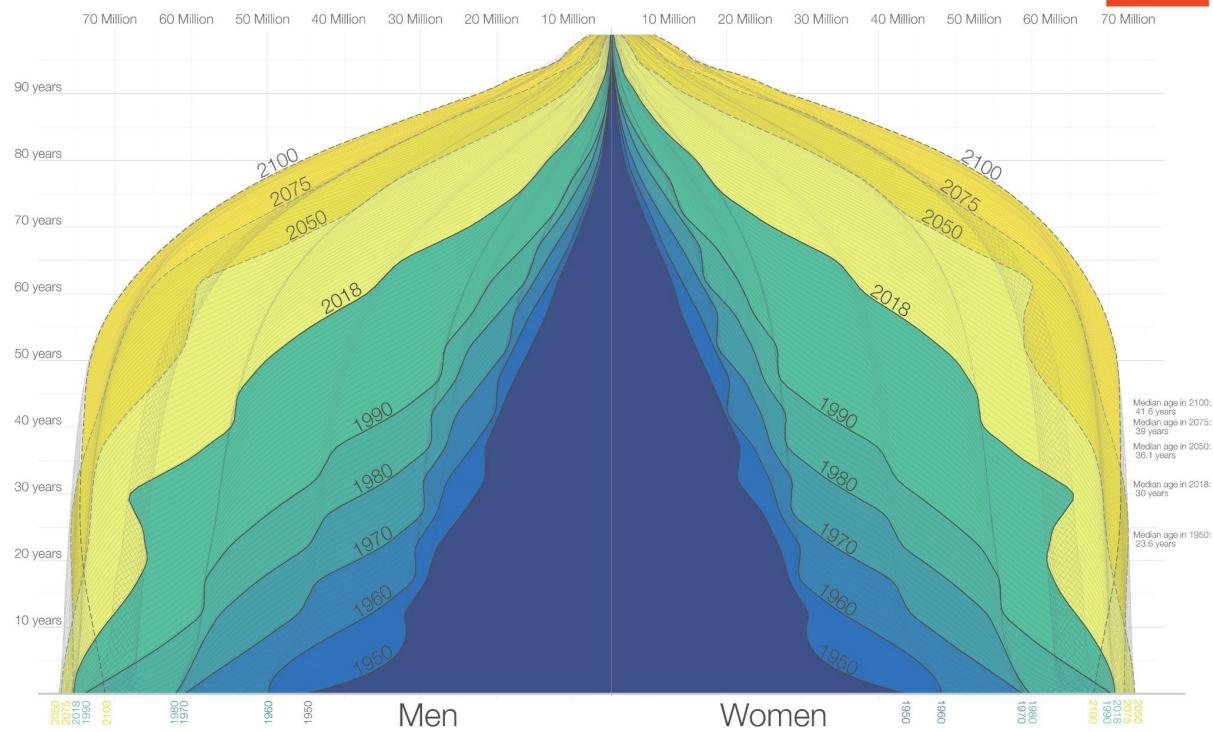
*The Fountain of Youth, 1546 by Lucas Cranach.*

Beyond the fundamental scientific question, aging is also an increasingly important concern for public health and demographic reasons. Indeed, it is the main risk (cancer, cardiovascular diseases, age-related diseases like Alzheimer or Parkinson, etc) and hazard (COVID19 [2], etc) factor of many diseases, in addition to be the main driver of a general state of frailty. This is problematic knowing that the population's average age keeps increasing every year (Figure 2), especially in wealthy countries. At the individual level, aging has been a major source of apprehension for at least 2500 years, with Herodotus reporting the existence of a mythological fountain of youth. Hence, understanding the origins and the molecular processes involved in this phenomenon is as interesting as it is important.

## The Demography of the World Population from 1950 to 2100

Shown is the age distribution of the world population – by sex – from 1950 to 2018 and the UN Population Division's projection until 2100.

OurWorld  
in Data



Data source: United Nations Population Division – World Population Prospects 2017; Medium Variant.

The data visualization is available at [OurWorldInData.org](http://OurWorldInData.org), where you find more research on how the world is changing and why.

Licensed under CC-BY by the author Max Roser.

*Figure 2: The demography of the world population from 1950 (blue shade) to 2100 (dark yellow shade) (From [3])*

In this introduction, we will first describe the concept of aging, the different theories proposed so far and the classical models to study it. Then, a specific focus will be made on the unicellular *Saccharomyces cerevisiae* and the various suggested aging pathways.

### 1.1. Definition of aging

Aging can be defined as “a persistent decline in the age-specific fitness components of an organism due to internal physiological deterioration” [4] or by an increase in the mortality rate with age. It is possible to measure parts of the biological decline with specific readouts, such as the world record, for human aging (Figure 3). However, such a definition of aging makes a generalization across species difficult and requires different assays for each model organism.

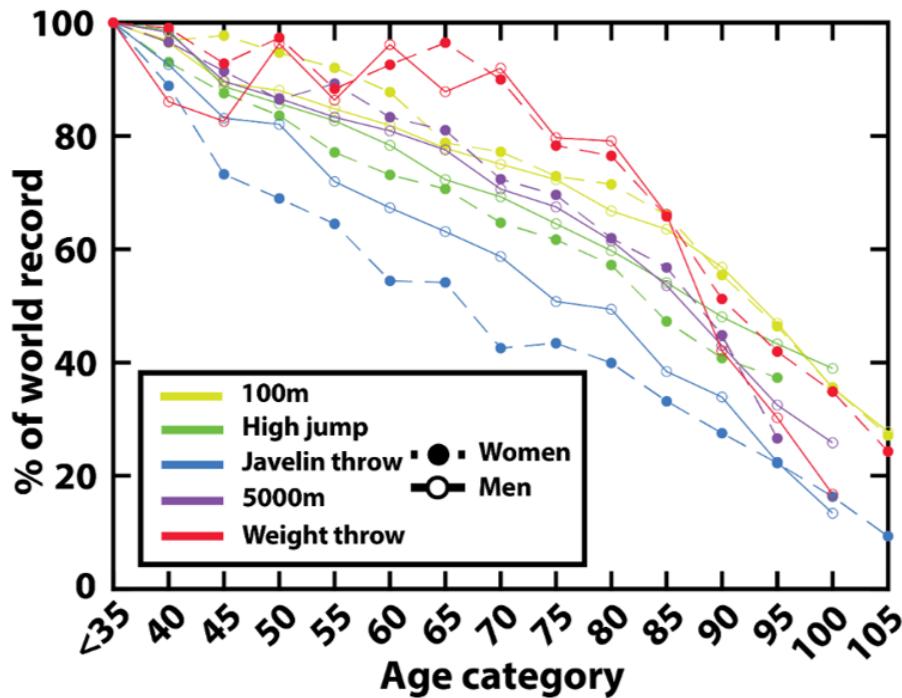


Figure 3: World records of different athletic sports from different age categories, normalized to the world record in the <35 years old category. (Data from [5])

The increase of the mortality rate, which is in fact a consequence of the first definition, is a more general readout and can easily be derived from the survival curve of a population. Indeed, let  $n$  be the number of individuals still alive at time  $t$  from a given population of  $N$  individuals at  $t = 0$ . The survival rate at a given time is  $s(t) = \frac{n(t)}{N(t)}$  and the hazard rate (or mortality) is  $h(t) = -\frac{s'(t)}{s(t)}$ , with  $s'$  the derivative of  $s$  relative to  $t$  (Figure 4).

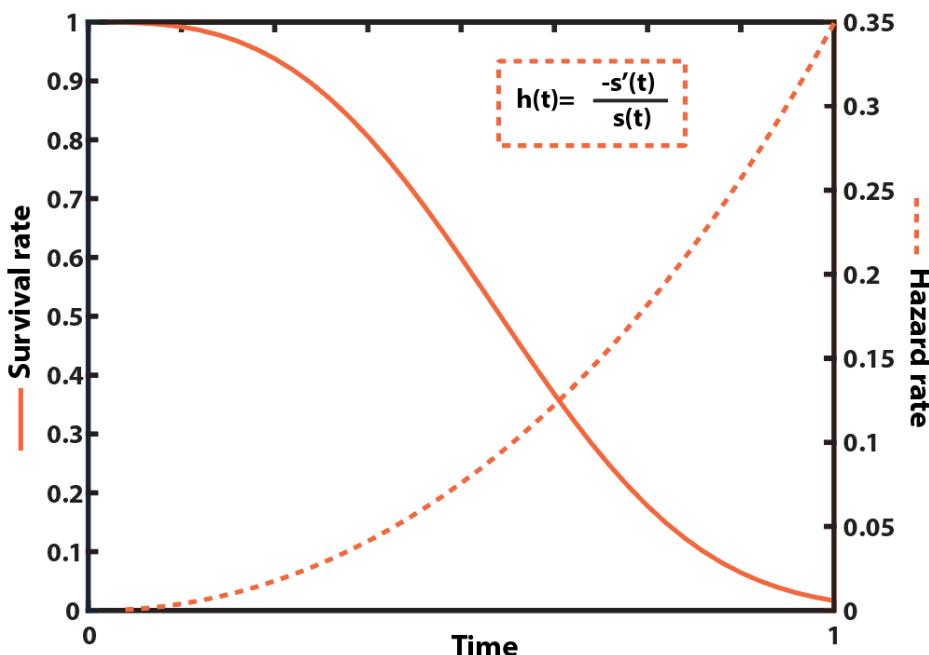


Figure 4: Theoretical survival and hazard rate

### Gompertz law

Taking this definition, we also observe that aging in humans exists and that the hazard rate increases exponentially with age (Figure 5) after the infantile period has passed.

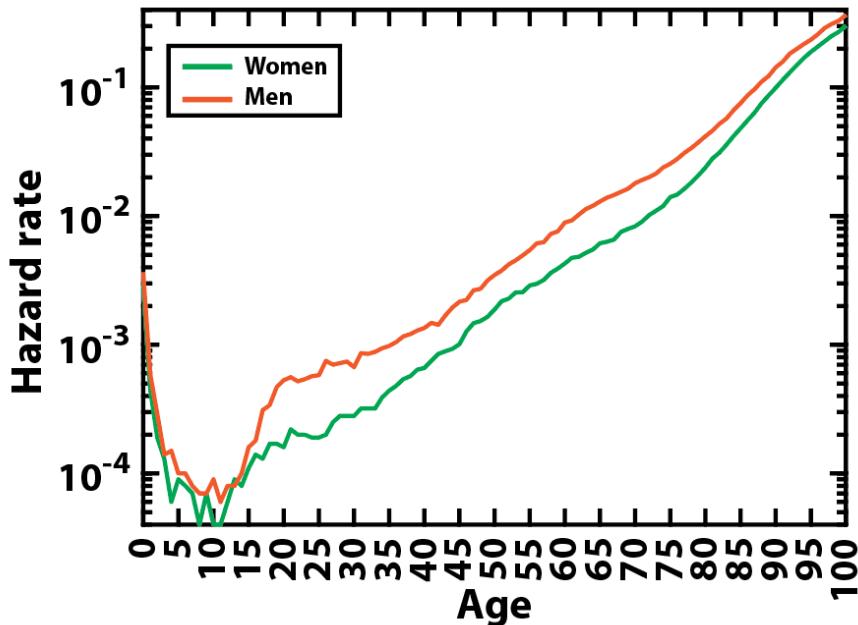


Figure 5: Hazard rate in the French population in 2019 (Data from INSEE).

Benjamin Gompertz first described this in 1825 [6], where he showed that the mortality  $h(t)$  was equal to  $\alpha * \exp(\beta * t)$ , with  $\alpha$  being the basal death rate and  $\beta$  describing how fast the rate of dying increases with age.

This law also describes the mortality of many mammals such as mice, rats, horses [7], but also of worms [8], fruit flies [9] or budding yeast [10], and many other animals [11]. Later on, William Makeham proposed a refinement of this law by adding a constant term to the hazard rate  $h(t) = \alpha * \exp(\beta * t) + \lambda$  to take into account age-independent death ( $\lambda$  is also called extrinsic mortality). However, this term is often negligible under a protected environment such as modern society or lab conditions.

Therefore, following a population can help us understand if individuals from a species are submitted to aging or not. However, it does not indicate which mechanisms are at stake nor how aging has arisen in the living.

## 1.2. Evolutionary theories of aging

Other approaches have emerged to try to understand aging, including evolutive theories. Georges Williams expressed in 1957 that "It is indeed remarkable that after a seemingly miraculous feat of morphogenesis, a complex metazoan should be unable to perform the much simpler task of merely maintaining what is already formed." [12]

Thus, despite its apparent drawbacks regarding Darwinian fitness, evolution has selected (or at least not discarded) aging, and a few theories have emerged to explain this selection.

### 1.2.1. Programmed aging

The first essay trying to explain the evolutionary origin of aging arose in the late 19th century from August Weismann with his theory of programmed aging "*When one or more individuals have provided a sufficient number of successors they, as consumers of nourishment to a constantly increasing degree, are an injury to those successors [...] natural selection, therefore, will weed them out.*" [13].

In other words, aging would be a mechanism to remove older, less fertile and less fit individuals from the species to benefit the younger and healthier ones. Some kind of programmed aging indeed exists in very specific contexts. For example, telomere shortening in an *in vitro* culture of mammalian somatic cells leads to the death of cells after a given number of divisions [14-16] (discussed later).

Nonetheless, the programmed aging theory lacks generality and was criticized for its many flaws. For example, the base of this reasoning is circular since aging is supposed to clear worn-out individuals, but is also causing this deterioration. Besides, if it is programmed, a set of genes controlling it must have evolved specifically for this function. Hence, it should be possible to stop aging by modifying these genes. However, despite having identified hundreds of longevity genes in different organisms, such as in *C. elegans* [17, 18], no combination of mutations has been able to stop it. If aging is programmed, then evolution has made this program very robust.

### 1.2.2. The selection shadow & the theory of mutation accumulation

Another evolutionary approach is based on the idea that natural selection declines progressively past a given age and that aging has arisen from the negligence of evolution. Indeed, different essays from the first part of the 20th century [19,20] used this idea to explain the high prevalence of the allele causing Huntington disease, which expresses after the age of 30, at which most individuals would have died pre-modern societies. Building on that,

Peter Medawar proposed in 1952 a more general model on how aging could have evolved in the wild.

It is based on the idea that most animals in the wild will die shortly after reaching sexual maturity and that deleterious phenotypes that are expressed later in life would not be affected by selection (in other words, be in the shadow of selection) (Figure 6).

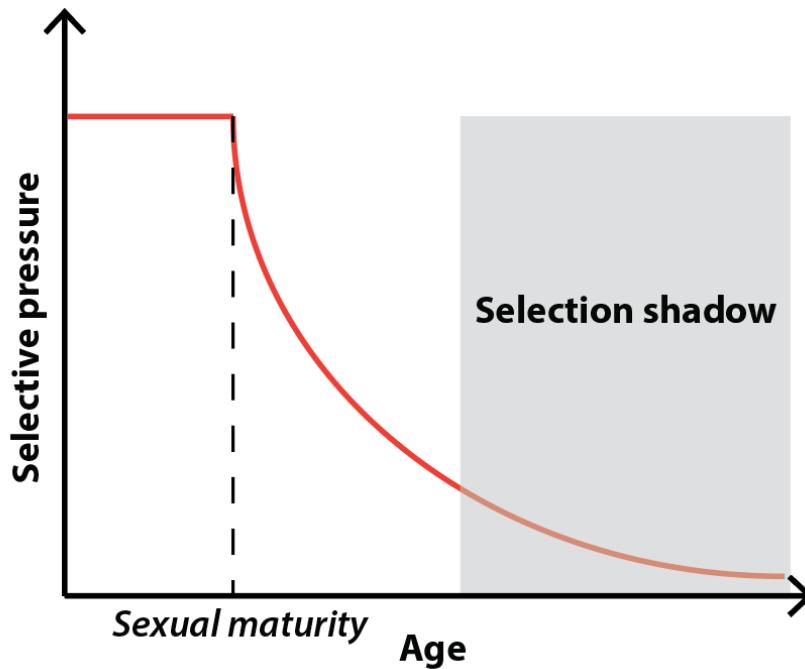


Figure 6: The Selection shadow concept

Hence, mutations that affect early life will be highly subjected to selection because reproduction has not occurred yet. In contrast, mutations expressed later could be passed to the offspring before they show any fitness problem.

Thus, species would accumulate mutations with late-acting deleterious effects in their genome, and this would cause aging. This theory is called the mutation accumulation theory and was later mathematically formalized by Hamilton [21].

This theory - though giving an attractive framework to consider gene actions in aging - raised several critics. Indeed, increasing evidence shows that aging is more common in the wild than previously expected [22,23] and influences the life tables of species [23-25]. Moreover, in social animals, knowledge transfer and material help such as parental care from the elders to the younger generation brings a fitness advantage [26]. For example, there is an increasing body of evidence that grandmothering in humans increases fitness by reducing mother hazards and child survival, which could explain why menopause (a consequence of aging) has been selected in humans [27-31].

Furthermore, this theory was also criticized because aging would still have a selective cost over individuals who do not age, even if it expresses late. Hence, it would ultimately be affected by natural selection [12].

Together, these critics suggest that aging is seen by natural selection. Then, why has it been selected?

### 1.2.3. Antagonistic pleiotropy

A refinement of the mutation accumulation theory was proposed by George Williams a few years later [12].

It states that if a gene has *pleiotropic* and *antagonistic* effects - one beneficial early in life (when selective pressure is high and survival and reproduction must be maximized), and one detrimental at a later age (such as the accumulation of damages) - then this gene will be selected and enriched in the population.

While direct evidence of the existence of such genes remains thin [32], they still exist. For example, in *C. elegans* and *S. cerevisiae*, competitive assays between long-lived mutants and wildtype (WT) showed that the mutant disappeared in a handful of generations due to an almost undetectable reduction in fertility [33,34,35].

Antagonistic pleiotropy can also mean an increase in fitness in varying environments. For example, *C. elegans* have shown that some long-lived mutants show no competitive disadvantage under normal conditions. Yet, the WT outcompetes the mutants once exposed to different feeding regimes (variation between starvation and feeding) [36].

Recently, more examples of antagonistic pleiotropy have been found and are discussed in [37,38].

A corollary of this theory is that since there is a trade-off between early reproduction and late fitness effects, genotypes with high reproductive capacity should be shorter-lived than those with lower fecundity and vice-versa [39]. This has been partly verified in field studies and in lab experiments, where a change in extrinsic mortality (thus, lower selective pressure at an early age) induces a change in aging [23,40,41]. On the contrary, selection experiments for long-lived individuals led to reduced fertility in Drosophila [42-45]. An extreme example of the trade-off between longevity and reproduction is the case of *Somniosus microcephalus* (Greenland shark) which can live more than 400 years but is thought to reach maturity at about 150 years old [46].

#### 1.2.4. The disposable soma theory

As an extension of these ideas, Kirkwood proposed in 1977 the *disposable soma* theory [47], which predicts that each species must maintain a given ratio between reproduction and maintenance of the existing structure. Indeed, organisms have limited resources (especially energy) and must allocate them between these two biological outputs in order to maximize their Darwinian fitness. Consequently, somatic cells would accumulate damages while germline cells are protected and immortal.

In line with that, some embryonic stem cells in murine models have a lower mutation frequency than somatic cells and a stronger oxidative stress detoxification system [48,49]. Similarly to the previous theory (and supported by the same set of evidence), it suggests that extrinsic mortality also drives this trade-off between maintenance and reproduction, with harsh survival conditions favoring rapid growth and sexual maturity over longevity [50]. Thus, aging would be an accumulation of damages due to the imbalance between soma maintenance and germline generation (see next paragraph)

A corollary of this theory is that aging must have appeared in the tree of life simultaneously as the distinction between the soma and the germline, *i.e.*, alongside asymmetric division. Indeed, if symmetrically dividing organisms were deteriorating over successive divisions, it would lead to the extinction of the whole lineage. This implies that symmetrically dividing organisms must keep a constant level of damage and allocate as much energy to division as they do to maintenance [51,52]. This idea is supported by data on symmetrically and asymmetrically dividing unicellulars (see p.22).

### 1.3. Molecular models of aging

Although these theories on the emergence of aging give a conceptual frame, they are not directly informative on which molecular players are at stake in this phenomenon.

The idea arising in the later theories is that damages and errors would accumulate with age in somatic cells, thus leading to the deterioration of tissues, as first suggested in 1963 by Leslie Orgel [53]. From then, many different types of damage accumulation have been described (Figure 7), such as (non-exhaustively) free radicals [54, 55], protein aggregates [56], telomeres shortening [15,16], mutations [57,58], DNA damages [59-61] and epigenetics alterations [62-65].



Figure 7: The hallmarks of aging, from [99].

In the following paragraphs, we will briefly review the main molecular theories of aging.

### 1.3.1. Free radicals

The free radicals theory of aging was proposed in 1956 and stood as one of the first models to explain how organisms lose fitness with time [54,55,66]. It states that cells accumulate free radicals (i.e., a molecule with a single unpaired electron from its outer shell) with time. These highly reactive species would damage the DNA and other molecules of the cell. It was later established that mitochondria produce Reactive Oxygen Species (ROS), which are free radicals, giving more substrate to this theory [66].

In line with that, ROS and other oxidative species levels increase during aging, even in mammals [67]. Besides, deleting the superoxide dismutase (an enzyme implied in oxidative stress detoxification) in yeast decreases the replicative [68,69] and chronological lifespan [70,71]. In *C. elegans*, increasing the level of superoxide dismutase has been reported to

extend lifespan [72]. However, reducing its level can also lead to increased longevity [73]. In *Drosophila melanogaster*, studies also reached opposing results regarding oxidative stress and longevity [74,75]. In ants, overexpression of the dismutase is not needed to reach extreme longevity in queens [76]. In mice, mutants lacking the superoxide dismutase have a decreased lifespan [77], but its overexpression does not lead to increased longevity [78]. Besides, reactive oxygen species also serve in signaling, and their average level might have evolved to ensure the proper functioning of the cell [79]. Moreover, hormetic effects exist regarding oxidative stress or antioxidants. Indeed, mild exposure to H<sub>2</sub>O<sub>2</sub> could have beneficial effects on longevity, potentially by overexpressing the detoxification machinery [80]. In contrast, excessive antioxidant supplementation can have negative consequences such as higher risks of cancers and increased mortality in humans [81-83]. The pleiotropic effect of oxidative stress in the cell and the way cells maintain their oxidative homeostasis might explain the discrepancy between the studies.

Overall, the free radical theory is one of the oldest molecular theories of aging and is still seriously considered and debated.

### 1.3.2. The somatic mutations theory of aging

Another early model proposes that the accumulation of mutations in somatic cells drives aging [57,58]. This is supported by evidence that the genome is constantly exposed to harmful reagents such as oxidative stress (see previous section), UVs and replication problems [84], which can create DNA damages (for example, single- or double-strand breaks). Endogenous DNA damages are widespread in cells since they occur tens of thousands of times per cell and per day in mammal cells (reviewed here [85]), which can lead to a mutation if not correctly repaired. In multicellular organisms, it can lead to the generation of a tumor or the altered function of the cell. In line with that, somatic mutations accumulate during aging in model organisms and humans [86].

Nevertheless, specific mutant mice defective in a protein for DNA mismatch repair have about a 100-fold elevated mutation frequency (and cancer) in all tissues, yet do not appear to age more rapidly [87]. Similarly, it has been recently reported in yeast and humans that mutation accumulation is not linked with aging [88,89]. Therefore, the somatic mutation theory of aging is strongly contested nowadays.

### 1.3.3. DNA damage and epigenetic alteration

Contrariwise, the DNA damage theory of aging has received substantial support since it was first expressed in 1967 [61]. If they can create mutations in dividing cells, these damages can

not be repaired and lead to the mistranscription of the affected genes in post-mitotic (or slowly dividing) cells. The accumulation of such abnormalities is considered a driver of aging. Experimental evidence indeed found increased levels of DNA damage in old cells and identified oxidative stress as a substantial cause [59,90-92]. In agreement with that, a very recent comparative study on 102 species of rockfishes, whose lifespan ranges from 11 to more than 200 years, identified the DNA repair pathways as a trait of longevity [93].

Interestingly, this model bridges with a more recent one, which states that the accumulation of epigenetic alterations drives aging. Indeed, the repair of DNA damages often leaves epigenetic marks such as DNA methylation or chromatin reorganization. These marks are enriched during aging, notably in mammal tissues [62-65,94-96]. Moreover, the DNA methylation state of human blood cells was shown to have predictive value on later-life mortality [97,98], which led to the famous term “epigenetic clock” of aging. Whether this clock plays a role in aging or is just indicative of the epigenomic state of the cell is not clear. Yet, preventing some of these epigenetic remodeling increases lifespan in yeast, flies, worms, and even mammals, according to a recent study [96,99-102].

Overall, the DNA damage theory of aging is still receiving attention from the aging community. Its link with the epigenetic alteration is not fully understood, and DNA methylation and chromatin reorganization can likely occur in a DNA damage-independent manner. This last theory is still young and emerging, and further study will tell us how much this hallmark of aging [103] is a cause of it.

#### 1.3.4. Telomere attrition

Telomere shortening is also an early theory of aging with the observation that somatic cells cultured *in vitro* experience an irreversible cell-cycle (termed *senescence*) after a given number of divisions, linked to telomere shortening in [14,15]. Yet, only a little evidence supports that it could cause aging in organisms. Indeed, normal somatic cells in tissue do not divide enough times for that [104,105], and telomere shortening could only be a mechanism of tumor prevention [106]. Besides, even though other studies report a global telomere shortening in old tissues [103] and an increased lifespan in mice with longer telomeres [107], it is still debated as to whether telomeres attrition is causal of aging [108,109].

#### 1.3.5. Decline in protein homeostasis

Since proteins are the basis of most cellular functions and their synthesis costs many resources (energy and amino acids), tight control of their content and quality is necessary. Thus, protein homeostasis is regulated by several processes such as protein production and degradation,

folding, and autophagy. In humans, proteostasis can change with age [110], and age-related disorders such as Huntington, Parkinson or Alzheimer's disease are caused by a loss of protein homeostasis [111]. Besides, increasing chaperone protein levels increase longevity in flies and worms [112- 115], and so does autophagy in mice [116].

### 1.3.6. Decline of repair functions

In addition to the previously suggested damages, repair and maintenance machinery such as the proteasome [117], chaperons [118], autophagy [119], and DNA damage repair [120] have also been reported to decline with age, amplifying the accumulation of damages. This is in line with the disposable soma theory (see p.10), in which aging arises from a rupture of homeostasis between repair and damages [50].

A link exists between the cellular, tissue, and organismal level. Indeed, senescent cells are enriched in old tissues of many organisms, including mice, apes [121], and humans [106,122]. Besides, these cells often trigger inflammation and senescence, in part due to their secretory functions (secretion of Senescence Associated Secretory Phenotype, SASP [123]). Hence, senolytics recently received much attention for their ability to kill senescence cells specifically. However, it is still not clear if these cells are a simple byproduct or a cause of aging. Consequently, if senescence at the single-cell level causes aging at the organismal level is still debated [124].

### 1.3.7. The network theory of aging

Yet, none of these processes can explain aging on their own. More generally, many hallmarks of aging have been identified [103,125], but no theory has emerged to link all the potential players and distinguish drivers from byproducts of aging.

Besides and as described all along this section, processes in one organism may not be at stake in another one. If a general theory of aging exists, it needs to be validated across many organisms.

Therefore, new concepts are emerging to try studying aging as a complex system with different interacting processes that have different dynamics instead of separate causes. This approach aims to link the many existing theories by considering the interactions and synergies between the molecular players implied in aging and across biological sub-systems (molecules, metabolic pathways, organelles, cells, tissue, and organism) [126]. It can be

termed the *network theory of aging* [127,128], or with a more recent terminology, the *system biology of aging* [129,130], and is discussed in the General discussion.

## 1.4. Universality of aging

Interestingly, aging is not universal across the tree of life. Many species have reported to not age at all (*i.e.*, to have a constant probability of dying, see p.4), and this phenomenon is termed *negligible senescence* [132].

Examples are the hydra (probability close to zero, *i.e.*, quasi-immortal [131], the red abalone, the collared flycatcher, or the great rhododendron (Figure 8). Other organisms experience *negative senescence* - namely a decrease in mortality with age - such as the desert tortoise or the white mangrove [11,133]. More recently, the famous *Heterocephalus glaber* (naked mole rat) has been reported as the first mammal not experiencing aging [134].

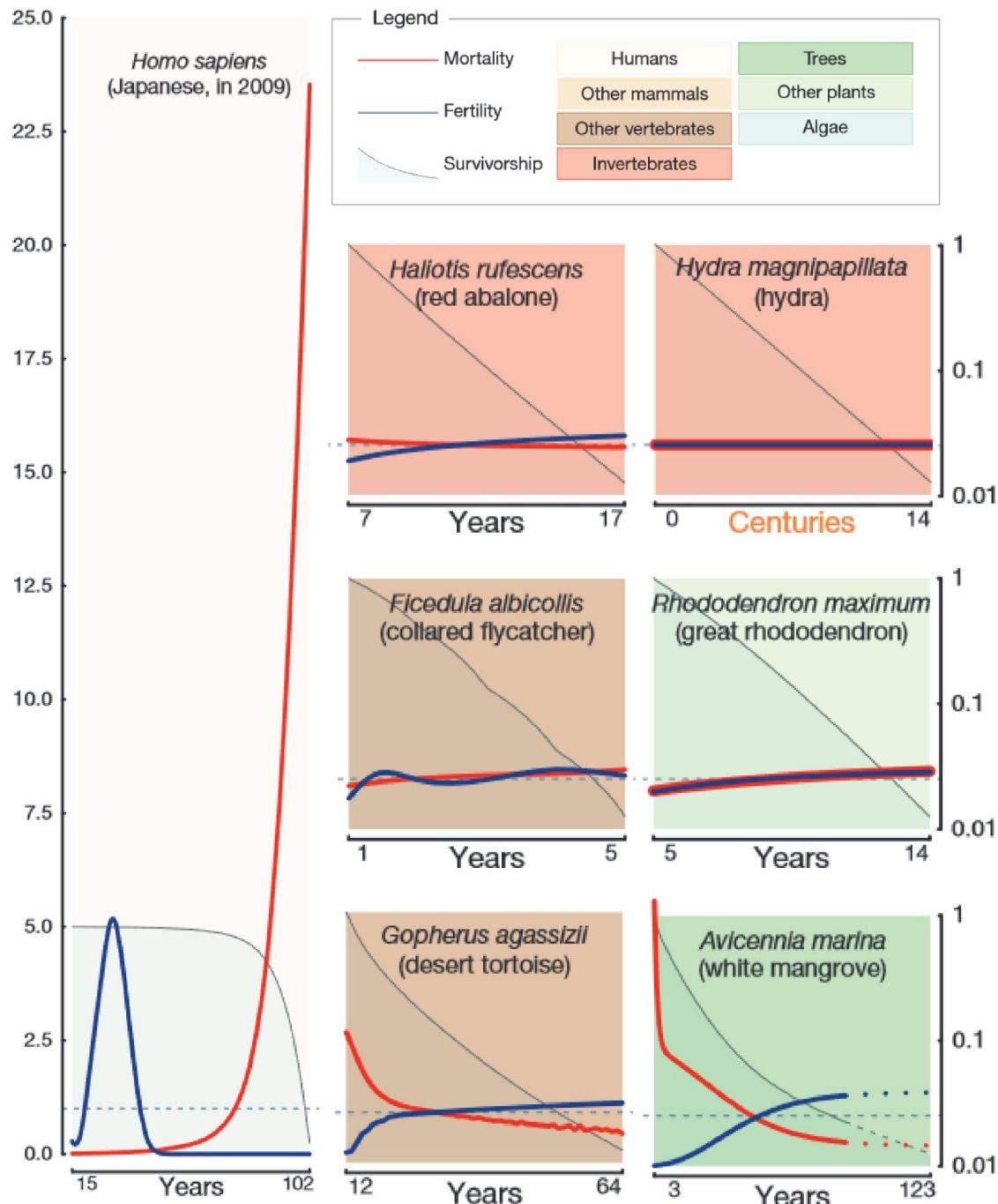


Figure 8: Mortality, fertility, and survivorship across several species. From [11]

Studying these models, especially in light of already known theories, can help us understand the different evolutionary and molecular aspects of aging.

## 1.5. Laboratory models

As previewed in the previous sections, aging can be studied at the cellular or organismal level. Besides, genes do not solely influence it since homogenic individuals can have different

lifespans. Thus, monitoring aging in laboratory conditions by varying the genetic background or the environment is a powerful approach.

### 1.5.1. Clonal aging

A simple way of studying aging is by observing cultures of dividing mammal cells *in vitro*. As discussed earlier (p.13), somatic cells can do only a limited number of divisions before entering senescence, and telomere shortening is mainly responsible for this arrest. In fact, since telomeres size is equal in cells dividing symmetrically and shortens at each division in somatic cells (lacking the telomerase enzyme), the whole culture stops dividing after about 60 divisions [14-16,106]. This phenomenon is termed clonal senescence (Figure 9) and is convenient to study since the whole process takes about ten days instead of years for mammal organisms.

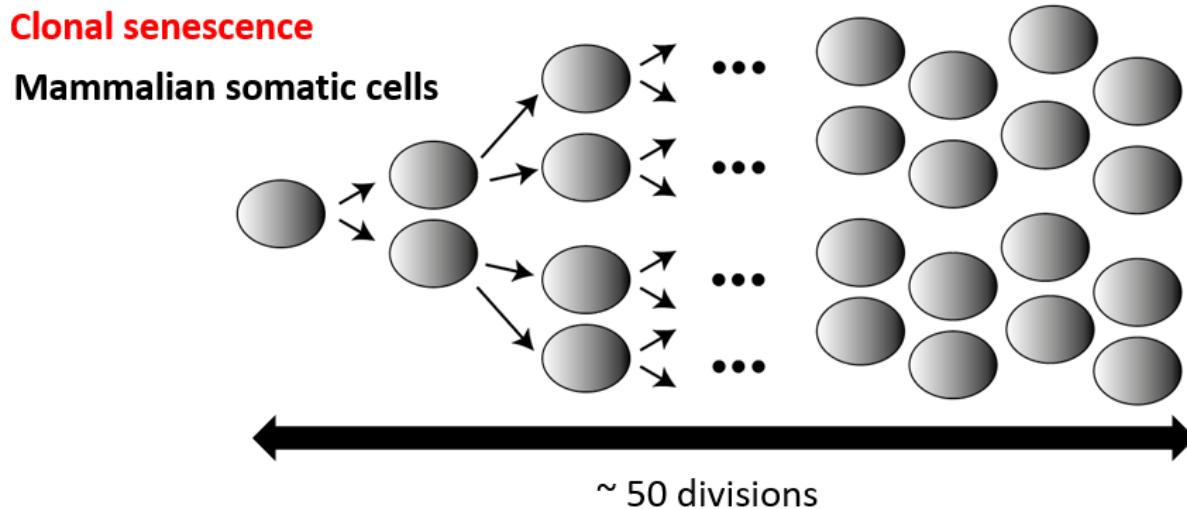


Figure 9: Schematics describing clonal senescence. A culture of cells originating from the same telomerase deficient cell will senesce after a given number of divisions.

Nevertheless, although it is interesting to study senescence and its involvement in development and tumor suppression, the relevance of such a model to study aging at the organismal scale is debated.

### 1.5.2. Organoids

An intermediate model has been emerging for the past few years, in which cells are cultured to grow in an organized 3D microenvironment to mimic micro organs [135]. These *organoids* are particularly interesting for morphogenesis and developmental research. They also happen to be useful for aging research since they age at the scale of a few months while having a tissue structure, therefore conserving the cell-cell interactions. Besides, they can be used with various cell types, especially derived from humans [136,137].

### 1.5.3. Model organisms

As stated before, it is essential to consider aging by integrating all the scales on which it has an influence.

Therefore, several model organisms have been extensively studied in the past decades.

For example, the fly *Drosophila melanogaster*, which has a lifespan of about 50 days under normal conditions, is a common model to study aging, and several mutants of longevity have been described [74,138-143]

Another valuable and popular model organism to explore the genetic influence of aging is the worm *C. elegans*, which lives about ten days and allows facile genetic screens thanks to the famous interfering RNA [144] tool. Again, many pathways affecting its longevity have been identified [17,18,145,146].

Concerning mammal models, the most used models are the mice *Mus musculus* [147] and the rat *Rattus norvegicus* because they offer an interesting trade-off between easiness of manipulation, aging kinetics, and similarities with humans (similar organs and diseases).

Aging can also be investigated using unicellulars such as budding yeast (*Saccharomyces cerevisiae*), which ages in a few days and is a historical model for this purpose [148,149]. This model organism will be thoroughly developed in the following sections.

Interestingly, in all of these organisms, blocking the main metabolic pathways (TOR, Sch9, PKA, and IGF) results in increased longevity [150]

Besides genetic manipulations, environmental modifications can also affect aging, with caloric restriction (CR) being the most widely used and documented. Indeed, it was found to positively impact longevity on most of the tested organisms. Already 90 years ago in the rat [151,152], and then in mice [153-155], worms [156], flies [9], budding yeast [157,158], fission yeast [159], and many other model organisms. In monkeys, it delays the onset of age-associated disease and increases lifespan (though it was debated during a few years, it now seems consensual) [160-162].

However, the range of effects of CR is still debated, and some could be due to the overfeeding of control groups or other analysis biases [163,164,331]. Furthermore, it is

unclear which CR regimes induce an effect (constant and low level of nutrients, or alternate periods of fasting) [155-165].

Caloric restriction is known to repress the growth metabolic pathways presented above. However, as these pathways impact most other biological processes, it is complex to understand, and the exact molecular mechanisms at stake remain unknown or controversial [166].

Nevertheless, working on different organisms and trying to identify the common pathways is interesting. In this sense, it has been shown quantitatively that the genetic components of lifespan are quite conserved across eukaryote species [167].

Thus, since unicellulars share many longevity pathways with other *taxa*, that they are simultaneously a cell and an organism, that they age very fast and are very convenient to manipulate, they seem to be of particular interest for aging studies.

#### 1.5.4. Unicellulars

Like in complex organisms, it is possible to measure aging in unicellulars, either by looking at their mortality rate with time or by measuring the decline of their physiological functions, such as the cell-cycle slowdown or growth rate. Interestingly, the division pattern varies among the classical unicellular models with the bacteria *E. coli* and the yeast *S. pombe* dividing symmetrically, while *S. cerevisiae* divides in an asymmetric manner.

##### 1.5.4.1. *Bacteria*

*E. coli* is a small (2 $\mu$ m long, 0.25-1 $\mu$ m diameter, Figure 10) rod-shaped bacterium that divides very quickly (~20min) under favorable conditions, giving two daughter cells.

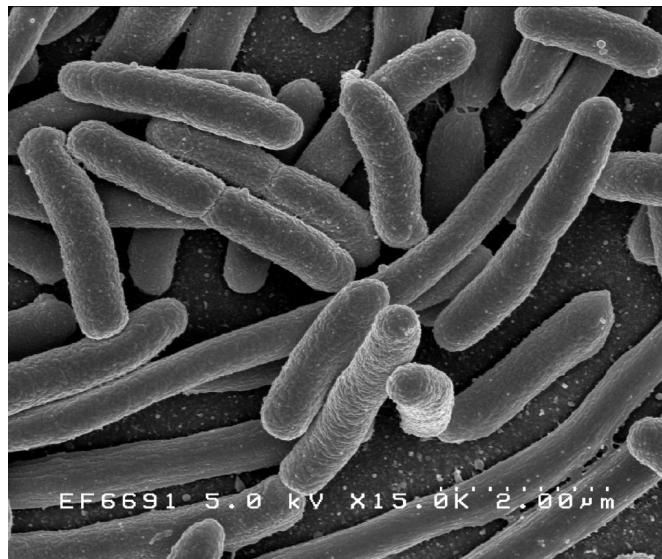


Figure 10: Electron microscopy image of *E. coli* cells. (Image from Rocky Mountain Laboratories, NIAID, NIH)

Historically, *E. coli* and other symmetrically dividing organisms were thought to not age for reasons explicated before (see p.10). In reality, the *E. Coli* division is not exactly symmetrical because after forming a septum and the septation, one daughter receives an old pole while the other gets a new one (Figure 11).

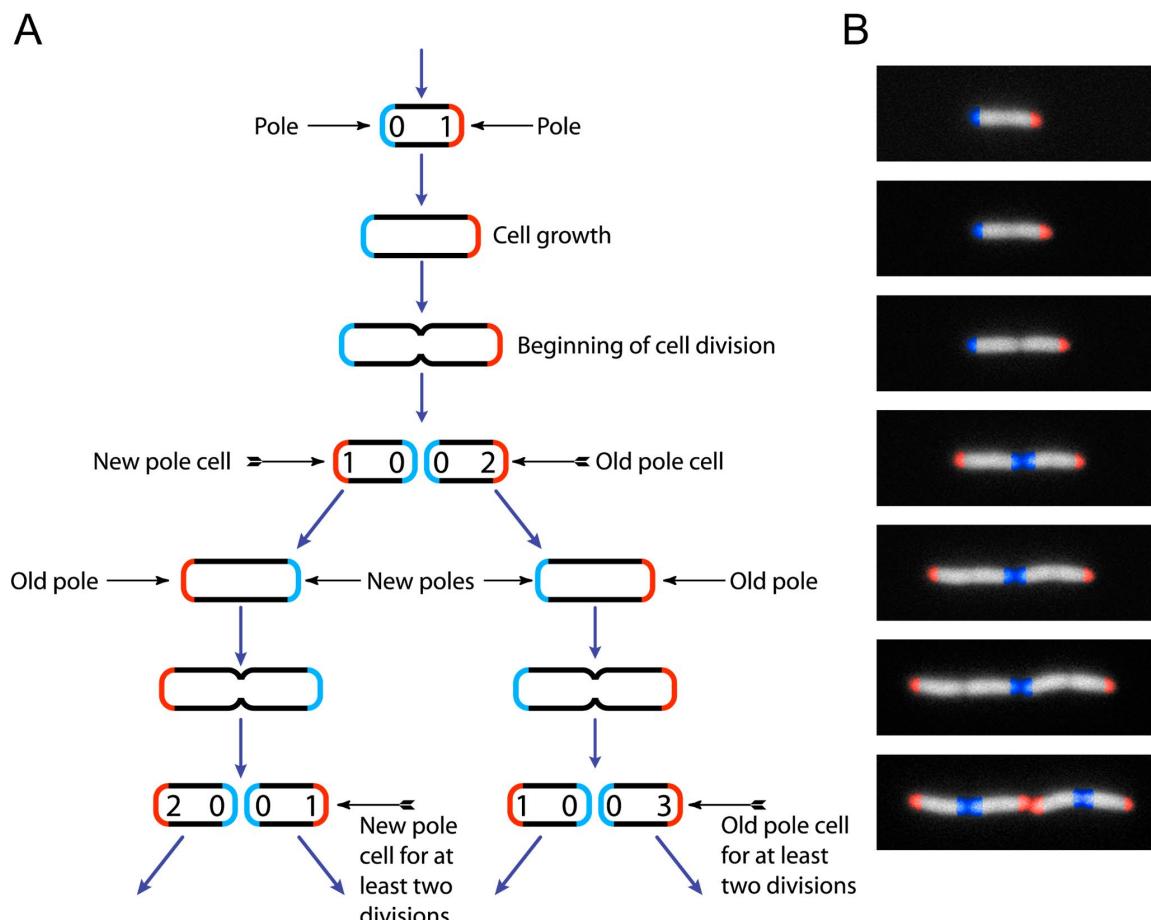


Figure 11: Division pattern of *E. coli*. (From [164]). (A): Schematics of the old and new pole distribution among the progeny. The numbers indicate the replicative age of the pole. The blue overlay: new poles, red overlay: old pole.; (B): Images of an *E.coli* dividing with the same color code as (A).

The first piece of work studying aging in *E. coli*, by following the first nine divisions of single-cells, concluded that the “old pole cell” was experiencing a decrease in division time after a few divisions [168] while new pole cells were gaining fitness during this interval.

However, a more recent study using microfluidics (discussed later, p.68) was able to track old pole cells for more than 100 divisions and showed that the division time of old and new pole cells was indeed evolving but reaching an equilibrium state - later identified as a stable attractor [169] - after a few divisions [170]. Besides, old pole cells were found to keep a constant growth rate throughout their lifespan after the first ten divisions (Figure 12).

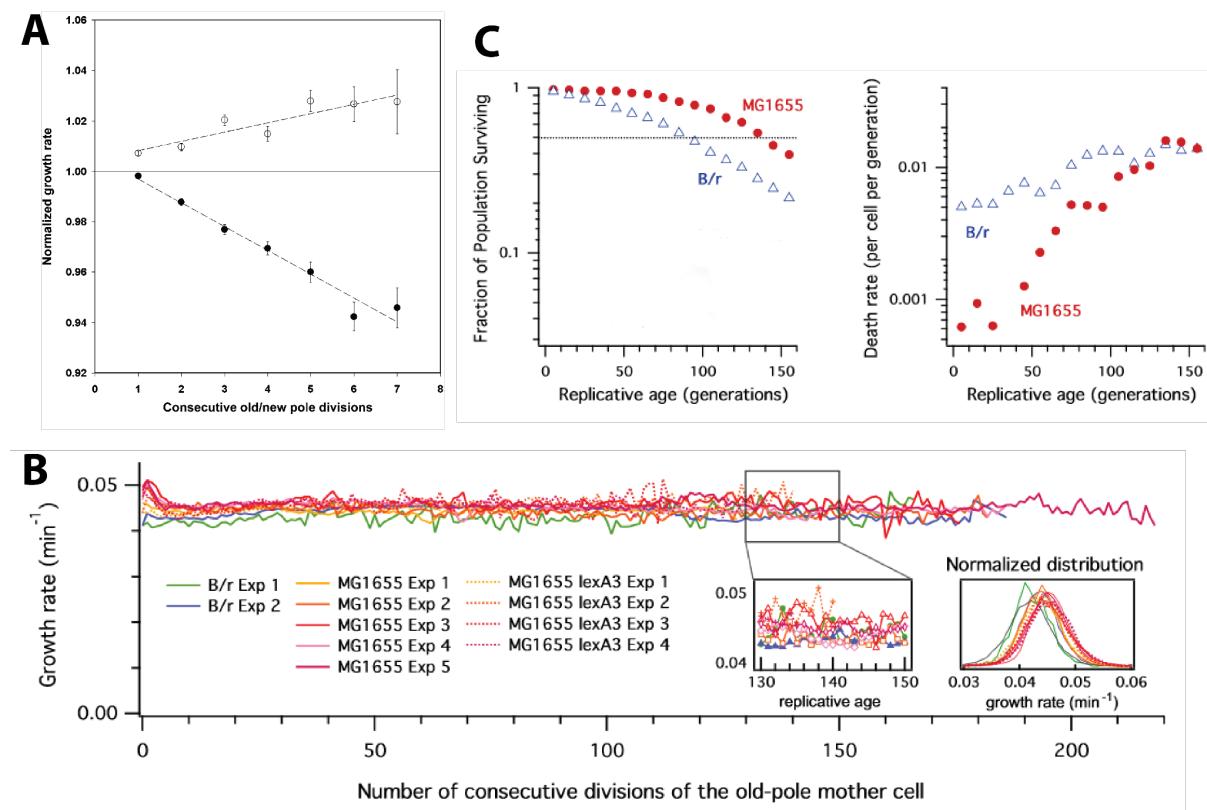


Figure 12: (A): Normalized growth rate of new (top) and old (bottom) pole cells during the eight first divisions (From [166]). ; (B): Growth rate of the old pole cell across different strains and replicates plotted versus replicative age of the old pole cell. (C): Survival rate (left) and death rate (right) of the old pole cells from the MG1655 and B/r strains.

Importantly, this study has also shown that the mortality of the old pole increases throughout the divisions.

Two interesting ideas emerge from this set of studies. Firstly, an increase in mortality can occur without a detectable decrease in fitness (such as growth rate). Secondly, aging can exist even in the case of seemingly symmetrical division.

How to explain this aging molecularly? First, the cell wall of the old pole is not renewed and is unable to avoid deterioration [17-173]. Moreover, protein aggregates have also been

proposed to segregate asymmetrically between the old and the new pole [174,175]. Even though more asymmetrically segregated factors could be involved and no causal link has been identified, this could explain the old pole lineage is aging.

#### 1.5.4.2. *Schizosaccharomyces pombe*

*Schizosaccharomyces pombe*, a rod-shaped yeast historically used to study cell-cycle and cell size control [176]–[182], divides in a similar fashion to *E. coli*, with each division giving rise to two daughter cells, “an old pole cell” and “a new pole cell” (Figure 13). Thus, if this unicellular eukaryote displays any sort of aging or not is of major interest.

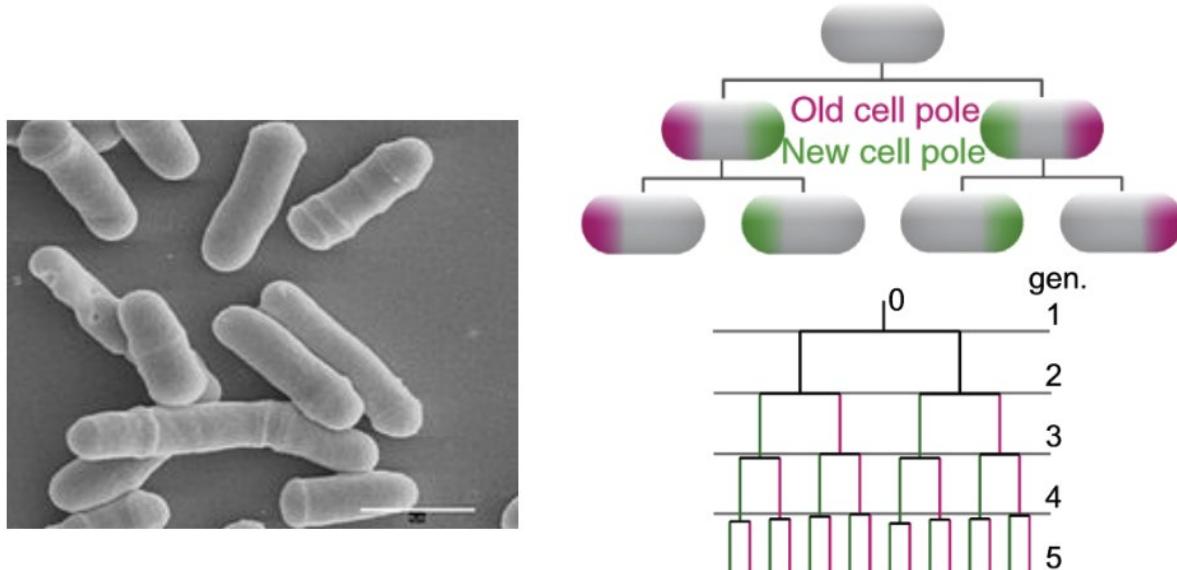


Figure 13: (Left): Electron microscopy image of *S. pombe* cells (scalebar: 10µm) from [179]; (Right): Division pattern of *S. pombe* cells and the repartition of the old (purple) and new (green) poles along with the divisions (from [180])

By following the old pole cell using microdissection techniques, it was first reported that the old pole was aging [185]. In contradiction, recent studies have shown that it was not the case [159,184,186]. Neither division time nor the death rate increases in the old pole lineage, and cells die without any aging-associated changes. One way to explain the difference with *E. coli* is that *S. pombe* is growing polarly, with newly synthesized cell wall materials being exclusively incorporated at the ends of the cells [187-189]. Instead, the new pole inherits a more significant proportion of old lateral cell walls and a birth scar[179,190]. Hence, the new pole lineage may experience aging, but no current method allows the tracking of the new pole for an extended number of divisions.

On top of that, the spindle pole body (SPB) and protein aggregates are also segregated asymmetrically and randomly [191] (or partly randomly in the case of aggregates). Indeed,

one cell inherits a new SPB or a protein aggregate while the other is born with the old SPB or no protein aggregate. However, none of these factors have been clearly shown to affect mortality or the division frequency.

So far, the distinction of lineages was based on the new versus old pole, new versus old SPB, or presence of a protein aggregate versus free of protein aggregate at birth, and no aging effect has been detected based on these markers. However, it is possible that a factor, transmitted asymmetrically and randomly, would cause aging in the lineage of the cells inheriting this factor, but that no one could not follow this lineage. Another option is that the generation of damages per division is slower than the division itself. Consequently, symmetrical damage segregation would lead to a constant average amount of damage in each cell, close to zero.

Again, new methods and further studies would be required to tackle these questions.

#### 1.5.4.3. Asymmetrically dividing cells

In other unicellular organisms, the division's output is two different entities - at least morphologically - making it easier to isolate a given lineage and measure its aging.

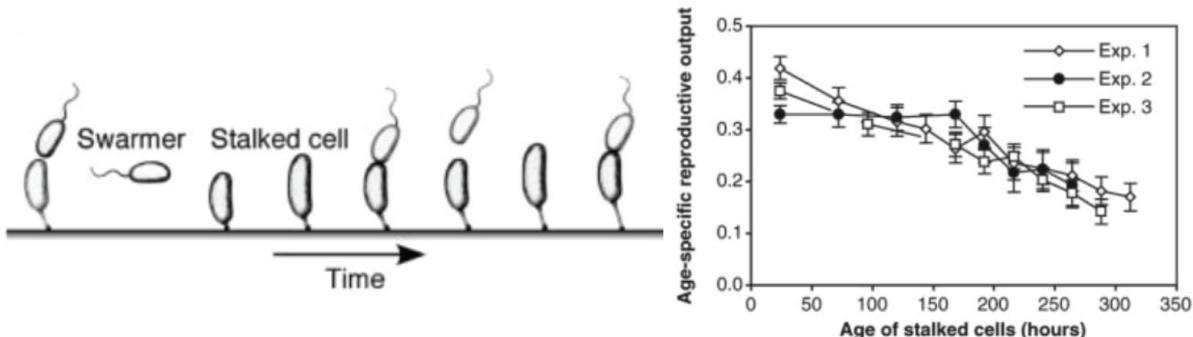


Figure 14: (Left): Schematics of the division of *Caulobacter crescentus*. (Right): Average division rate of single stalked cells versus replicative age. (From [192])

For example, in the bacterium *Caulobacter crescentus*, only the "stalked cells" attach to a surface. It then divides and gives rise to a "swarmer" cell that freely swims until it finds a substrate to attach to and differentiates into a stalked cell (Figure 14). In this context, it has been established that the division frequency of a given stalked cell was decreasing with time, whereas its progeny recovered a normal reproductive output [192]. Interestingly, young cells born from old stalked cells had the same division time as young cells from the beginning of the experiment, indicating a rejuvenation of the progeny of stalked cells.

Similarly, in the budding yeast *Saccharomyces cerevisiae* (Figure 15), the result of the successive divisions is a mother lineage and a daughter lineage, which are distinguishable by

several properties such as the morphology or protein expressed. After the division completion, the daughter will grow and become a mother itself. From that, it was found that mother cells can only undergo a finite number of divisions before dying, while the progeny is rejuvenated (described in detail in the next section).

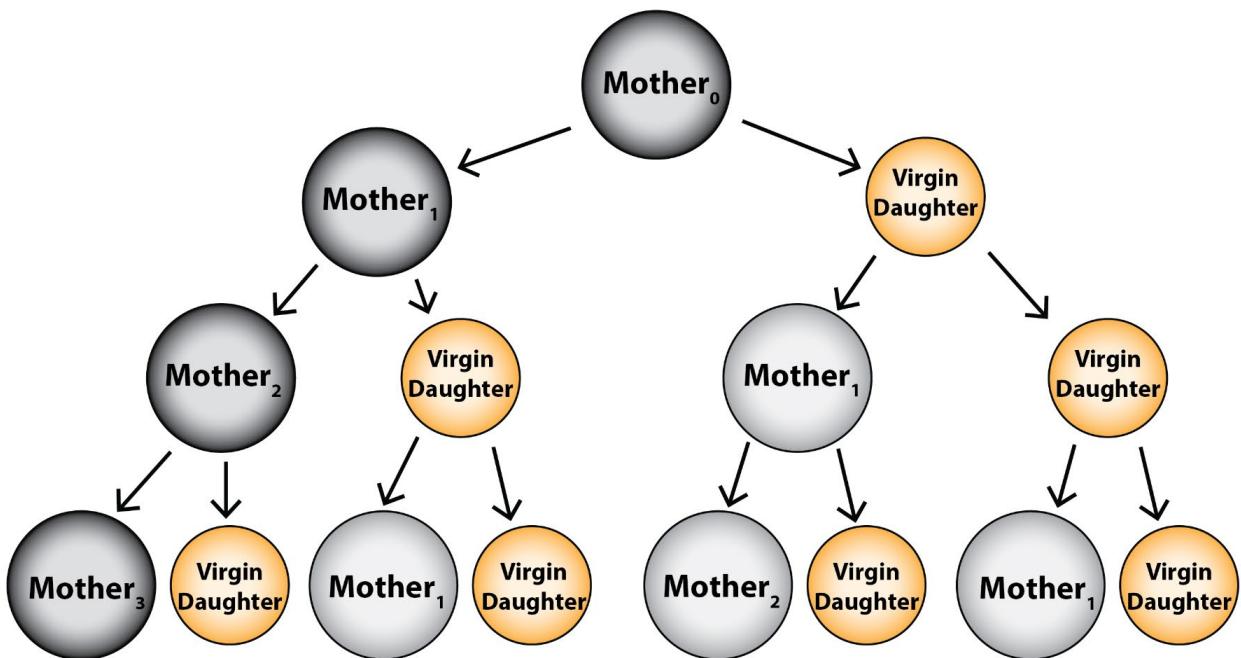
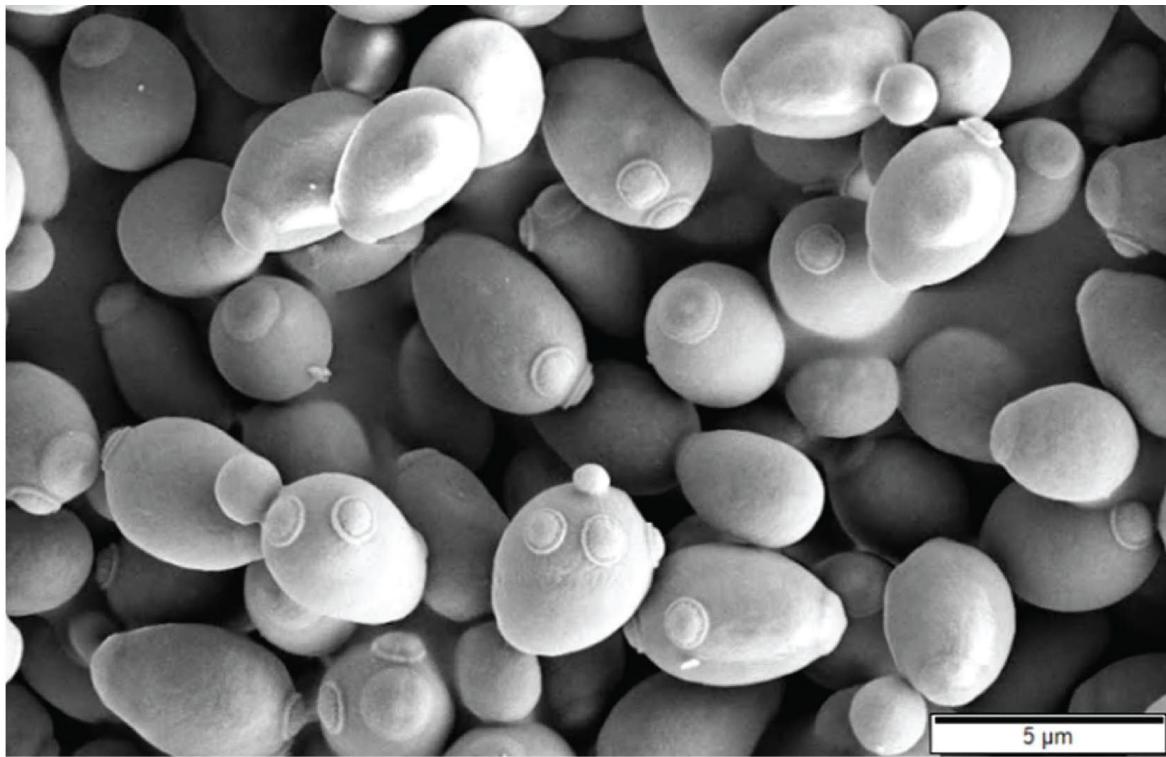


Figure 15: (Top): Electron microscopy image of *S. cerevisiae* cells (from [188]).; (Bottom): Phylogeny of an original mother cell (dark grey). The indices represent the replicative age.

This model is particularly interesting to study aging for several reasons. Firstly, it perfectly illustrates the disposable soma theory, where the mother cell (*i.e.*, the disposable soma) produces healthy daughter cells at the expense of accumulating damage. Indeed, it is thought that mother cells accumulate damages that are not passed to the daughters (see next section), which is another strategy (compared to *S. pombe*, for example) to ensure that the offspring survives and reproduces to maintain the lineage. Besides, it can also be a model for stem cell replicative aging, where the mother lineage mimics the differentiating lineage while the daughter lineage represents the stem cell lineage [194,195].

Secondly, though the relevance of this model to study human aging is debatable [196,197], many molecular pathways and hallmarks of aging remain conserved with higher eukaryotes [198]. Moreover, it is very convenient to manipulate thanks to its unicellularity, to the fact that the mother lineage is easily isolable from the daughter lineage, and to its short lifespan when replicating (a few tens of hours). In addition, even though there is increased ease to generate mutants in other model organisms thanks to CRISPR-Cas9 and other new genetic technologies, budding yeast remains one of the easiest for mutant and genetic variety generations. Indeed, it is compatible with many genome editing techniques (classical and new), can adopt sexual reproduction, and offers exhaustive mutant collections (deletion and GFP [199]). Furthermore, the species *S. cerevisiae* contains more than 1000 natural isolates [200], so studying the similarities and the differences regarding aging across the isolates can be helpful to understand the genetic drivers of longevity evolution [201-204].

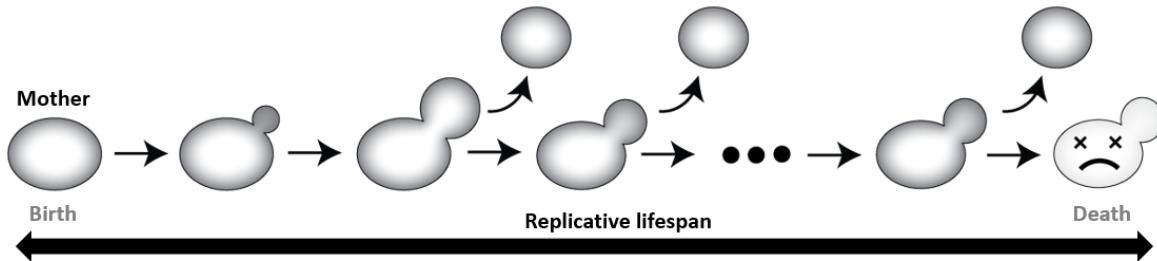
For these reasons, our lab uses this model organism to study aging, and the following sections of this manuscript will focus on it by calling it "yeast" for simplicity.

## 2. Aging in budding yeast: different models

One strength of yeast when studying aging is the multiplicity of approaches. For example, one can focus on the time a yeast cell survives in a non-dividing state, which mimicks post-mitotic cells' aging in higher organisms such as neurons [71]. Technically, this chronological lifespan is assayed by measuring the viability of a culture of cells left in the stationary phase at given timepoints (typically, by replating cells on rich media and counting the number of colonies forming). From that, several longevity mutants were reported, such as mutants of the SCH9 and TORC1 pathway (metabolic pathways controlling growth which are strongly conserved across organisms, see p.44). Nevertheless, the parallel with post-mitotic cells is

debatable because post-mitotic cells are metabolically very active while yeast in the stationary phase are dormant cells [205].

The second widely used model is to observe mother cells dividing from birth to death, and in particular, to count how many daughters are generated before death (termed Replicative lifespan, **RLS**). This replicative aging approach will be the focus of the rest of this chapter.



*Figure 16: Schematics of the replicative lifespan of an *S. cerevisiae* cell*

Finally, it is also possible to mimic clonal aging (which happens in telomerase deficient mammal cells, (see p.13 for detailed definition). In fact, in certain mutant strains, the culture fails to propagate indefinitely, which is the case for telomerase deficient cells in mammals) [206-208].

## 2.1. The dogma of replicative aging

Concerning replicative aging, it has been known for 60 years that yeast mother cells can undergo a finite number of divisions before dying [148].

The replicative survival curve of yeast mother cells is sigmoidal (or Gompertzian or a Weibull distribution), not only in lab strains, both haploid and diploid, but also in natural isolates [209,210]. This demonstrates that aging occurs in budding yeast (see p.4 for aging definitions).

Besides, yeast shows a decline in physiological functions with age (described later), which also conforms to the definition of aging.

Then, the central hypothesis to explain replicative aging is that a toxic factor would accumulate in mother cells, leading to their death [211].

Interestingly, daughter cells recover a full replicative lifespan, which is called rejuvenation. This is not true for daughters from very old mothers, but in this case the progeny of the daughter eventually recover its full replicative potential [212].

From that, we can define the dogma of the replicative aging paradigm as [213] (Figure 17): Aging occurs in the mother lineage due to aging factors that 1. Accumulate in the mother, 2. Are not transmitted to the daughter and 3. Are toxic for the cell, directly or indirectly, and modulating the level of the factor should impact lifespan.

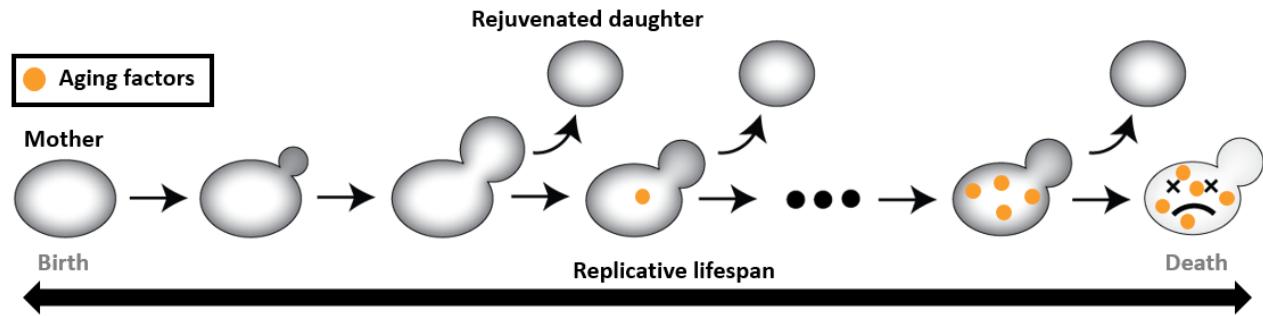


Figure 17: Schematics of the replicative aging dogma

This field of research focuses on identifying aging factors, determining how they are toxic, and by what mechanisms they asymmetrically segregate.

Hence and similarly to general theories of aging (see p.10), many different hallmarks of aging and candidate aging factors have been proposed [194,198,214-216](Figure 18). In the following sections of this introduction, we will review a few of these aging phenotypes, starting with changes at the whole-cell level, before describing changes at the organellar and molecular level, and finishing with a focus on a particular aging factor.

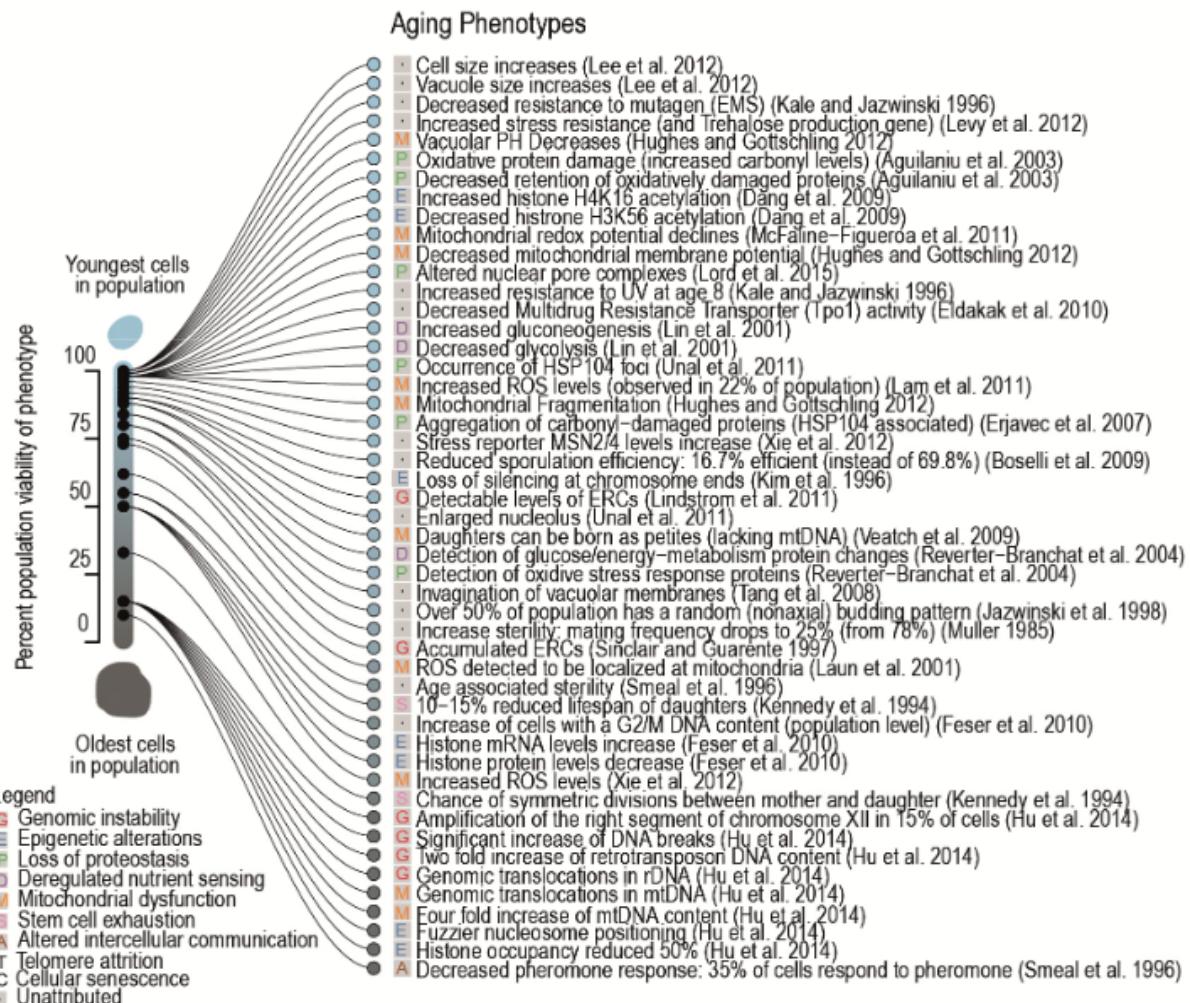


Figure 18: List of replicative aging phenotypes in yeast, sorted by category and by the approximate percentage of viability at which age-related phenotype occurs in the population (Figure from [198])

## 2.2. Bud scars and budding pattern

After each cytokinesis, a chitin ring remains in the cell wall of the mother, at the place of the former bud neck. These “bud scars” can be revealed by simple staining and counted to determine the replicative age of a cell [217] (Figure 19). They accumulate in the mother cell and are not passed to the daughters (daughters only inherit a *birth scar*) [149,218]. Hence, they have been proposed as aging factors by early studies [148,219] as a source of decreased metabolic exchange with the external media and obstruction for new bud necks.

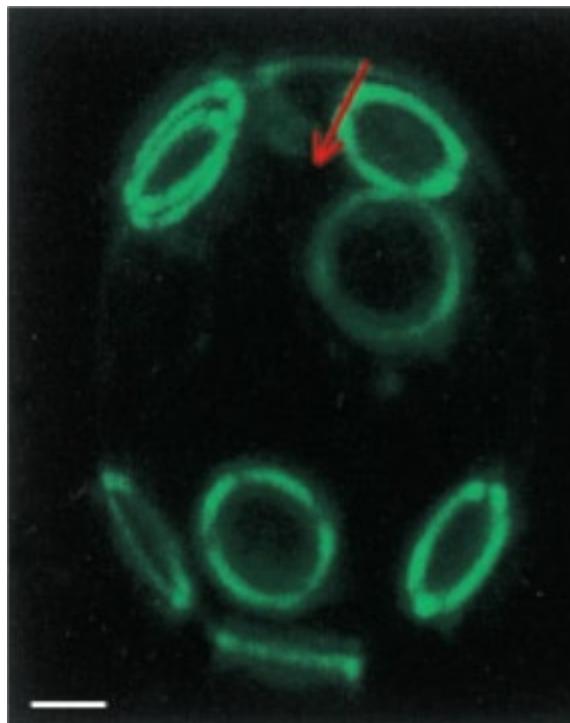


Figure 19: Bud scars of a mother cell, stained with a FITC-labelled wheat-germ agglutinin. Scale bar: 1.25 $\mu$ m  
(From [217], ignore the red arrow))

However, the toxicity of these scars has never been established. Furthermore, it is now known that the ratio between the scars surface and the total surface decreases with age, and artificial increase of cell volume fails to increase replicative lifespan [212].

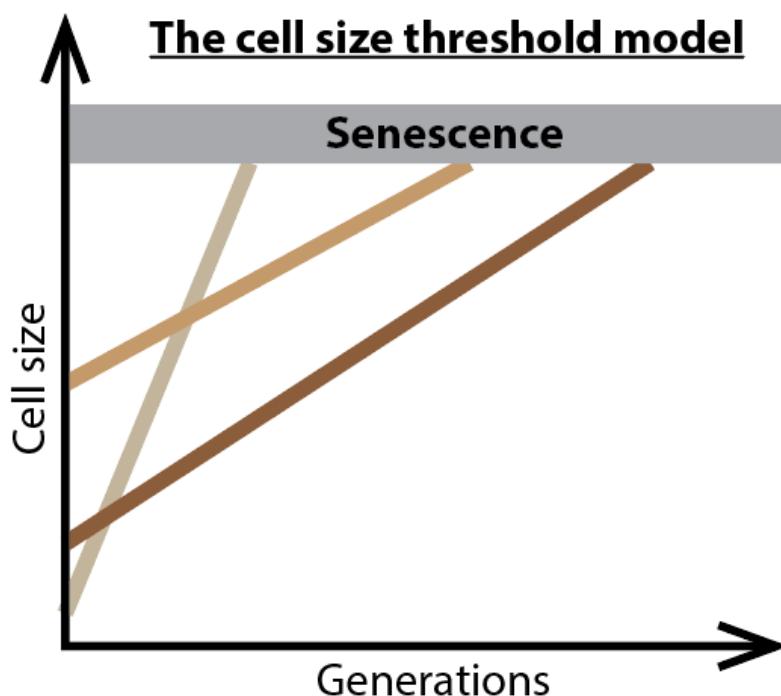
Another hallmark of aging relative to budding is the budding pattern of cells. Indeed, its polarity is normally tightly controlled for the cell to bud in the same direction, but often becomes random with age [220]. Similarly, the morphology of the bud changes during aging, with buds becoming more elongated or more round [221-223]. Yet, these phenotypes are more likely to be a pure byproduct of aging instead of a cause.

### 2.3. Hypertrophy and crowding

Another striking morphological feature of yeast aging is the increase in cell size throughout lifespan, as reported by many studies [221,224-226]. In particular, quantitative measurements show a doubling in average size between birth and death (in apparent surface, not volume) [221,226]. Enlargement is mainly due to the cell constantly producing matter directed to the bud during all the cycle phases, except in G1 (absence of bud) when only the mother grows. Therefore, it is possible to modulate cell size by using mutants of the G1 or by arresting cells in G1 for a certain time.

Remarkably, the cell size of the daughter cell lineage is quickly reset, even from big mothers. Thus, the increase in cell size would be an aging factor if it is toxic. The first study to test this hypothesis used a pheromone to block cells in G1 for a certain time before releasing them and measure their RLS [212]. However, no effect on the lifespan was detected on arrested cells compared to unarrested cells. From then, other studies using artificial volume increase proposed that, on the contrary, the increase of cell size is a driver of replicative aging and named this model the *hypertrophy theory of aging* [227-229]. In line with that, a correlation exists between size and lifespan in some mutants affecting the size [225,230].

Moreover, the replicative lifespan negatively correlates with the size at birth [225,231] among isogenic strains, although this result is debated [234]. The ideas that emerge from this model is that there is a critical size upon which the cell cannot maintain its homeostasis and that what determines longevity is the size at birth and the growth per generation (in other words, how fast the cell will reach this threshold size, Figure 20) [230,232].



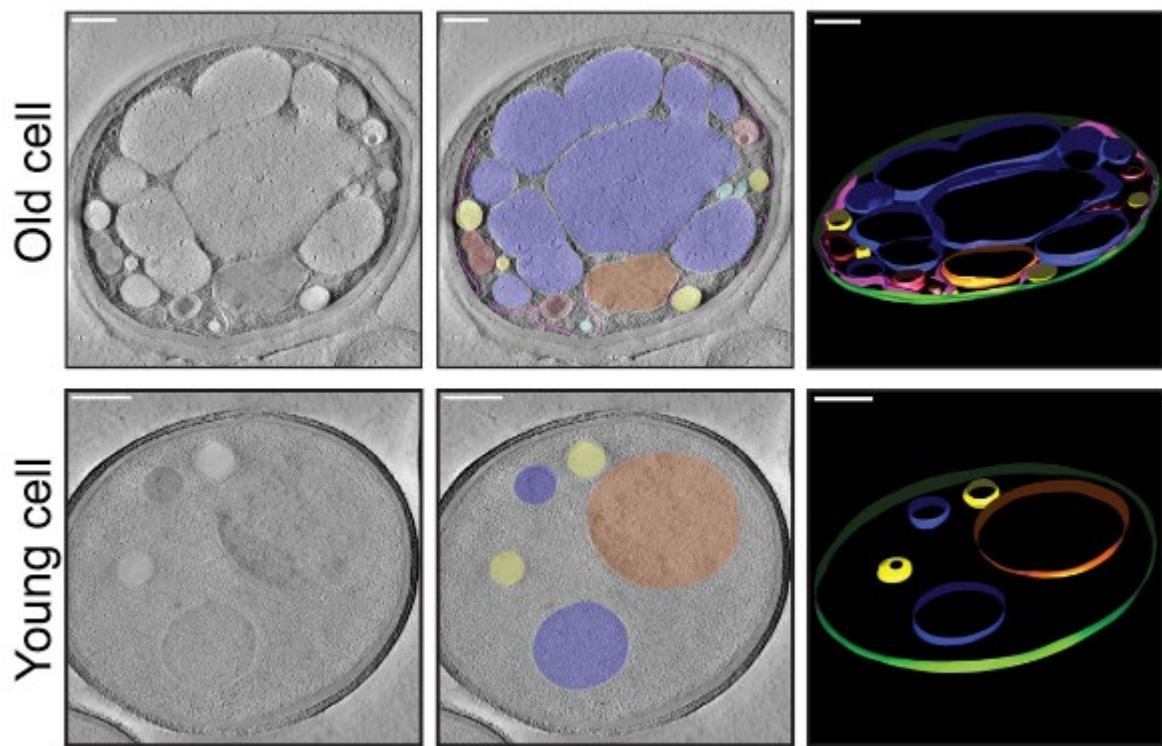
*Figure 20: Schematics of the size threshold model of aging. Cells (3 brown lines) are born at different sizes (among an isogenic population or across mutants) and grow at different rates. What determines lifespan in this model is the time to reach a threshold size.*

On the other hand, other studies show that mother cells die at a wide range of sizes [221,233,234] and that the size at birth does not correlate with lifespan [234,235], contradicting the threshold volume theory. Furthermore, long-lived mutants are not particularly mutants of size [236,237]. Besides, if cells growing faster and with a high metabolic activity have a reduced lifespan (and vice-versa) [234,235] does not necessarily

mean that this effect on lifespan is due to hypertrophy. Indeed, it could be that these accumulate more damages per generation.

Beyond these discrepancies, the major weakness of this theory is that it is mostly correlative and lacks mechanistic insights. Only recently, a study found that the loss of scaling between the protein & RNA biosynthesis and the volume was due to a limitation in the transcriptional and translational machinery [229]. This reduction could be due to the activation of a general stress response, leading to the dilution of the cytosol and the impairment of several processes like cell-cycle progression. However, the G1 arrests performed in this study led to cell volumes way beyond what is usually reached during normal aging (by a factor 2), which questions the physiological relevance of such strong perturbations. Moreover, another study tracking cells throughout their lifespan in physiological conditions found that the cytosolic macromolecular crowding was only slightly reduced - if not unchanged - during aging (although a correlation exists between change in crowding and lifespan) [238].

Apart from the cytoplasm, most organelles also become larger with age, such as the vacuole (see p.38) and the nucleus (see p.45). Insightful studies comparing cellular structures at the Correlative Light and Electron Microscopy between young and old cells [238] showed that the distance between organelles (also called organellar crowding) was strongly reduced during aging.



*Figure 21: Single slices of tomograms without (left panel) and with an overlay to emphasize organelles (middle panel). 3D isosurface rendering (right panel) of tomograms of young or old cells. Nuclei (orange), vacuoles (blue), lipid droplets (yellow), mitochondria (red), ER (magenta), and plasma membrane (green). Scale bars: 500 nm. (Figure from [238])*

This could affect cytoskeletal dynamics or the movement of structures above a specific size (for instance, ribosomes or lipid droplets), their interaction with organelles or the cell membrane. Besides, the increased surface area of organelles could lead to more interaction with their immediate environment (adsorption or molecule exchanges), hence disrupting their homeostasis.

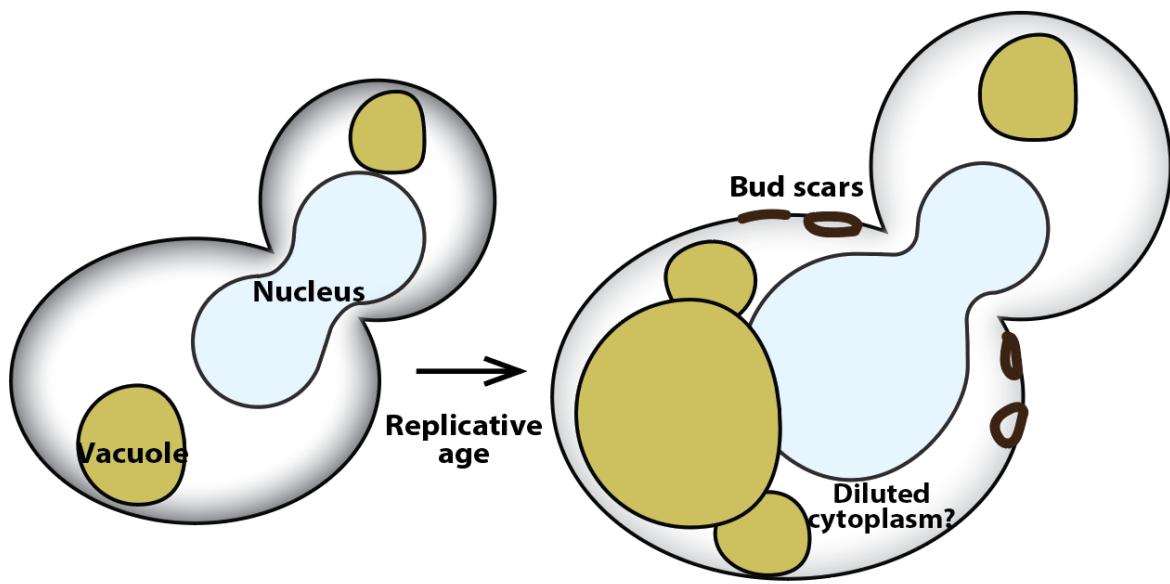


Figure 22: Schematics of the evolution of the physical state of the cell with replicative age. Young cells (left) represent the normal state, while old cells (right) have bud scars, larger cytoplasm and organelles.

Altogether, the fact that cell size increases during aging and resets in daughter cells is consensual (Figure 22). Yet, it is still not clear and openly debated whether the **hypertrophy of the cell and its organelles is a cause, a consequence, or just an unrelated effect of aging** [230,232,233,239]. However, since cell size affects many processes such as nutrient sensing and transcription regulation [240,241] - two processes known to be involved in aging (discussed in later sections) - the hypertrophy theory of aging should not be ignored. Of note, the control of cell and organelles size during aging has recently seemed to regain interest, not only in yeast but also in mammal cells [229,242-244,244b].

#### 2.4. Division time

Early studies already noticed an increasing division time at late ages [211], later validated by timelapse microscopy [221,245,246] (Figure 23).

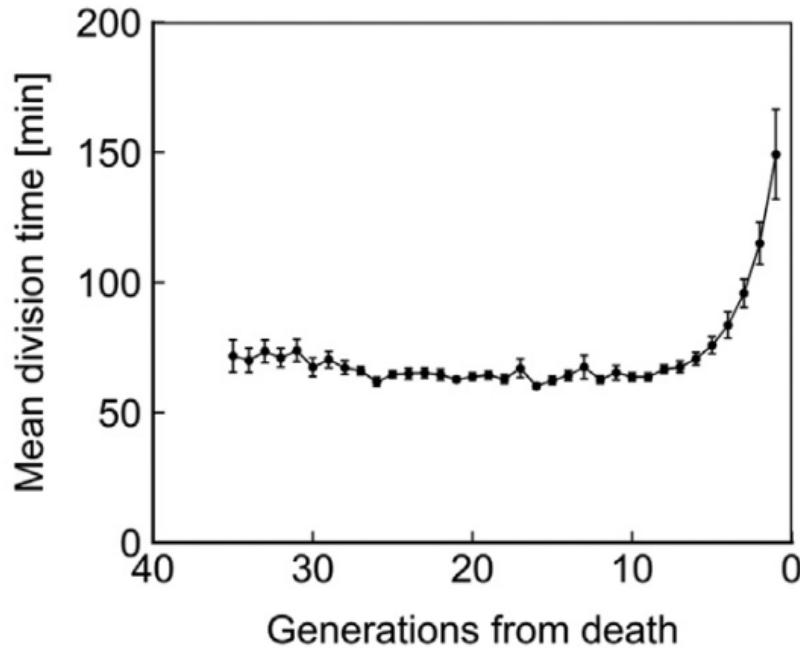


Figure 23: Averaged division time of 50 cells with age, aligned from their death (From [247])

Remarkably, daughters recover a normal division time at birth, unless they are born from an old mother, and in this case, it takes a few divisions of the cell to be back to normal [211]. This suggests that some aging factors affect the division time of the cell and all the cell cycle stages seem affected [248]. For example, the G1/S transition is delayed, which is thought to be caused by an inefficient induction of the G1/S transition gene cluster [248]. In particular, the induction of CLN1 and CLN2 (G1 cyclins in budding yeast) becomes less efficient as the cell approaches death. Similarly, the S-phase cyclin CLB5 is induced less efficiently in old cells. Such defects could be due to accumulation and longer residency in the nucleus of Whi5 (a Cln2 and G1/S transition repressor) of old mothers. However, more insights are needed to understand the causes of this cell-cycle lengthening properly.

Nevertheless, instead of antagonizing lifespan, longer cell cycles could occur to let the aged cells repair accumulating DNA damage [249]. On the other hand, old cells spend more time in G1, making them grow larger (if their growth rate is not decreased) and could drive the hypertrophy described earlier.

Overall, the cell cycle becomes longer with age and resets in daughter cells. Yet, the mechanisms underlying this phenotype are not really identified, nor is it clear as to whether it participates in the worsening of the cell fitness or if it is a compensatory mechanism.

## 2.5. Response to environmental changes and sterility

Another well-observed general phenotype of old cells is their difficulty in responding to environmental stimuli. For example, they become less responsive to sexual pheromones, which leads to sexual sterility [250-252]. Similarly, the ability of diploid cells to form spores strongly decreases with age [253].

Overall, the loss of homeostasis of the general processes described in these paragraphs seems to be the consequences of aging rather than causes (though they could worsen the cell's fitness). Therefore, we must describe which organellar and molecular processes fail to better understand the bigger picture of the aging decay.

## 2.6. DNA damage, mutations, aneuploidy, and loss of heterozygosity

An increase in DNA damages is a conserved hallmark of aging across the tree of life, as seen in the introduction. In yeast, RAD52 foci and other DNA damage markers are more frequent in old cells [248]. Besides, old cells have decreased capacities in repairing single and double-strand breaks (DSB) [254].

In diploid cells, DNA damages, and in particular DSB, can lead to the loss of heterozygosity (LOH) from a gene that becomes homozygous, and it was shown that the rate of LOH increases with age [255-257].

Similarly, missegregation of chromosomes, aneuploidy, and multinucleated cells [226,248], [258], most likely due to spindle pole body problems (such as multiple SPB per cell) [226,248,258].

Of note, telomere attrition, which is also a genomic problem discussed as a potential cause of aging in mammals, is not happening in normal yeasts [259], although telomeres stability has been implicated in yeast aging [260,261].

Nevertheless, it is widely accepted that DNA mutations are not a cause of aging in yeast since these defects are inheritable and that daughters cells do not display them. Besides, the accumulation of DNA mutations does not correlate with age in yeast [88]. Therefore, the genome most likely remains intact until extreme late ages.

Overall, although DNA damage could be a source of aging, DNA mutations, aneuploidy, and LOH cannot be considered as such since they are propagated to the progeny.

## 2.7. Mitochondria

Mitochondria have been a major center of attention for aging, not only in yeast [262-264] but also in mammals [265-269]. These complex organelles play a significant role in the homeostasis of the cell since they control many metabolic pathways, in addition to producing Reactive Oxygen Species (see p.41).

Of note, mitochondria have their own DNA, and some cells lose it (often called  $\varrho\theta$ , or *petite* cells in yeast) [262]. Interestingly, the fraction of petite cells is highly variable (up to a factor of 100) from one strain to the other [257-270], and many lab strains suffer from a high percentage of this defect<sup>1</sup>.

Nevertheless, cells are still viable without mtDNA since respiration is not essential for survival, and the cell can still divide if it can fermentate sugars. However, in this case, the whole lineage is affected [246,262,272,273]; that is, all the daughters also become petite.

As a consequence, the loss of mtDNA does not fit the definition of aging factor. Besides, it has been shown that the probability of this event was not affected by age and was rather purely stochastic [246].

Beyond the petiteness, it seems that mitochondria can be damaged and oxidized during aging but be retained in the mother [274]. Active mechanisms that rely on an actin retrograde transports [275,276] and other molecular complexes [277] are involved in the retention, but how the defective mitochondria are filtered from the healthy one is still unclear. A differential movement speed between oxidized and healthy mitochondria during the actin-mediated transport could explain the filtering [276].

Nonetheless, knowing this risk of transmissible loss of mtDNA, studying mitochondria and aging in yeast can lead to inexact or biased conclusions because of the difficulty to differentiate between transmissible loss of mtDNA and asymmetrical mitochondrial damages. For example, it has been proposed that mitochondria's potential decreases with the cell's age and that the mitochondrial network goes from a tubular to a fragmented morphology to end forming aggregates until their death [264] (Figure 24).

---

<sup>1</sup> This questions the relevance of studying natural phenomena in domesticated yeast strains. Indeed, lab strains have evolved towards a more efficient fermentation over a less efficient respiration and stress tolerance [271], and have a different lifespans compared to natural isolates [10].

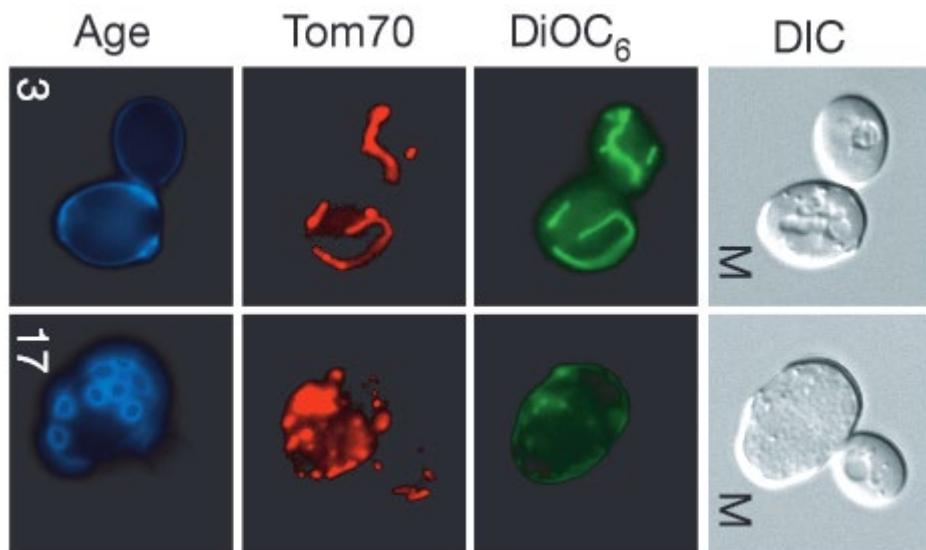
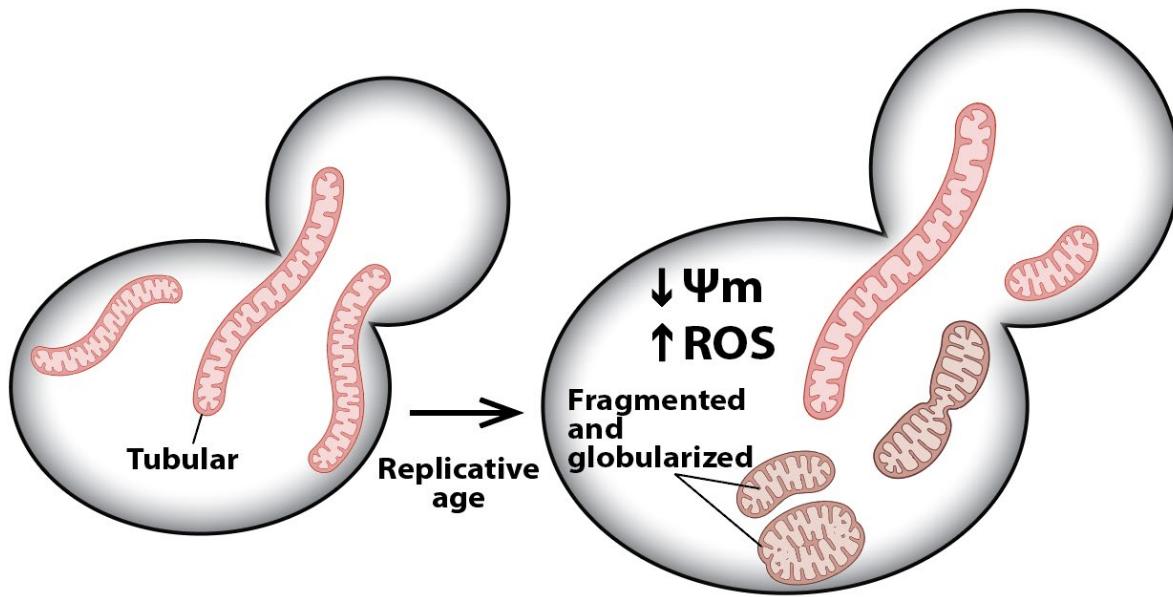


Figure 24: Bud scars (blue), mitochondrial network (red), mitochondrial potential (green), and DIC images of a young (top) and old (bottom) cell. (From [264])

Although mitochondrial fragmentation occurs during respiration [278] and that cells are suspected of going from a fermentative to a respiratory metabolism during aging [279], mitochondrial aggregation can be a sign of petiteness [278]. Thus, this famous study perhaps just describes petiteness (age-independent and transmissible defect) and not mitochondrial aging.

With this being clarified, it is still relevant to understand how mitochondria homeostasis declines with age. Many studies have pointed out the role of iron homeostasis in this phenomenon. Indeed, iron is crucial for the mitochondrial respiratory machinery, and defects in the iron-sulfur (Fe-S) cluster (a form of iron in the cell) pathways have been described as causal for petiteness and mitochondrial decline during aging. In line with that, reinforcing these pathways with mutants leads to increased longevity [262,274,280,281]. Similarly, heme metabolism has been described as a critical factor of mitochondrial decay and aging, with its increase leading to higher RLS [282,283].

How defective mitochondria are toxic to the cell remains unclear since these organelles are central to many metabolic pathways of the cell. The most proposed mechanism is that damaged mitochondria could contribute to the loss of the redox homeostasis by producing more ROS [284,285], which in turn could damage proteins, DNA and lead to genomic instabilities (see p.41). Besides, since the Fe-S clusters are often co-factors of DNA repair enzymes, declining mitochondria could drive genomic instabilities [255].



*Figure 25: Schematics of the evolution of the mitochondrial state of the cell with replicative age. Young cells (left) represent the normal state with functional tubular mitochondria, while old cells (right) have fragmented and globularized mitochondria with a reduced potential.*

Altogether, mitochondria decline with age and are partly causal for aging Figure 25, but it is difficult to distinguish this age-related decline from the accidental loss of mtDNA present in the laboratory strains. Furthermore, the precise mechanisms of rejuvenation and toxicity remain unclear.

Interestingly, mitochondrial functions have been extensively linked to the homeostasis of another cell organelle: the vacuole [264,280,281,286,287].

## 2.8. Vacuole, cytosolic pH, and mitochondria homeostasis

The vacuole in fungal is an acidic organelle that possesses degradative and storage capacities, in addition to its role in pH- and ion-homeostasis [288,289]. In this sense, it has many similarities with the vacuole in plants or with the lysosome in mammal cells. In the context of aging, this organelle enlarges as the cell ages [221,238,290]. It has been proposed that the morphology of the vacuole has an impact on the replicative lifespan since mutants deleted for vacuolar fusion proteins produce fragmented vacuoles and have a decreased lifespan [290,291], while overexpressing these proteins lead to an increased lifespan. However, this result should be taken with caution since these proteins also affect sterol and ergosterol biosynthesis and could thus influence longevity through other pathways.

Apart from their morphology, the pH of the vacuole has also been described as involved in aging [264,280]. Indeed, acidity in the vacuole is high in young cells and starts dropping very early, whereas daughter cells recover an acidic vacuole. The vacuolar pH is mainly controlled by the V-ATPase, a proton pump encoded in part by VMA1. Overexpression of Vma1 delays the pH drop in the vacuole of old mothers and increases lifespan, placing vacuolar alkalinization as an aging factor.

Interestingly, it turns out that the pH increase in the vacuole is responsible for the mitochondrial declines described in the previous section. Indeed, changing the acidity of the vacuole also alters its ability to store (through proton-dependent amino-acid transporters) essential or toxic amino acids for the mitochondria [264,281].

These results seem to indicate that vacuolar homeostasis, and in particular vacuolar pH, can impact other organelles' homeostasis. However, why this pH fluctuates with aging is still not consensual. It has been proposed that the cell's cytosolic pH increases with age due to an enrichment of Pma1 (an efflux pump of protons) at the membrane of old cells and that daughter cells recover a normal Pma1 concentration and a normal pH. Therefore, the lack of protons in the cytoplasm would, in turn, lead to a loss of acidification of the vacuole since less protons can be pumped into it.

However, this study was, in reality, measuring the cortical pH of the cell and not the cytosolic pH. Indeed, more recent studies tracking cells throughout their lifespan described contrarily that the cytoplasmic pH of cells was dropping with age (from 7.1 to 6.7) as well as the isoelectric point of the proteome [238,292]. They also propose that the alkalinization of the vacuole [293] could be due to a loss of functional V-ATPases, as suggested by a large-scale study on protein stoichiometry [294].

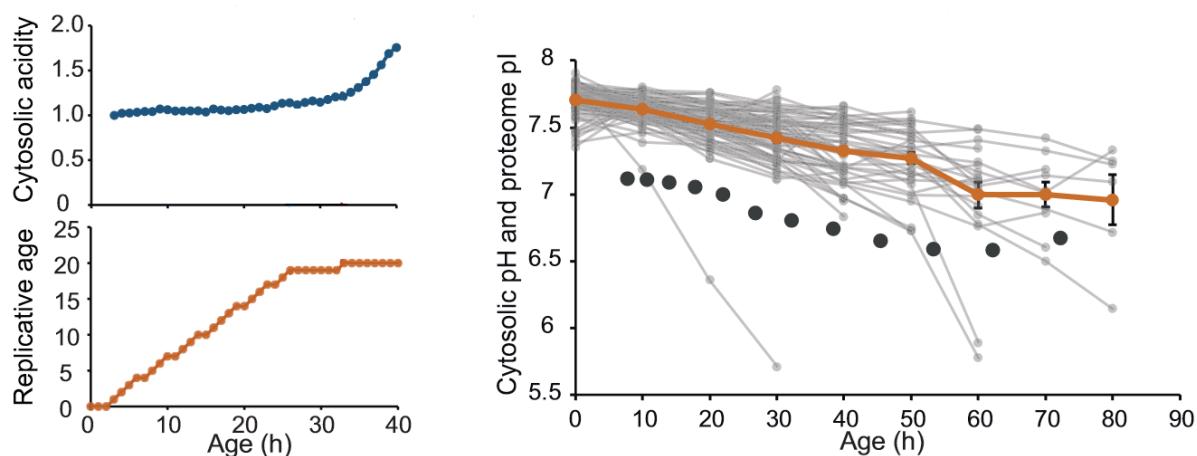


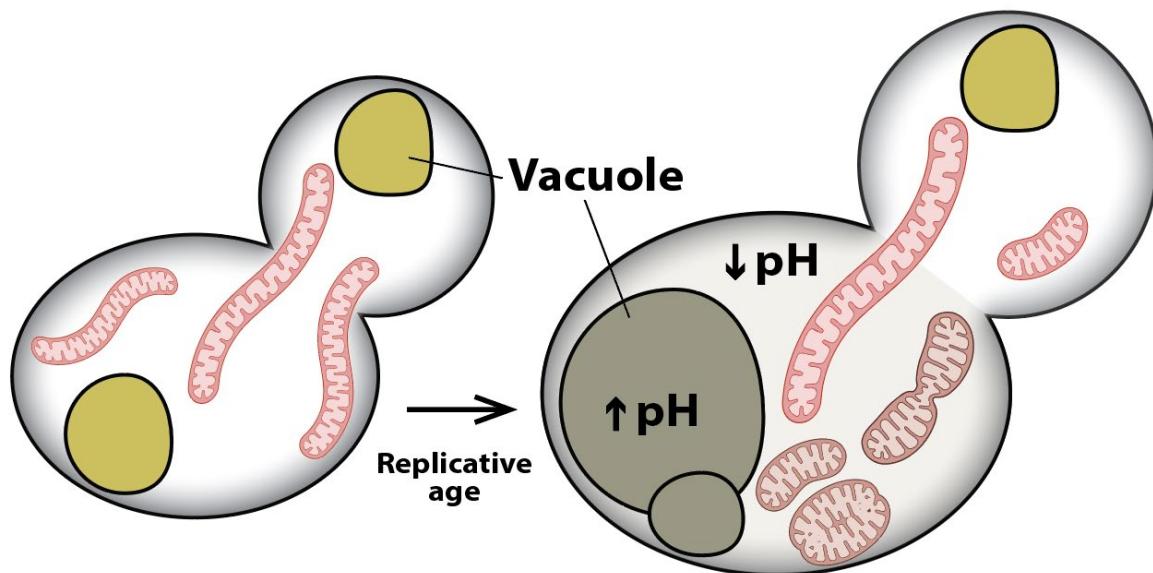
Figure 26: (Left)/ Trace of a single cell's cytosolic acidity and replicative age along time. (Right)/ Cytosolic acidity (single traces in grey, mean, and SEM in orange & black) and estimated proteome pI (black dots) along time.

Further suggesting that vacuolar and cytosolic pH are linked and influence lifespan, a recent report shows that overexpressing VPH2 (a protein required for assembly of V-ATPases) is delaying the cytosolic pH drop during aging, as well as increasing lifespan [292]. Hence, it could be that the loss of vacuolar homeostasis drives the drop in cytosolic pH, and not the contrary.

If vacuolar pH seems to influence lifespan, it is ambiguous for cytosolic pH. Indeed, the drop in cytosolic pH is a late event in life and is not predictive of longevity [238,295]. It could be the direct effect of a metabolic slowdown and lower energy availability occurring in late ages [226,295] since the major proton pumps work with ATP. In line with that, starvation or lower energy states of the cell can cause acidification of the cytoplasm [295-297].

On the other hand, the identity of the cytosol and vacuole are converging during aging, both in size and in pH, which could participate in the multiple problems enunciated previously, such as the proper compartmentalization of amino acids and damaged proteins.

Furthermore, any change of intracellular pH (in any direction) could profoundly impact cell physiology. Indeed, pH influences proton gradient and proton-dependent transports (see previous paragraphs) but also redox potential, enzyme activity, protein folding and solubility, phase separation [296], and many other general processes [298].



*Figure 27: Schematics of the evolution of the vacuolar, mitochondrial, and acidity state of the cell with replicative age. Young cells (left) represent the normal state with small vacuoles and functional tubular mitochondria, while old cells (right) have larger and less acidic vacuoles, which lead to fragmented and globularized mitochondria.*

Overall, intracellular pH modification is a consensual hallmark of aging and is reset in daughter cells. Vacuolar alkalinization seems to be an aging factor since correcting it

leads to an increased lifespan, but it is less clear if cytosolic pH is a cause or a consequence of aging (Figure 27).

## 2.9. Reactive Oxygen Species

As discussed earlier (p.11), the link between Reactive Oxygen Species (ROS) and aging has been extensively debated since 1956 [54] in many different organisms, and the conclusions are not consensual.

In yeast, it has been shown that old cells have increased ROS levels [245,285,299]. Moreover, mitochondria from old mothers have a lower redox potential and produce more ROS compared to their daughters [275]

However, some reports show that cells with increased ROS live longer under particular conditions [300] or that the redox state of the cell does not correlate with its lifespan [295]. Besides, mild exposure to oxidative stress is shown to have a hormetic effect by increasing the lifespan [80,301]. **Therefore, ROS could have a hormetic role and whether or not their natural accumulation is causal or consequential of aging, and protective or toxic, is not clear [302,303]<sup>2</sup>.**

## 2.10. Carbonylated proteins and protein aggregates

Since ROS levels are high in old cells, one could wonder how this could affect proteostasis. Indeed, upon oxidation, proteins can be cleaved or carbonylated [304] (among other modifications), especially by ROS during aging [305].

Carbonylated proteins and other denatured proteins tend to form aggregates. They are known to interact with chaperon proteins (such as Hsp104 [306]) in charge of the disassembly of these aggregates. Interestingly, carbonylated proteins and Hsp104 foci are found in aging cells [307-312].

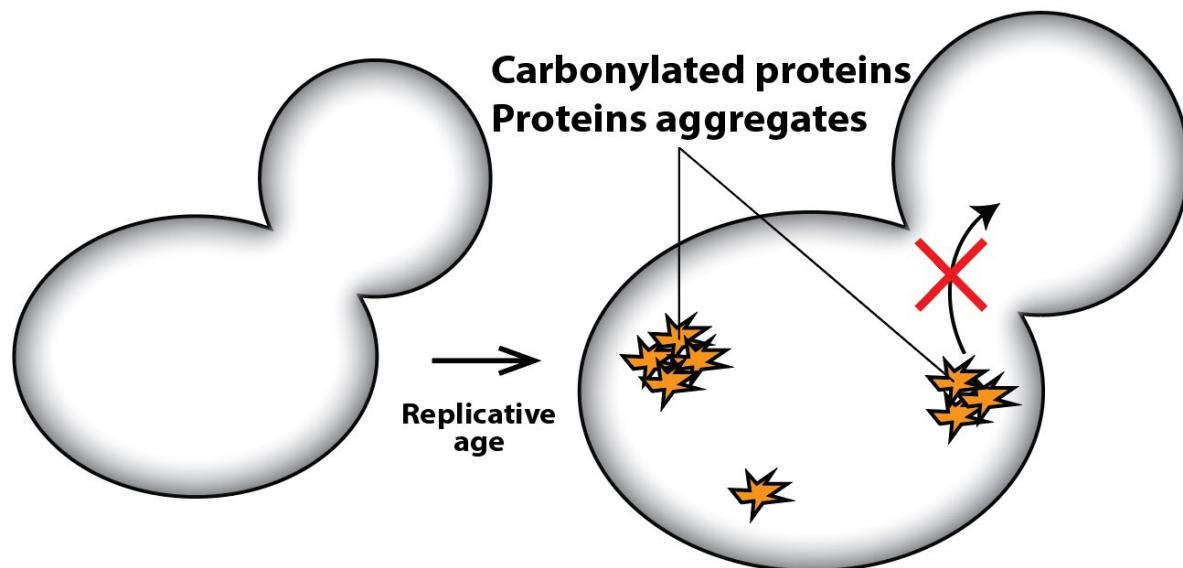
These proteins segregate asymmetrically in the mothers, and different models have come up to explain this asymmetry. For example, it has been proposed that this asymmetry is dependent on the actin cytoskeleton and the polarisome, where protein aggregates would be retrogradely transported from the bud to the mother [308,311,313,314]. On the other hand, the geometry of the cell division (growth directed to the bud and low diffusivity between the mother and the bud) and the slow and random movement of these aggregates could explain by themselves the lower amount of aggregates in the bud [310,315].

---

<sup>2</sup> I know, it starts to be repetitive

Besides, it has been shown that Hsp104 is required for asymmetric segregation and that mutants lacking this gene are short-lived. Therefore, it is still unclear if Hsp104 aggregates (and other protein aggregates) act as an aging factor or if they are protective for the cell by sequestering otherwise toxic content [195,316].

Very recently, P-bodies (protein aggregates formed by RNA-binding proteins), known to form under stress or low pH, have been found enriched during aging. Interestingly, their quantity correlates with the remaining lifespan, and cells that leak P-bodies to their bud tend to live longer [292].



*Figure 28: Schematics of the evolution of the presence of protein aggregates with age. Young cells (left) are devoid of these aggregates, while old cells (right) have enriched levels of them, which are not passed to the progeny.*

Overall, protein aggregates accumulate in old cells and are not passed to the daughter (Figure 28). However, if these aggregates are toxic or if they participate in the maintenance of cell homeostasis is not consensual.

## 2.11. Proteostasis and protein stoichiometry

As seen previously (p.13), protein homeostasis is implied in the aging of complex eukaryotes. In yeast, it is mediated by chaperones (as discussed in the previous paragraph) but also by proteasomal activity and autophagy.

A comprehensive study that performed proteome- and transcriptome-wide analysis of aging mother cells [294] showed that the stoichiometry of protein complexes is affected during aging.

Though the cause of this mismatch is not fully explained, the accumulation of P-bodies during aging (detailed in the previous section) - sequestering certain mRNA away from the translation machinery - could play a role in this loss of stoichiometry.

Moreover, old cells also show a decoupling between protein and transcript levels for biogenesis machinery genes such as translation regulation, ribosomes, and tRNA synthesis genes [294].

Interestingly, mutants with increased proteasomal activity have one of the longest RLS described in the literature so far [236,317], much clearer than mutants with improved chaperon activity. This result is to be nuanced since they are also thought to have fewer rDNA instabilities and less ERCs (another aging factor, discussed later) [318] and could therefore act on two different causes of aging simultaneously. However, increasing the proteasomal activity could solve the loss of stoichiometry and the imbalance between proteins and transcript, which would explain the longevity of such mutants.

Similarly, deletion of ribosomal proteins, translation initiation factors, or caloric restriction, all affect protein translation and lead to robust lifespan extension [167,236,319]. Interestingly, pre-ribosome contents have also been noted as upregulated during aging in different studies [226] but do not show loss of stoichiometry, suggesting that they are still functional and active.

**Altogether, loss of proteostasis and excessive translation are a strong hallmark of aging - though all the underlying mechanisms are not yet defined.**

## 2.12. Epigenetic alterations

In yeast, epigenetic control of DNA expression is solely mediated by the chromatin state, which is mainly controlled by histone content and acetylation since there is no known DNA methylation pattern in this organism [320] (as opposed to drosophila or mammals).

During aging, acetylation of H4 at lysine 16 (H4K16) increases, notably at the rDNA and subtelomeric regions, and deacetylation of H3K56 decreases [321]. Of note, the H4K16 acetylation state is mediated by the sirtuin Sir2, an NAD<sup>+</sup>-dependent histone deacetylase, which levels decrease during aging [321]. In line with that, deletion or overexpression of Sir2 greatly reduces or increases lifespan (respectively), though this effect is also due to modified recombinations in the rDNA (discussed later) [99,322]. Similarly, specific deletion in the chromatin-modifying SAGA complex greatly enhances longevity [323].

Additionally, histone levels are also reported as going down during aging, which could be partly due to increased DNA damages in old cells (see p.35) since it can trigger histone degradation [99,258]. This phenotype does not seem to be a pure byproduct of aging but to be part of its cause, since increasing histone transcription, translation, or blocking histone degradation, increases lifespan [99,258] while decreasing the amount of histone negatively affects lifespan [324].

However, these results are not consensual since several studies report an increase of histones H2B levels with aging [226]. In addition, partial deletion of histones H3 and H4 (hence, theoretical decrease in histone content) promotes longevity, though this has been attributed to hormetic activation of the chromatin architecture defect response [325].

Moreover, not only are the histone state and content thought to be modified during aging, but also the nucleosomes, which show a decreased abundance in the chromatin as well as increasing mispositioning [326]. Yet, whether this is an aging factor is contested because deletion of ISW2, a chromatin remodeling enzyme, decreases nucleosome levels and increases lifespan [327].

Contrariwise, a recent study using ATAC-seq assays showed that the nucleosome occupancy was globally unchanged with age [318] and that the previous measurements were most probably biased by the dead cells from the population (since dead cells lose their nucleosomes).

Despite these discrepancies, pervasive transcription of non-coding RNA [318,328-330] and general transcriptional induction of all genes [326] seems to happen during aging.

**Altogether, epigenetic alterations and loss of silencing appear to be a hallmark of aging, even though the causality of these phenotypes relative to aging is debated.**

### 2.13. Nutrient sensing and metabolism

Nutrient sensing and metabolic pathways are the most conserved players in aging (see p.18). In yeast, aging is accompanied by a change in carbon metabolism, with cells decreasing glycolysis while increasing gluconeogenesis, energy storage, and respiration [279,303,331]. The fact that cells grow larger with age leads to a decrease in surface-to-volume ratio, and it has been proposed that fluxes with the environment - such as nutrient uptake - could be reduced and trigger starvation cues [279,332,333]. This is supported by proteomic analyses in which proteins of the starvation response are enriched in old cells [294]. Besides, the

growth rate of cells decreases in late ages [226], which could be a consequence of nutrient availability.

However, and as for many aging phenotypes, if this is a cause of aging or just a consequence remains unclear.

Besides, it seems that the nutrient-sensing pathways such as TOR are somewhat hyperactivated during aging. In line with that, deletion of TOR1 or SCH9 (a protein kinase downstream of TOR1) increases lifespan [150], as well as caloric restriction [157,231,334]) or caloric restriction mimetics in strains deleted for a glucose transporter [334]. Of note, a meta-analysis argues that caloric restriction does not elicit such a robust effect on lifespan in yeast and that the previous positive results were due to analysis biases [335].

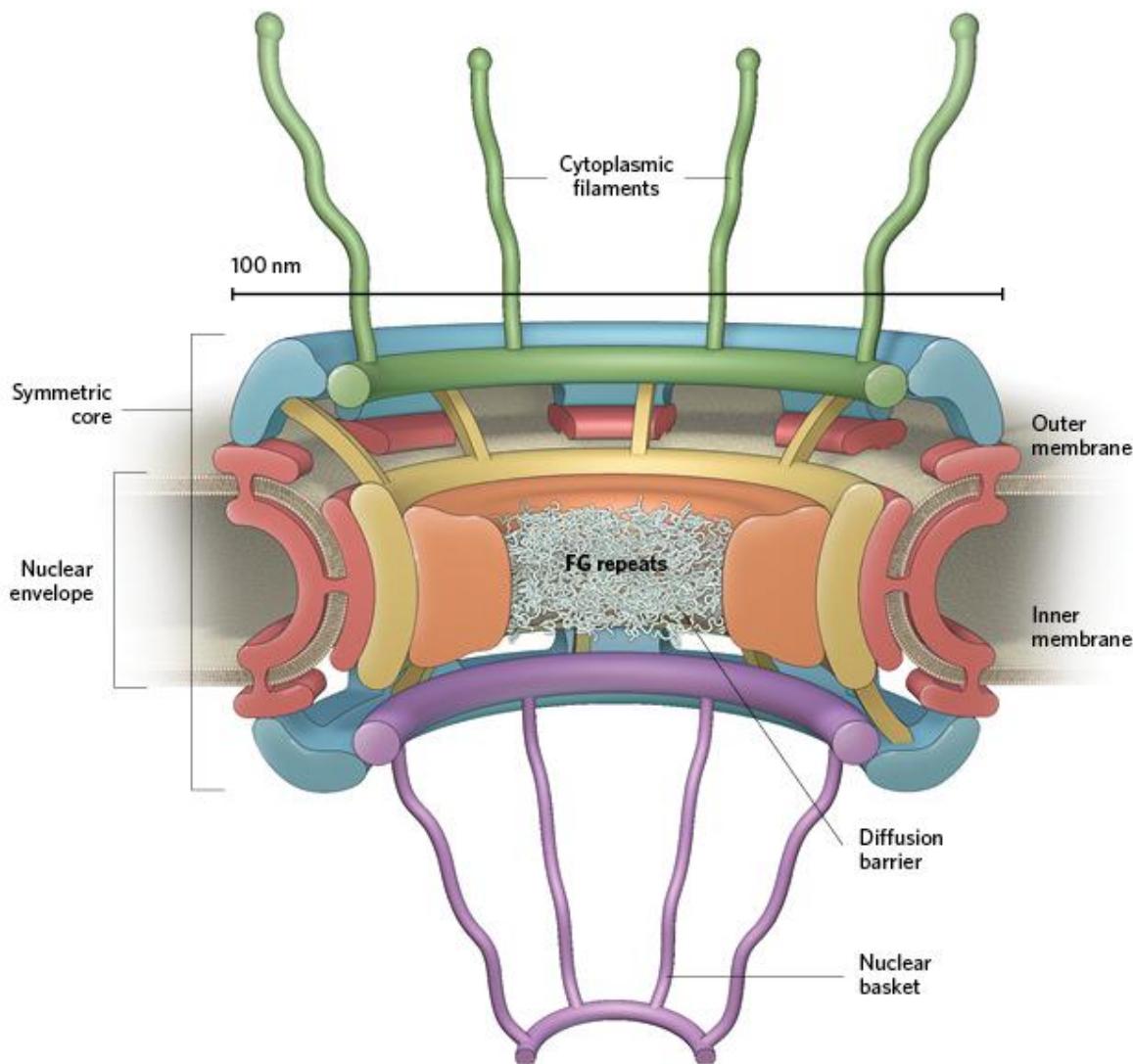
To explain their lifespan extension properties (if they exist), it is proposed that since these pathways control ribosome biogenesis and protein translation, their deregulation could be linked with the loss of protein stoichiometry and proteostasis described in the previous section. Moreover, they regulate histone remodeling [325,327] as well as the rDNA stability [336], which has been extensively pointed as involved in yeast aging (discussed p.48).

**Altogether, nutrient sensing and metabolism seem to be central in the modulation of aging and are deregulated during this process. Yet, their strong pleiotropy makes it difficult to conclude about their effect on specific pathways.**

## 2.14. Nucleus and nuclear pores

The karyoplasmic ratio (also called nuclear-cytoplasmic ratio, abbreviated N/C ratio here) is the quotient of the nucleus volume divided by the total volume of the cell. This value is very tightly regulated under normal conditions and conserved in many contexts, not only in budding yeast but also in *S. pombe*, mammal cells, and Xenopus [337-340]. Its homeostasis is thought to play a role in the global homeostasis of the cell since it dictates, and is dictated by, the flux of matters between the two major compartments of the cell [339,340].

Despite this tight regulation, the size of the nucleus increases with age, and at a faster rate than the cytoplasm [226]. This increase of the N/C ratio is accompanied by an increase of nuclear content, ranging from histones to fluorescent proteins with a simple Nuclear Localization Signal (NLS) [226,341]. Of note, a known regulator of the N/C ratio is the transport of matter in and out of the nucleus (called nucleocytoplasmic transport)[339,340], which mainly goes through the Nuclear Pore Complexes (NPCs)(Figure 29).



*Figure 29: Schematics of one nuclear pore complex (Figure from [342])*

The NPC is one of the largest complexes in yeast in terms of protein numbers and size [343] and has several crucial functions required for cell homeostasis. It is primarily known for allowing exchanges of proteins and RNA between the cytosol and the nucleoplasm, and this exchange is mediated by karyopherins (importins and exportins). However, NPCs also have gene regulation functions with their ability to interact with chromatin and therefore promote efficient transcription [344] by bringing the transcriptional, splicing, and nuclear export machinery into close functional context [345,346]. Similarly, NPCs, and in particular their nuclear basket, can filter pre-mRNA from mature mRNA and therefore play a role in splicing quality control [347].

Along the same line, they also seem to be involved in DNA repair (including rDNA) [348-351], and more generally, they can exert spatial control on DNA by tethering it to the nuclear membrane.

In yeast, a growing body of evidence suggests that NPCs also play a role in cell identity between the daughter and the mother. In fact, they get specifically deacetylated in daughter cells which then impact other cellular processes such as the cell cycle [352,353]. Moreover, studies suggest that old or damaged pores are kept in the mother [194,354,355], further contributing to the segregation of biological material between mother and daughter and to the asymmetry in general.

In the aging context, it has been established that the NPC homeostasis was destabilized, not only in yeast but also in other cell types (mitotic and non-mitotic) [341,356-358]. For example, the NPCs stoichiometry is one of the most affected complex during yeast replicative aging [294]. This could partly be explained by the fact that some components of the pores, such as the core scaffold, are very long-lived, while others (such as FG-Nups) have a high turnover [359]. Consequently, a change in protein homeostasis would differentially affect these different components.

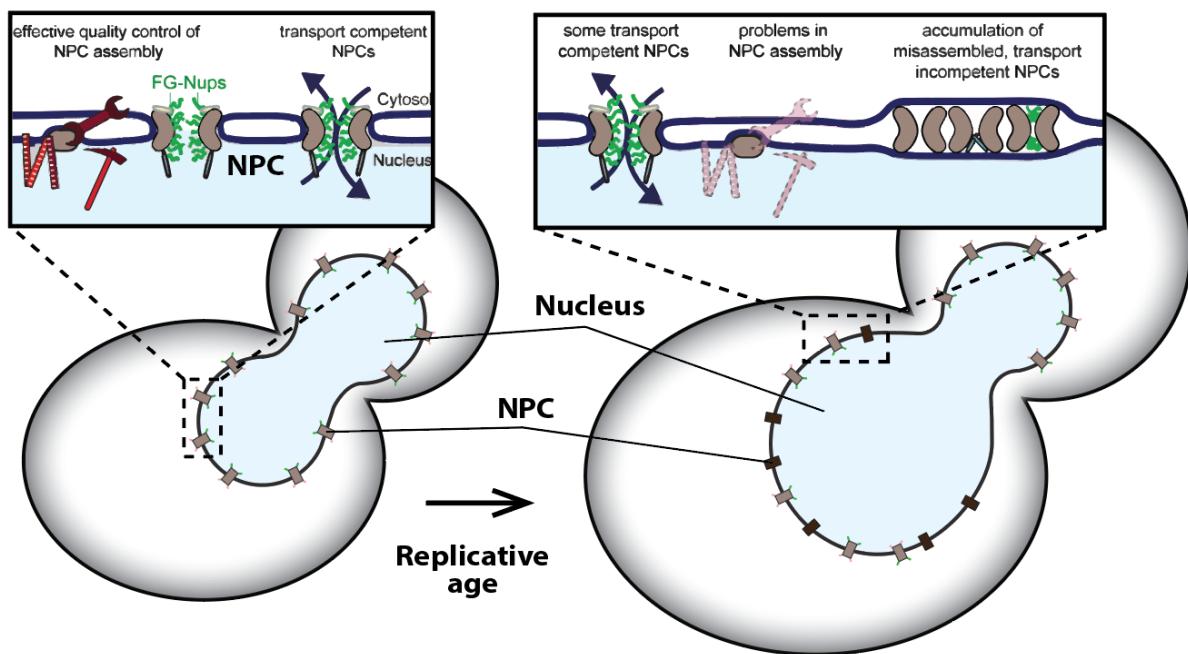
Moreover, NPC assembly, quality control, and maintenance machinery are also decreasing during aging [341], which leads to more pores being misassembled and not cleared from the nuclear envelope of old mothers, thus accumulating. Indeed, signals of NPC assembly problems are detected in old cells three times more frequently than in young cells. In addition, it seems that old misassembled pores are covered by the nuclear envelope (NE), leading to local herniation of the NE [341].

Notably, a decrease in several nucleoporins (components of the NPCs) was correlated with a shorter lifespan, while deletion of some other nucleoporins led to increased longevity [341,360,361]. Furthermore, affecting specific nucleoporins can, in turn, have beneficial effects by tuning down other pathways implied in aging, such as translation [360,361].

If NPCs lose their integrity during aging and if this loss is toxic to the cell, how to explain the daughter cell rejuvenation? Several studies show that NPCs from the mother are not passed to the daughter because of a diffusion barrier present during mitosis at the nuclear membrane (discussed p.45) [354-356]. Hence, old and damaged pores can be retained in the mother, and daughter cells are born with new functional pores.

Then, how are the NPCs damaged during aging? It has been proposed that some nucleoporins are carbonylated during aging (due to a high level of ROS, for example) [362], but recent studies and modeling suggest that it might not be the case for several nucleoporins [341,363].

This destabilization could then come from the binding to the pores of rDNA circles (discussed later), which lead to the loss of the nuclear basket from the NPC [358]. In line with that, proteomics on old cells found that several proteins from the nuclear basket were missing (though, not precisely the same) [341,357]

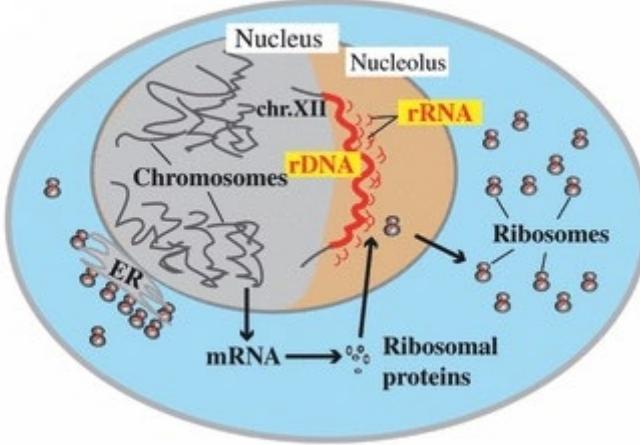


*Figure 30: Schematics of the evolution of the nuclear state of cells with replicative age. Young cells (left) represent the normal state with a normal N/C ratio, functional pores, and active NPC assembly and quality control machinery. Old cells (right) have a larger nucleus with defective or remodeled pores (adapted from [238])*

Altogether, NPC remodeling and destabilization can be considered as an aging factor (Figure 30). They are increasingly present in old cells but not in daughters, and alteration of the NPCs' stoichiometry can positively affect the lifespan. Besides, this loss of NPC integrity could explain the increased N/C ratio and compartmentalization in the nucleus.

## 2.15. Nucleolus and rDNA

Another source of deregulation in the nucleus could arise from the nucleolus. This crescent-shaped sub-compartment of the nucleus contains the ribosomal DNA (rDNA) and is the place of rRNA transcription, processing, and parts of the ribosomal assembly [364,365] (Figure 31).



*Figure 31: Schematics of the nucleolus place and role in the cell. This crescent shape subcompartment (brown) of the nucleus (grey) contains the rDNA (thick red line). rRNA (small red lines) are also transcribed in the nucleolus. Besides, the mRNA of ribosomal proteins is exported to the cytoplasm to be translated. Then, the ribosomal proteins are imported to the nucleolus to form mature ribosomes. Then, ribosomes are exported to the cytoplasm to translate various proteins, mostly at the endoplasmic reticulum (ER)(From [366])*

In the context of aging, it is found to increase in size, to become fragmented, and the transcriptional activity happening there is also strongly upregulated [226,258,367,368]. Moreover, more DNA recombinations happen at this particular locus, which is thought to be causal for aging (see following paragraphs).

This makes it not only a hallmark of aging but also a significant center of aging deregulation since it is where the ribosomes biogenesis starts, and that ribosomes are highly implied in aging (see p.44).

Interestingly, this hallmark is conserved across many organisms and predictive of longevity [367,369,370].

For these reasons and because we will partly focus on the rDNA in the results part, a particular elaboration will be made in the next section.

#### 2.15.1. The architecture of the rDNA locus

In most eukaryotes, the rDNA locus is composed of long tandem repeats [371,372]. In yeast, it is made of 100-200 repeats [373] of size 9.1kb and located on chromosome XII. The number of repeats is not fixed and can vary between mutants, strains, and culture conditions [374]. Importantly, each repeat comprises a 35S and a 5S gene coding for the precursors of the ribosome subunits. The RNA Polymerase I transcribes the 35S (abbreviated Pol I here) to form different rRNA precursors after processing, while the 5S is transcribed by Pol III.

The metabolic cost of rRNA and ribosomes synthesis is immense since 60% of all the ribonucleotides are consumed by Pol I for the sole transcription of the 35S, and 50% of the total activity of Pol II is dedicated to ribosomal proteins. [375,376]. Hence, the transcription of a single rDNA gene is not sufficient to allow fast cellular growth, which explains why the rDNA locus is composed of several dozens of repeats [377]. Interestingly, the number of rDNA repeats can rapidly adapt by recombinations (in a matter of tens of generations at the population level [240]), depending on the environment [378] (which can be considered as an evolutive adaptation). Besides, the repeats are transcriptionally controlled, with approximately half of them being transcribed by Pol I at a given time and the other half being silenced by chromatin organization [377,379-381]. Altogether, this shows that the rDNA locus is very adaptable at the timescale of the cell division but also in the longer term to ensure tight regulation of the rRNA levels and fit cellular demands. This also means that the ribosome biogenesis (and in particular, rRNA transcription), is very dependent on the nutrient availability in the cell [336,382-384]. For example, the TORC1 complex (whose repression increases lifespan, see p.44) controls many steps of the ribosome biogenesis and, in particular, the Pol I transcription of the 35S [385,386] and the Pol III transcription of the 5S [387].

These particular properties are in part conferred by sequences present on two intergenic spacers (IGS1 and IGS2) that lie in between the 35S and the 5S (Figure 32).

For example, in the IGS1, the bidirectional promoter known as E-pro, transcribed by Pol II and silenced by Sir2 (Sir2 is a histone deacetylase, see p.43), is thought to regulate rDNA recombination by modulating the cohesion of sister chromatids [329,330] (discussed in the next section). Each repeat also has an origin of replication (or Autonomously Replication Sequence, ARS) for efficient replication of the whole rDNA locus. In the IGS2, a Replication Fork Barrier (RFB) sequence, active when Fob1 binds to it [388-390], prevents collisions between the transcription and the replication machinery.

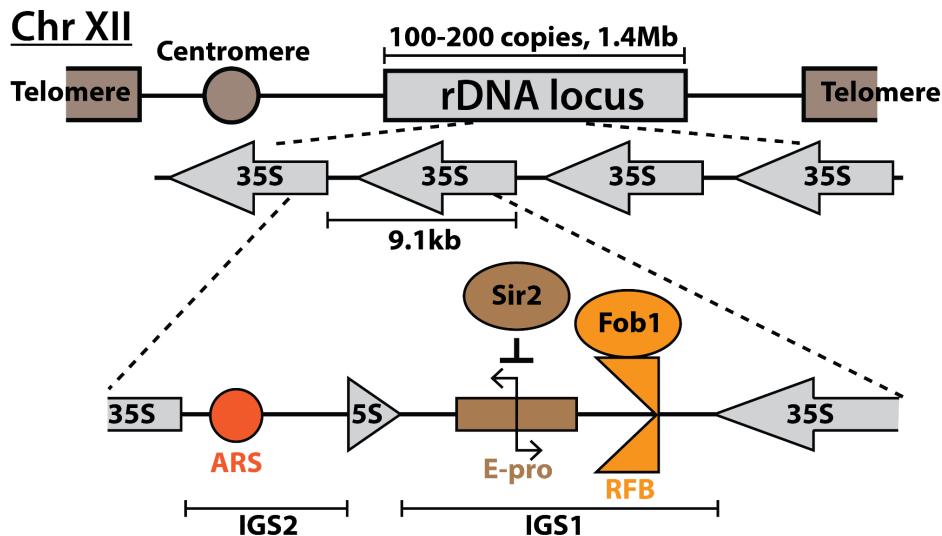


Figure 32: Schematics of the architecture of the rDNA locus

### 2.15.2. rDNA silencing

An important regulatory part of the rDNA passes through the Pol II transcription of the IGS1 and IGS2. Initially, these spacers were called NST1 and NTS2 (for Non Transcribed Spacer). However, it is now known that they are naturally transcribed by Pol II, producing non-coding RNA (ncRNA) [391-394], and in particular from the E-pro promoter. IGS1 and IGS2 are mainly silenced by Sir2 [389,395] via its histone deacetylase function, and of note, **Sir2 does not directly regulate Pol1 transcription or silencing** [396,397]. Interestingly and non-intuitively, the rDNA silencing of Pol II requires Pol I transcription [394,398,399], though only a part of the mechanism is understood ([400] for more details).

As to Pol I transcription, the repeats are transcriptionally controlled, with approximately half of them being transcribed by Pol I at a given time and the other half being silenced by chromatin organization [377,379-381] (as enunciated previously). Adding more repeats does not modify the number of active genes (*i.e.*, not silenced), and modifying the number of active genes does not modify the number of genes being transcribed. Altogether, this suggests that rRNA synthesis is controlled by the chromatin state of the repeats and by the initiation of Pol I onto them [381].

Nevertheless, what controls these two processes remains partially unknown. Relative to the chromatin state, its control is very complex and will not be detailed here. Briefly, several complexes are known to affect the acetylation, methylation, or ubiquitination of different histones at the 35S, such as Nop1 or Torc1 [401-403].

Concerning the activation of Pol I, several proteins such as Rrn3 and Net1 are known to promote its transcription of the 35S gene [404-407].

However, more research needs to be done to unveil the exact molecular mechanisms of these regulations and understand how the cell decides to transcribe some repeats and silence others.

**Altogether, the transcription and silencing of the rDNA repeats are tightly controlled, not only in its coding sequences but also in the intergenic one. Interestingly, a link exists between the transcription of the repeats and their ability to change their number.**

#### *2.15.3. rDNA plasticity*

The rDNA is the most unstable region of the genome because of its highly repetitive nature, its high recombination rate, and because it is heavily and constantly transcribed (except in anaphase) [374,408]. This high recombination rate gives the ability to the rDNA array to modify its number of copies to fit the cell needs (in a matter of hundreds of divisions) but can be detrimental and toxic in specific contexts, such as replicative aging.

The recombination mechanisms are well described. Indeed, recombination occurs after a DSB, most of them (but not all [409]) being caused by a stalling of the replication fork at the RFB formed by Fob1 [388-390,410] (Figure 33, top).

The repair can be done (mainly) in three ways: equal sister chromatid recombination, unequal sister chromatid recombination (USCR), and intrachromosomal homologous recombination. The first one uses the copy at the same location on the sister chromatid, and in this case, the repair does not change the number of copies (Figure 33, left). In the case of USCR, the broken end invades into a misaligned sequence on the sister chromatid, and the repair results in amplification or deletion of rDNA copies [411,412] (Figure 33, middle). This is the primary way the rDNA array changes its copy number [372,373,408]. **Similarly, the broken strand can recombine with itself at another repeat (Figure 33, right), which will lead to the formation of an rDNA circle, also called Extrachromosomal rDNA Circle (ERC).**

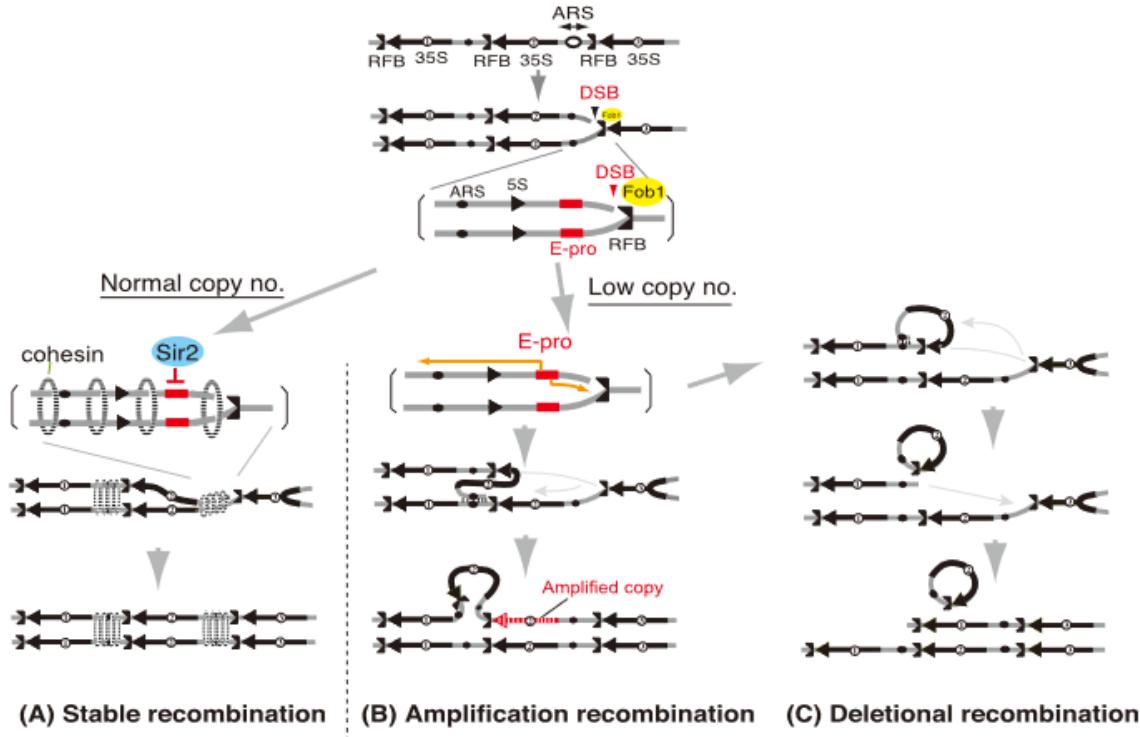
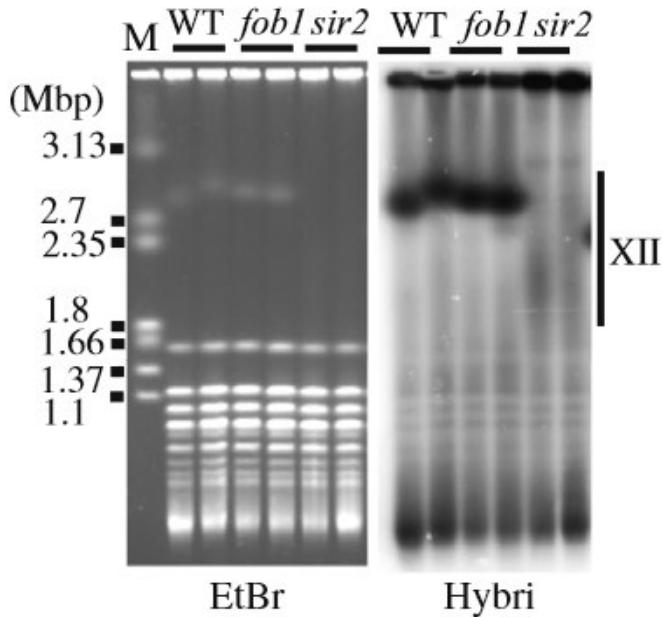


Figure 33: Schematics of the different scenarios after a DSB in the rDNA, mostly occurring at the RFB (top). (From [374])

Interestingly, the cell is able to sense its number of copies and control it since halving the copy number in a strain results in a gradual expansion of the array to get back to equilibrium in a Fob1 dependent manner [410]. Of note, more recent reports show that ERCs can reintegrate into the genome to help modulate the rDNA copy number as well [378,413]. How the cells “count” their number of copies and active copies is a fascinating question that is still not fully answered [414,415].

If these recombination mechanisms confer expansion and contraction properties to the rDNA array, it can also create instabilities in certain contexts. The rDNA instability refers to the heterogeneity of the rDNA array size among a population that can be measured by the sharpness of the band of a gel generated by pulsed-field electrophoresis (Figure 34). Screening the deletion mutant database using this technique reveals that 10% of the genome impacts rDNA stability [374,416].



*Figure 34: Total DNA gel revealed by EtBr (left) and rDNA revealed by a specific hybridization probe (right). WT and fob1 conditions have sharp bands, while the sir2 bands are smeared and undetectable due to the size variation among cells (From [417])*

#### 2.15.4. The link between rDNA transcription, silencing, and recombination

A close link exists between Pol I & Pol II transcription and rDNA recombinations.

First, the silencing activity of Sir2 at the ncRNA is necessary to repress the expansion of the array [372,415]. The main explanation is that transcription at the E-pro - which is repressed by Sir2 - stimulates USCR and intra-chromosomal recombinations by removing cohesin from the DSB [330,418-420](see Figure 33). Additionally, Pol I transcription on the repeat seems necessary for the RFB to be in place and the DSB to appear [409].

To summarize the main points of this section, rDNA recombinations are a way for the cells to control their rDNA copy number. Transcription of the E-pro promoter, normally silenced by Sir2, is linked with modifications of the rDNA size and ERC excision after a DSB that mainly depends on Fob1 association with the RFB.

#### 2.15.5. rDNA instability during aging

Back to aging, the rDNA silencing of Pol II targets and Pol II transcription impacts lifespan, with increasing H4K16 acetylation or decreasing Sir2 levels negatively impacting longevity and vice-versa [321]. This is thought to be mediated by unwanted variation in the rDNA array size (called rDNA instability) as well as the production of ERCs.

The rDNA instability theory of aging [417,421,422] suggests that the rDNA copy numbers variates during aging, which would, in turn, alter the expression of important genes (typically,

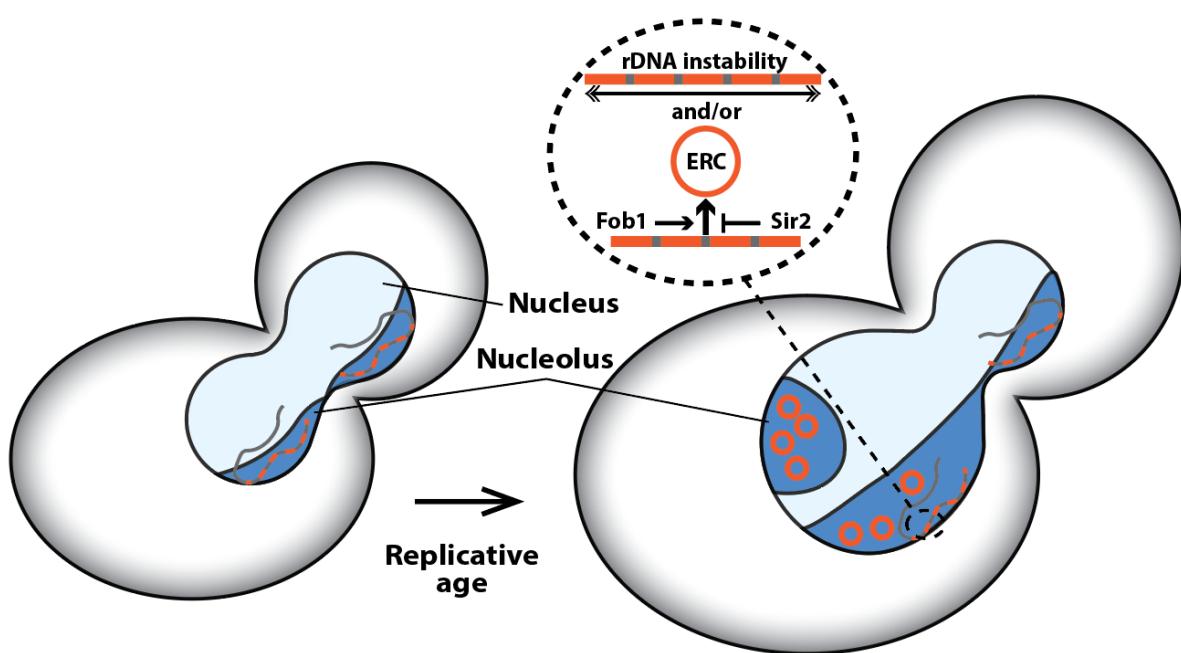
ribosomal genes). In addition, rDNA instability would trigger global genomic instabilities and increase replication stress, leading to senescence [330,421,422]. If this theory is seducing because a correlation between the rDNA stability and certain longevity mutants exists, it lacks an important criterion. According to the definition of an aging factor, if rDNA instability causes aging, it should be present in the mother but not in the daughter. However, the same DNA (and in particular, rDNA) segregates to both cells, and even if putative mechanisms have been proposed to solve this contradiction [366,421], further experiments will be required to back this model.

Apart from variabilities in the rDNA size, several other rDNA-related phenotypes are observed during aging. For example, old cells show more Pol I transcription, suggesting a global loss of silencing of Pol I transcription and an increase of its activation [226]. Pol II silencing also decreases at the rDNA locus, with an accumulation of ncRNA transcripts from the two IGS being detected [328,330,423] as well as an increase in H3K16 acetylation [321]. Besides, cohesin is lost during aging, which could participate in the increase of USCR observed during aging [330].

Finally, ERCs are also enriched in old mother cells [226,424].

**How these nucleolus and rDNA deregulations - namely a larger and fragmented nucleolus, greater variation in the rDNA array size, a higher number of ERCs, a higher Pol I transcription, and a higher Pol II transcription of IGS1/2 - are linked during aging remains unclear and debated (Figure 35).** Nevertheless, it seems that the rDNA regulation is a core feature of the process of replicative aging, which is confirmed by Quantitative Trait Locus analyses across very different budding yeast strains [202,210].

From that, another model of aging implies the rDNA has received extensive backing, namely: the ERC theory of aging.



*Figure 35: Schematics of the evolution of the nucleolus and rDNA state with replicative age. Young cells (left) represent the normal state, while old cells have a bigger nucleolus, rDNA instabilities, and ERCs (right).*

## 2.16. Extrachromosomal rDNA Circles

As briefly enunciated in the previous section, ERCs are created after a DSB repair caused by a stalling of the DNA polymerase at Fob1 if the recombination occurs when the sister chromatids are not held together by cohesin (which is mediated by Sir2 silencing). Interestingly, ERCs can be observed using gel electrophoresis and have been shown to accumulate in old mothers and be an aging factor [424]. Indeed, the *fob1Δ* mutant as well as mutants overexpressing Sir2, accumulate less ERCs during aging and are long-lived [322,425]. On the contrary, the *sir2Δ* mutant accumulates many ERCs and is short-lived. More recently, ERCs accumulation has been monitored at the single-cell level and their level correlates with the RLS [226]. Importantly, their level is reset to zero in daughter cells [226]. **Overall, ERCs accumulate in old cells, are not transmitted to the daughter, and are toxic for the cell.**

In the following sections, we will see how ERCs accumulate in old cells and the putative mechanisms behind their toxicity.

### 2.16.1. Accumulation of ERCs

So far, we have described how ERCs are excised from the genome but not how they accumulate in the mother cell. In fact, since each rDNA repeat (and therefore, each ERC) contains an origin of replication (ARS, see p.49), each ERCs can replicate at each division.

Recently, a study tagging each ERC with a LacO LacI-GFP system and tracking them throughout aging showed that the doubling time of the ERC pool takes 1.5 divisions, suggesting that two-thirds of the ERCs are replicated at each division [226].

Besides, since the daughter cells are born free of ERCs (except daughters of very old mothers) [226], it means that the ERCs are highly retained in the mother and thus, that their number increases exponentially.

Therefore, how to explain this highly asymmetric segregation? Previous mathematical modeling predicted that a retention of 0.99 was needed to recapitulate experimental survival curves [426]. Later on, a diffusion model suggested that freely diffusing plasmids are not likely to go through the bud neck since it is a small area only opened during the short window of anaphase and that plasmids are not highly diffusive. Nevertheless, such a model can only account for a retention frequency of 0.75-0.90 [427].

This suggests that another mechanism of segregation is at play. Indeed, yeast has a closed mitosis during which a septin-dependent lateral diffusion barrier is formed around the outer nuclear membrane. This barrier strongly impairs the diffusion of proteins embedded in the outer nuclear membrane [428], such as NPCs (Figure 36). Indeed, using Fluorescence Loss in Photobleaching (FLIP) on tagged nucleoporins showed that the pores from the mother are not passed to the daughter, thanks to a septin-dependent diffusion barrier [354]. Similar results were obtained by tagging an artificial plasmid, suggesting that ERCs could bind to the NPCs. In line with that, weakening the barrier with mutants such as bud6 $\Delta$  increases the leaking of old NPCs and of tagged plasmids to the daughter. Moreover, this mutant displays increased longevity that does not depend on Fob1, still experience excisions but accumulate less ERC, suggesting that the ERCs are leaked to the bud. Altogether, the retention of the nuclear pores of the mother cell could be the way cells retain ERCs in the mother (Figure 36).

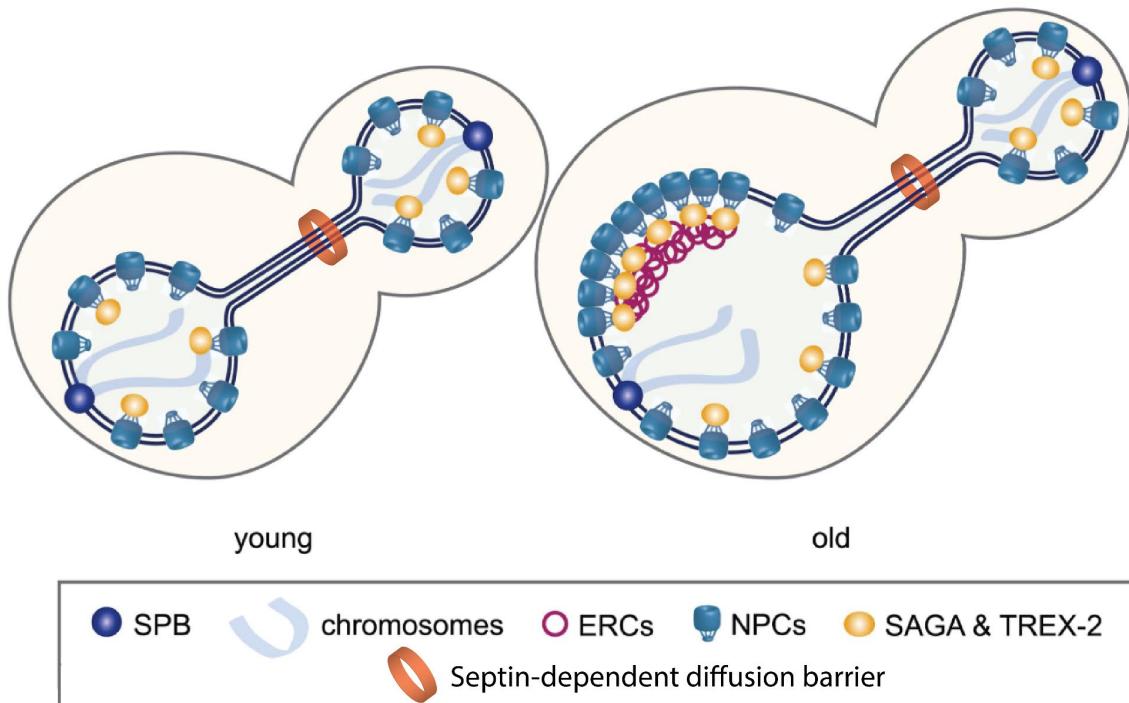


Figure 36: Schematics of a model of ERC retention in the mother. ERCs attach to the NPC with the SAGA & TREX2 complexes, and NPCs cannot pass the septin-dependent diffusion barrier. Hence, ERCs stay in the mother (adapted from [194]).

Further refinement of this model [354,356] showed that the circles need to be bound to stable parts of the NPCs in order to be able to not diffuse to the bud. Indeed, anchoring the circles to the nuclear basket (Mlp1 or Nup2, for example) leads to their free diffusion in the bud [429], whereas tethering to more stable parts ensures proper retention in the mother [356]. What mediates this anchoring is also partially known and involves the SAGA and TREX-2 (which associates with the NPCs) complexes. Indeed, deleting specific proteins from SAGA leads to the leaking of circles to the bud, while artificial tethering to the pores bypasses the need for these proteins for proper retention.

From then, how are these circles recognized by the cell compared to other transmitted plasmids? Beyond the fact that the presence of a centromere sequence leads to equal segregation, a long-standing observation is that plasmids with an ARS are highly retained in the mother cell (only 1 out of 10 leaks to the bud) [430] and that the stronger the ARS, the higher the retention [431]. In line with that, previous ChIP data indicate that ARS are targets of SAGA and that SAGA targets preferentially non-centromeric circles [356,432]. Thus, SAGA has a high affinity with non-centromeric and autonomously replicating plasmids.

Beyond the context of aging and ERCs, this could be a general mechanism for yeast to prevent the spread of extrachromosomal DNA (foreign or endogenous) in the population by retaining replicating circles in the mother.

Altogether, this suggests that asymmetric segregation of ERCs could be partially mediated by anchoring non-centromeric circles to the NPCs of the mother, which are not passed to the daughter. Yet, this model relies on experiments with artificial model plasmids and has never been assessed with ERCs directly.

### *2.16.2. Toxicity of ERCs*

In addition to the fact that *fob1Δ*, *sir2Δ* and other mutants affecting the number of ERCs have a corresponding lifespan, the most substantial evidence of the toxicity of ERCs comes from the original study [368]. There, the authors used an ectopic plasmid containing an rDNA repeat as well as an excisable centromere (using a LoxP and Cre-recombinase system). When the plasmid has the CEN, it segregates symmetrically between the mother and the daughter, and the cells have a normal RLS. Excising the CEN leads to the accumulation of the plasmid in the mother and to a reduction of lifespan, hence proving the toxicity of having many ERC. However, some discrepancy remains since modifying the ARS of the rDNA, which influences ERC accumulation, does not always correlate with RLS [417]. Indeed, cells engineered with a weaker ARS are short-lived, even though cells with a stronger ARS are also short-lived, more than cells with the weaker ARS. Therefore, having a weaker ARS or an ARS that is not adapted to the rest of the array could induce detrimental effects, independently of ERC accumulation. Conversely, another study showed that exchanging ARS between different strains leads to a change in lifespan, with having a stronger ARS decreasing RLS and vice-versa [202], and mutants decreasing the ARS efficiency are also long-lived [424].

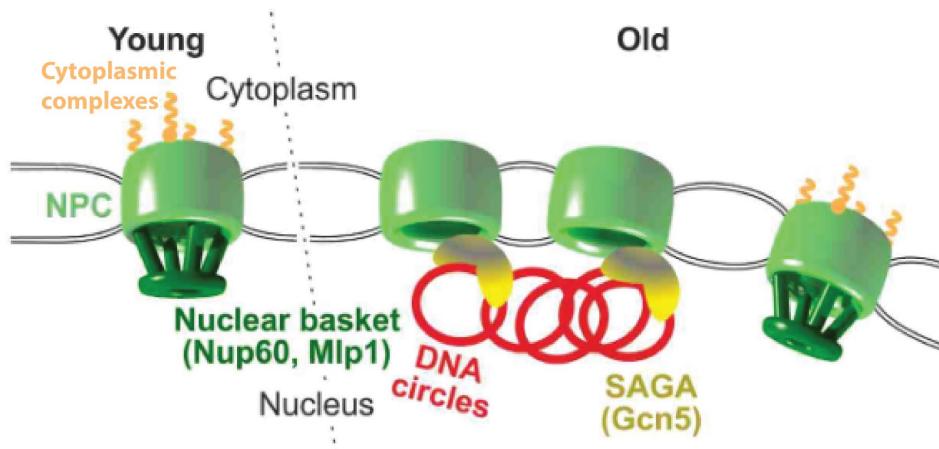
If the ERC accumulation is widely admitted as to be toxic for the cells, the reason behind this toxicity remains unclear.

Simple experiments with an empty plasmid containing an ARS show that these circles of DNA are sufficient to induce a reduction in lifespan, which is rescued by adding a CEN to the plasmid [424,433]. Hence, it is possible that accumulating plasmids causes aging, independently of if they contain rDNA sequence or not. To explain that, it has been proposed that ERCs could titrate important factors away from their usual action sites, thus disrupting the cell homeostasis. For example, ERCs could bind with histones, chromatin modifiers, and other parts of the silencing machinery, thus leading to the observed global derepression of expression. They could also induce longer DNA replication, as suggested by the fact that yeast mutant over-amplifying 2μ DNA circles possess a longer S-phase [434].

If the sequence plays a role in the toxicity (though the few available data argue it is not the case), ERCs could also titrate important transcription factors for cell cycle and growth. In fact, the DNA sequence of these circles contains binding sites for the Swi4/Swi6 (SBF) and the Mbp1/Swi6 (MBF) transcription factors, on which depend the transcription of genes from the G1/S regulon (Wittenberg and Reed 2005). In line with that, it has been shown that reducing the number of ERCs in the mother during its lifespan makes it recover a normal Cln2 level activator of S phase). However, this result is weak since the authors used a nucleolar marker as a marker for ERCs, and did not check if the reduction of nucleolar size (interpreted as ERCs number) was linked to a reduction in cell-cycle duration [248].

Besides, since deletion of SBF and MBF lead to hypertrophy (in part due to a longer G1 phase) [338,435], titration of SBF and MBF by ERC could explain the cell-cycle slowdown observed in old cells, as well as their hypertrophy.

Another model proposes that the tethering of ERC to NPCs leads to their remodeling. Indeed, a recent study shows that plasmids-bound NPCs lose their nuclear basket due to the SAGA complex acetylating parts of its nucleoporins [358] (Figure 37). In line with that, blocking this acetylation results in a longer lifespan (even though the increase is very mild).



*Figure 37: Schematics of young VS old pores. Pores from young cells are intact while certain pores in old cells tether ERCs, which leads to the loss of their nuclear basket (adapted from [358]).*

Basket-less NPCs are known to be present under physiological conditions and have specific functions. Recent studies even suggest that basket-less pores are the default assembly state [436]. However, having more ERCs could disrupt the equilibrium between pores with and

without baskets, which could in turn disrupt the nuclear homeostasis (theory further discussed in the general discussion).

ERCs are a credible aging factor since they accumulate with age, are segregated asymmetrically, and are toxic for the cells. Yet, many questions remain unanswered: how the ERCs are toxic is still putative and if ERC excision is the result of the loss of regulation from other processes (such as rDNA stability) [257], or if they are also the cause of these losses, is still not clear.

## Introduction II: Methods to study replicative aging in yeast

Studying replicative aging in budding yeast is a challenging task. Indeed, since the daughter cells are rejuvenated and that the daughter lineage grows exponentially, the fraction of cells of age N is equal to  $\frac{1}{2^N}$ . Therefore, separating the mother cells from their progeny is necessary to avoid such dilution (Figure 38).

This introduction will review the different strategies to tackle this challenge that exist since the birth of the field in the 1950s.

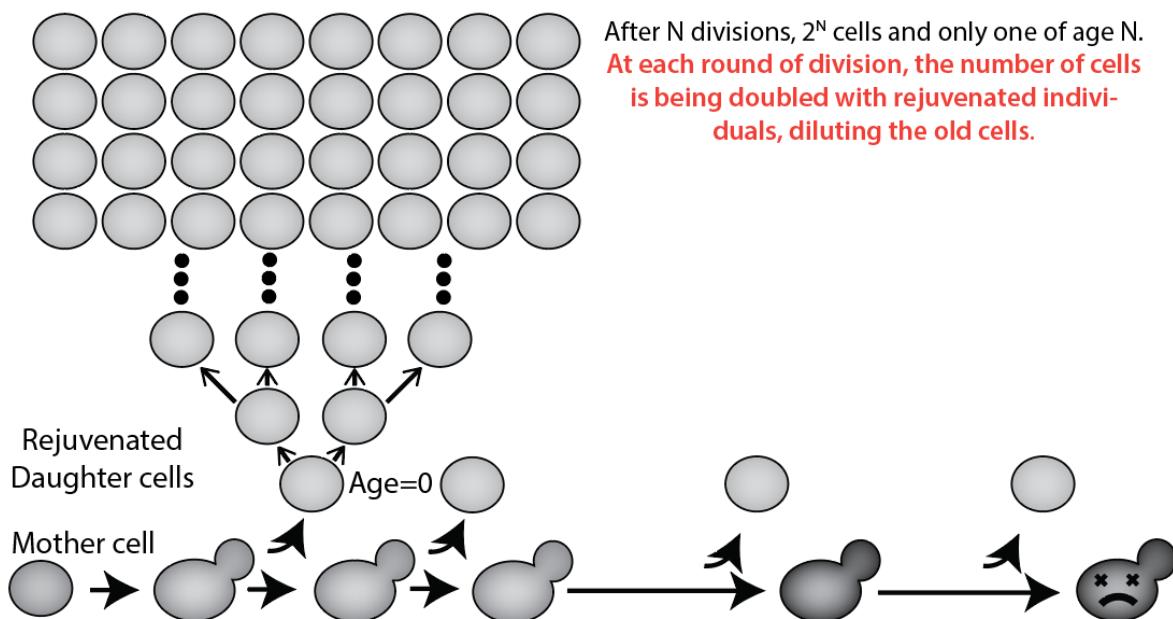


Figure 38: Schematics describing the exponential growth of a yeast population and the exponential dilution of cells of old cells.

### 1. Microdissection

The historical method consists in manually removing the daughter cells from a mother placed on a culture pad using a microneedle under a microscope after each division (Figure 39) [148,149].

## Conventional dissection

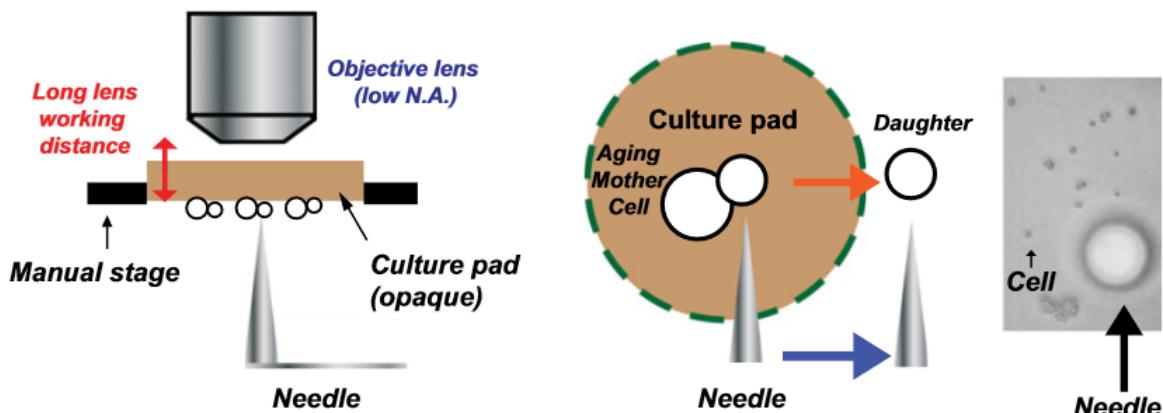


Figure 39: Principle of the microdissection manipulation. A microneedle under a microscope (left) can remove the successive daughter cells from the mother (middle). The diameter of the needle is ten times bigger than the cell (right). (From [221])

With that, one can assess the replicative lifespan of cells by counting how many divisions are performed before dying and plot the survival curves of populations (Figure 40). In this sense, it has been very instructive to identify longevity genes thanks to deletion mutants and to find molecular processes involved in aging [236].

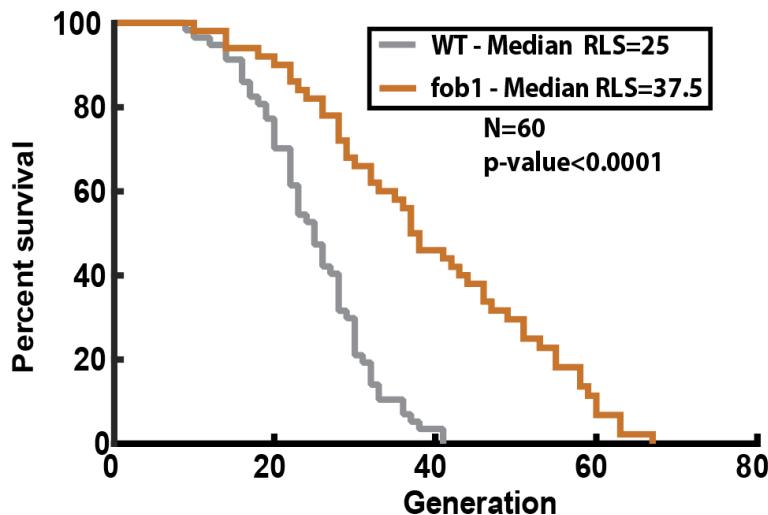


Figure 40: Replicative survival of a wild-type population VS a *fob1* $\Delta$  population. Each time a cell dies, the percentage of cells alive is decreased. (Data from a personal experiment)

However, the replicative lifespan of the cell is the only information that can be extracted from such assays, and understanding aging requires more physiological and dynamic metrics. Moreover, it has a very low throughput and is tedious (measuring the RLS of 60-100 cells takes between one and four weeks for an experimenter). Indeed, the needle used to remove cells is more than ten times larger in diameter than the cells themselves, and cells need to be put at 4°C when no experimenter is available to remove the progeny.

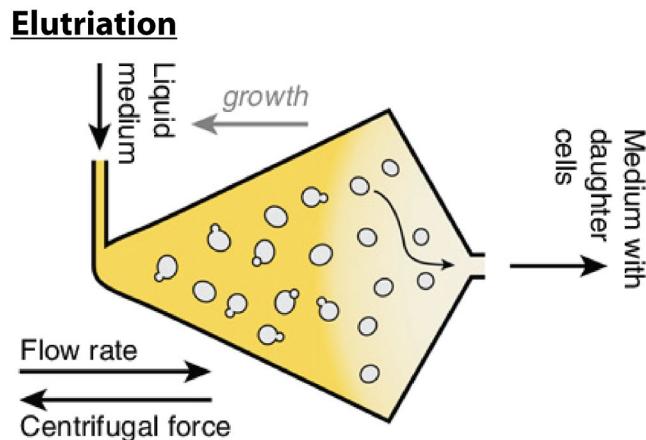
## 2. Populational enrichment of old cells

To complement this method and do populational assays (for instance, biochemical or omics), other approaches have been developed in order to enrich a population with old mothers.

### 2.1. Density gradient and Elutriation

The historical enrichment method takes advantage of the size difference between young and old cells. Practically, it consists in making a population of cells migrate through a sucrose gradient during centrifugation, which leads to different bands (just like a DNA-gel during electrophoresis) of cells of the same size. By selecting the bands and repeating this migration process, one can separate millions of cells (up to  $10^8$  cells) of the same age ( $\pm 2$  generations) with an accuracy of 90% [224]. Yet, this method is quite labor-intensive since it requires several rounds of purification for several days, is vulnerable to contaminations, and requires keeping the cells at 4°C overnight.

A similar technique using the morphological differences between daughters and old mothers, called elutriation, has been developed and improved several times to enrich a population with old mothers [285,437,438]. It involves applying a certain centrifugal force to the cells while exposing them to a flow in the opposite direction. Consequently, the trade-off of forces will have the small cells being removed by the flow, while the bigger ones will remain in the chamber (Figure 41). This method is less labor-intensive than sucrose migrations since cells can be grown and separated in the elutriation chamber in a continuous manner. Nevertheless, inspection and adjustment of the flux is still needed, in addition to vulnerability to contaminations

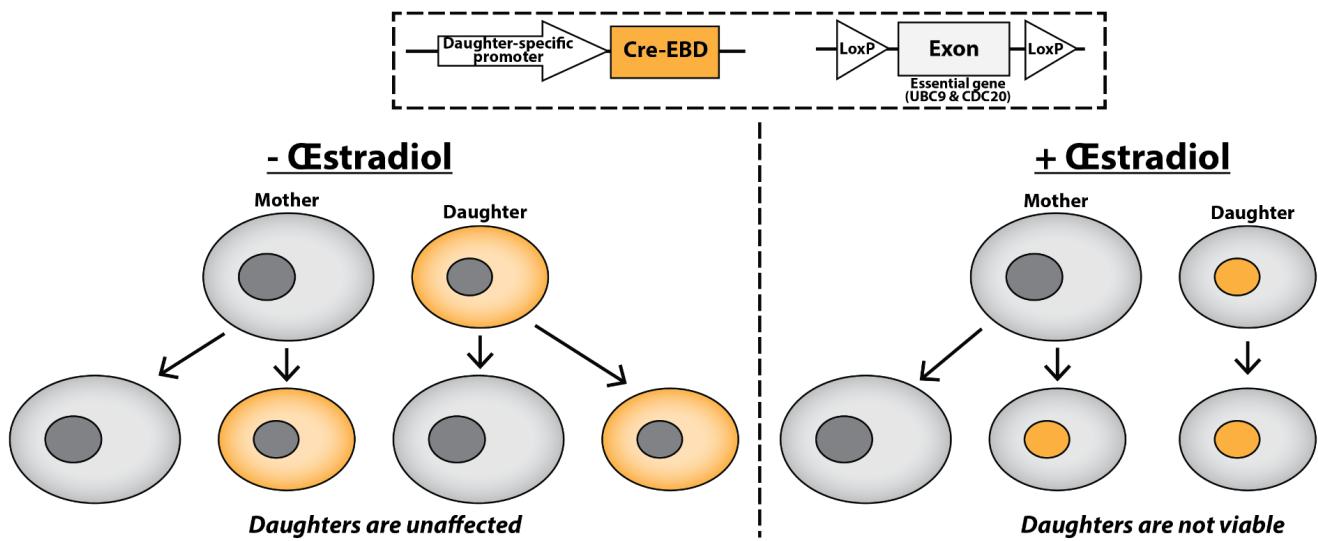


*Figure 41: Principle of the elutriation enrichment of old cells. A trade-off from two forces, centrifugal and hydrodynamic, can separate cells according to their size. (From [194])*

Interestingly, these methods allow the harvesting of daughter cells from a population of mothers of a given age to perform subsequent analysis, such as size and replicative lifespan measurement, or question the presence of a given potential aging factor.

## 2.2. Mother Enrichment Program

Another way of overcoming the exponential dilution of original mother cells is to utilize the fundamental asymmetry of budding yeast. Indeed, some genes are specifically expressed in virgin daughter cells just after their birth. By hacking this system, it is then possible to differentially express proteins in the daughter compared to the mother, which can be used to kill the daughter. With this idea, the Gottschling lab has developed a strain that expresses a Cre recombinase fused to an Estradiol Binding Domain (EBD) under a daughter-specific promoter. In the absence of estradiol, the protein is cytoplasmic in the daughter and does not exist in the mother. However, when in contact with estradiol, the protein translocates to the nucleus and is able to cut essential genes flanked by artificially engineered LoxP sites [439](Figure 42).



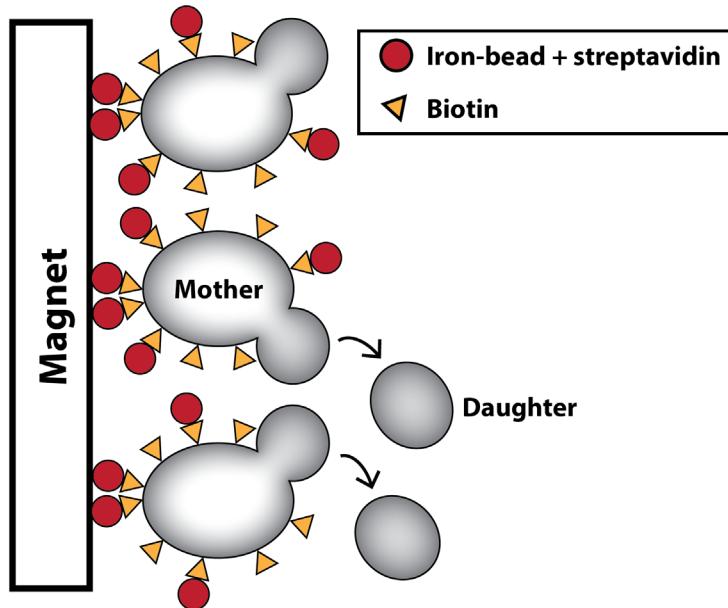
*Figure 42: Principle of the Mother Enrichment Program. Upon addition of estradiol, a Cre recombinase, only expressed in daughter cells, enters the nucleus to excise essential genes, which prevents the cell from dividing.*

Therefore, it is possible to render newborn cells unviable without affecting mother cells, transforming an ordinarily exponential growth into a linear growth, hence limiting the dilution of the mothers by their progeny.

Nevertheless, this *Mother Enrichment Program* (MEP) comprises certain caveats. First, it requires genetic manipulations of the strains of interest. Furthermore, it was found that some daughters do not undergo senescence immediately, especially if arrested during M-phase, and continue producing a few daughter cells before division arrest. Similarly, cells escape the selection by getting mutations in the Cre or EBD sequence at a rate  $\varrho$  of  $1.4 \times 10^{-6}$  per division [439], which further limits the yield. Consequently, this genetic trick is often combined with other selection methods to allow efficient enrichment.

### 2.3. Cell wall labeling

Another method to enrich mother cells from a culture is to label them with iron beads using a biotin-streptavidin system. Since the cell wall of new daughter cells is newly synthesized [440] and thus, unlabeled, passing cells through magnetic columns [251] or directly culturing them on magnets [294,318] allows automatic separation between the original tagged cells (mothers) and their daughters (Figure 43).



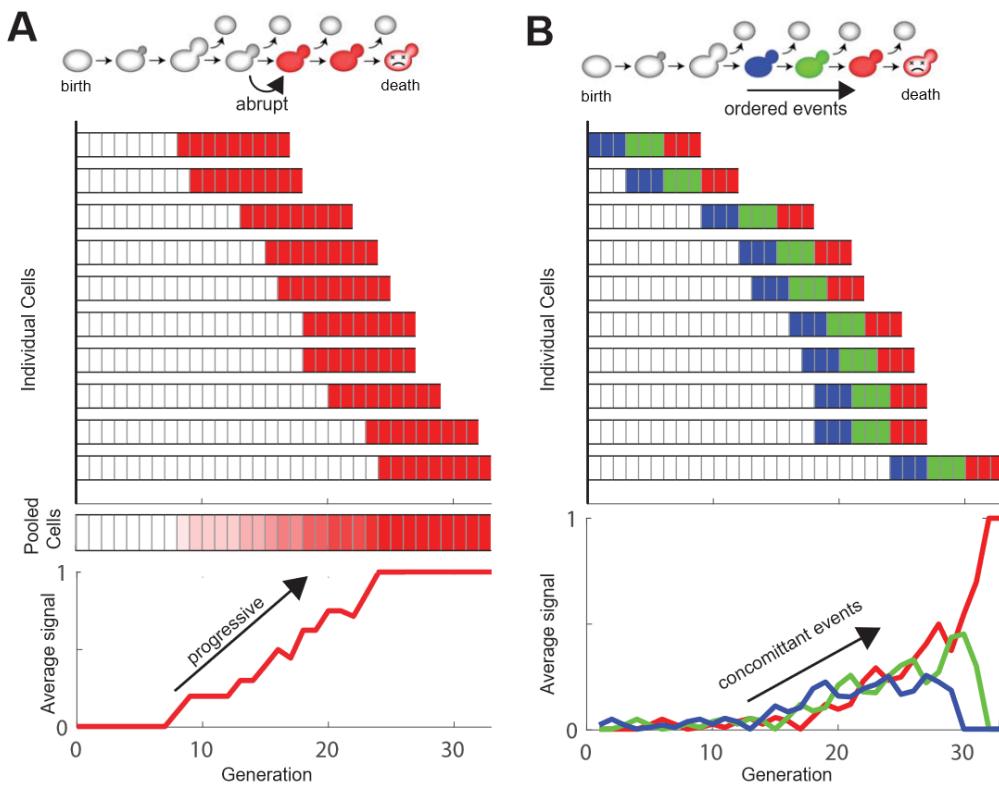
*Figure 43: Principle of the magnetic enrichment of mother cells. The cell wall of mothers is decorated with iron beads which allow them to attach to a magnet, while daughter cells are born with a new cell wall and do not attach to the magnet.*

Similarly, using the principle that daughter cell does not inherit cell wall from the mother, it is possible to select old mother cells by labeling the cell wall of progenitor cells with a fluorescent tag instead of iron beads, to stain their budscar at the end of the experiment, and to sort the population at the FACS using the two fluorescent channels [441].

These methods, often combined with the Mother Enrichment Program, are the state-of-the-art method to produce a population of old cells before conducting further assays on it.

## 2.4. Conclusions on traditional assays

Populational assays and genetic screens using microdissections have been extremely informative and influential in describing which biomolecular processes are changed during aging. However, these approaches fail to capture the proper dynamics of the processes and can only tell if they are affected - on average in the population and in time - or not.



*Figure 44. Schematics describing averaging biases. (A): The heterogeneous occurrence of an abrupt event (represented in red) at the single-cell level will give a progressive signal at the population level. (B): Similarly, an ordered series of events (represented by the colors) will look like a progressive and parallel accumulation of events (From [226])*

Indeed, due to the cell-cell heterogeneity of most phenotypes during aging (at least in time), averaging biases can lead to misinterpreting an abruptly occurring phenomenon into a progressive or a series of ordered events into concomitant parallel processes (Figure 44).

Therefore, another way of studying replicative aging - while avoiding population biases - is to extract temporal signals directly from single cells.

### 3. Capturing single-cell dynamics

For a decade, the development of new technologies such as microfluidics has allowed the isolation of cells of interest as well as precise control of their environment, not only in budding yeast (described above) but in bacteria [170,174], fission yeast [159,186,442], *C. elegans* [443], or mammal cells [444,445]. When coupled with timelapse microscopy, this approach allows tracking cellular responses to various environments at the single-cell level, using direct light and epifluorescence with mutant strains harboring proteins with fluorescent tags. In the context of replicative aging, they permit the isolation of mother cells of interest in order to follow them throughout their lifespan. The next paragraph will briefly describe how these

microfluidic devices are fabricated, which designs are used for replicative aging, how to track aging cells, and how to analyze their images.

### 3.1. Microfabrication and soft-lithography

Many different techniques exist in order to create microfluidic devices. The most common one is called soft-lithography [446] and consists of pouring a polymer on a mold that will, after chemical treatment (such as the addition of a cross-linking reagent), take the negative shape of the mold (Figure 45, third column). Therefore, the mold has to contain structures of interest.

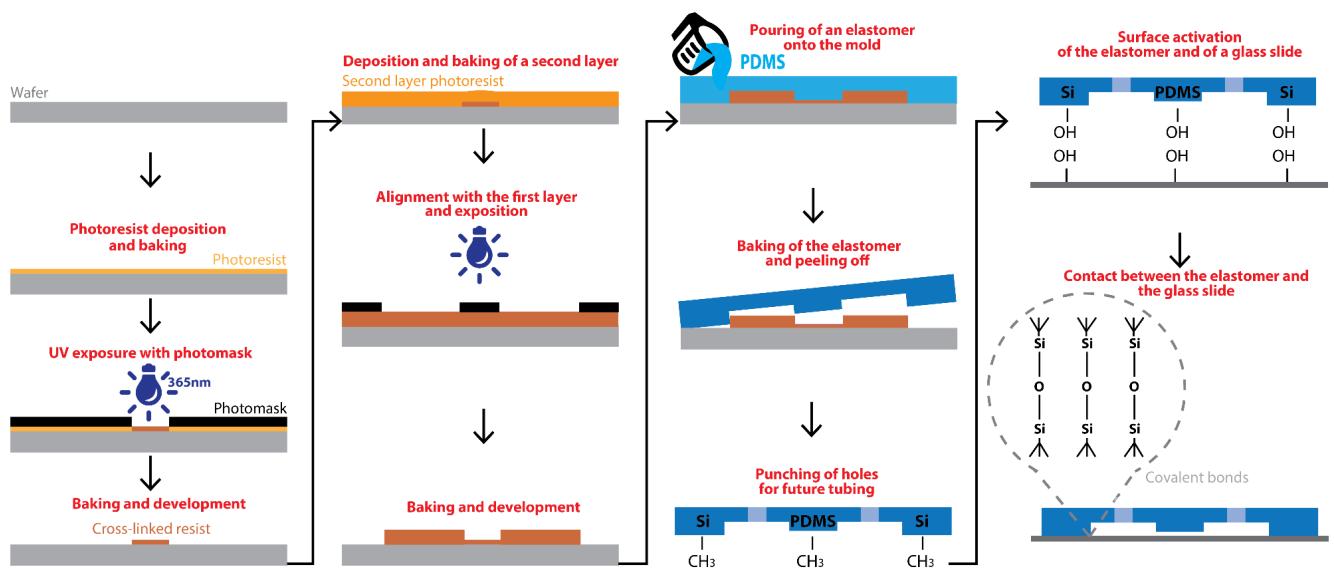


Figure 45: Schematics representing the main steps of the photolithography (two first columns) and of the softlithography and plasma activation (two last columns) for the preparation of microfluidic devices.

To create these structures, again, several approaches are possible depending on the resolution and size requirement. If 3D printing and micro-milling can reach a resolution of 50 $\mu\text{m}$  in specific contexts, going lower often requires lithography techniques. Direct photolithography involves depositing a photosensitive resist on a substrate before exposing it to a given wavelength through a mask containing transparent and opaque parts. Exposed resist will release cross-linking agent, become solid and adhere to the substrate after heating, while unexposed resist can be removed using a solvent (Figure 45, first column). In theory, the resolution of such a technique is then only limited by the diffraction of light. In practice, one can achieve microstructures with a 1 $\mu\text{m}$  resolution if the thickness of the resist does not exceed 10 $\mu\text{m}$ .

Once the mold is done and the chip cast, baked, and peeled off, one can pierce holes for the future tubing and seal the device to a substrate such as a glass slide, using (for example) plasma activation to create covalent bonds between the polymer and the glass (Figure 45, last column).

### 3.2. Microfluidic designs for replicative aging

Using this approach, several microfluidic devices have been developed to study replicative aging.

Similar to enrichment techniques, their objective is to isolate mother cells from their exponentially growing progeny, but this time to observe them throughout their lifespan.

To do that, the first devices “glued” the cells to a coverslip using a biotin-streptavidin system before applying a gentle flow of media to remove the successive daughters [222]. This pioneering system is however relatively inefficient to retain cells over an extended period of time and can be toxic to them.

Therefore, it has quickly been replaced by mechanical trapping, for example by trapping the cells under pads (see “Micropads” in Figure 46) of polydimethylsiloxane (PDMS). In fact, if the ceiling has the proper height, the newborn daughters can be removed by the flow since they are smaller than the trapped mother [221,245]. These devices, which are still used nowadays by a few labs [248], also suffer from low retention of the mothers with age, are hardly adaptable to different cell sizes, and have low throughput.

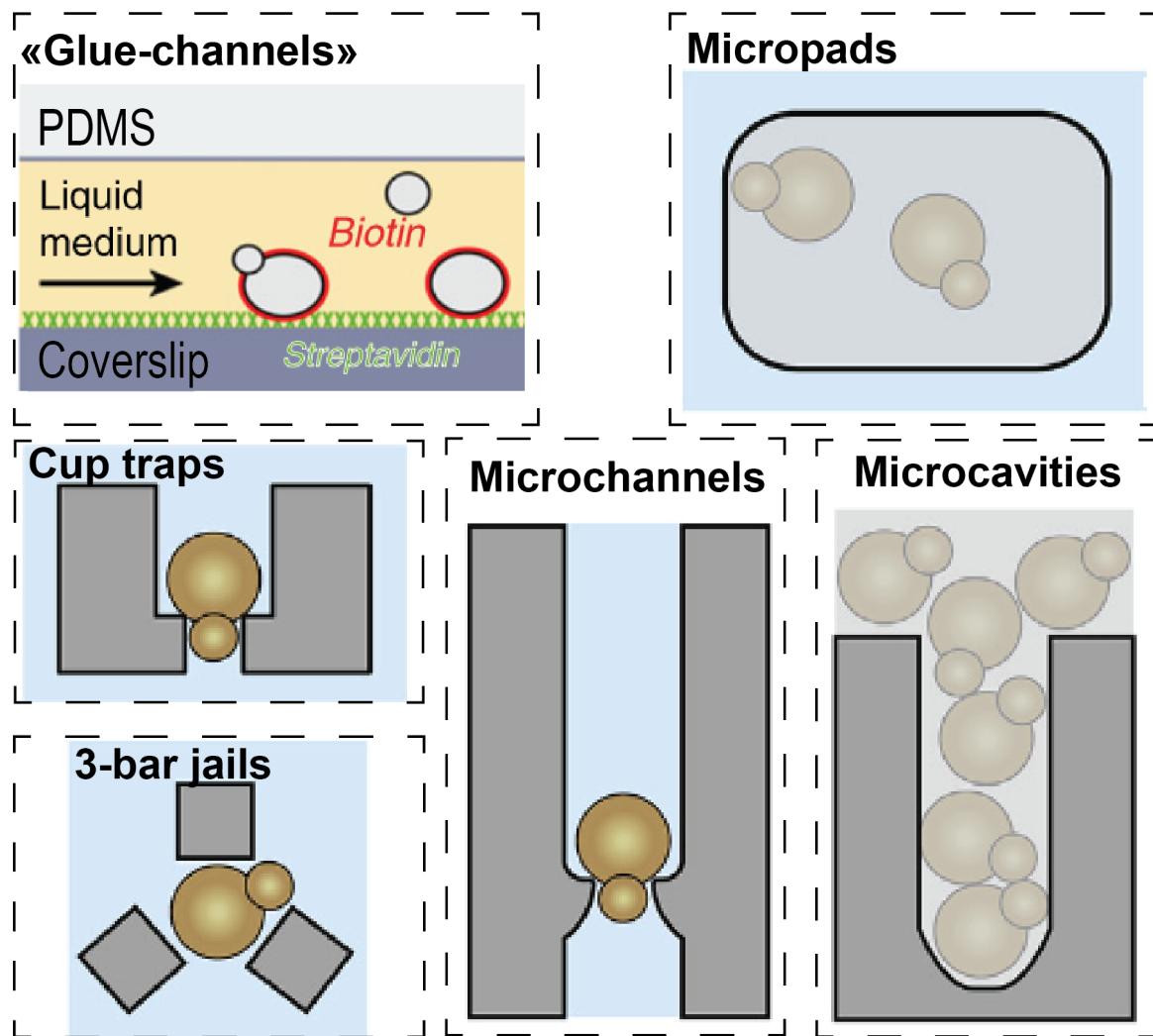


Figure 46: Schematics from the different microfluidic devices used to perform replicative lifespan assays in yeast  
(Adapted from [194], [447])

Inspired by the mother machine (introduced p.19), devices with cavities have been developed to work with budding yeast [246](see “Microcavities” in Figure 46). In this device, mother cells are trapped at the tip of the cavity and the successive daughters are pushed out of the cavity and removed by a lateral flow. It comes with a few caveats such as the requirement to use strains that always bud in the same direction. Moreover, the budding pattern sometimes becomes erratic during aging, leading to the loss of the mother cell, replaced by a daughter. Taking that point into account, microcavities with a hole have been developed [328], with the drawback that the number of traps is reduced in such devices (100 traps/device)(see “Microchannels” in Figure 46).

Alternatively, devices with an array of inflow traps are also used [231,247,448,449] in which the mother cell is blocked in-between pillars of different shapes. Trapping mother cells in between cup-shaped structures (see “Cup trap” in Figure 46) or chevrons is straightforward, efficient, and easy, but the retention is not perfect since big cells tend to leave the trap or be

replaced by their daughter. Using three pillars (see “3-bar jail” in Figure 46) solves this problem but is much more challenging to trap the original mothers since a cell has to come outside the jail and bud inside to inoculate it [449,450]. With inflow traps, the progeny is automatically dissected by the constant flow of media.

Depending on the strategy, the trapped cell will be guaranteed virgin [246,449] or might have any age. However, since the age distribution of an exponentially growing population is composed of 50% of virgin cells, 25% of cells with an age of one division, and 12.5% of cells with two divisions, most of the cells are virgin or very young when trapped at the beginning of the experiment (Verified experimentally: [231]).

Overall, microfluidic devices can isolate mother cells throughout their lifespan and avoid an exponential dilution by their progeny. Interestingly, the culture media is tightly controlled and can be changed in a matter of seconds to submit cells to stress, drugs, or to change their carbon or nitrogen source.

From that, they can be used to enrich a population with old cells before harvest (like enrichments methods presented in the previous section) (see Appendix 1), or Appendix 1: Microfluidic enrichment of old cells and sorting according to their senescent state to track them longitudinally during the aging process.

### 3.3.Longitudinal tracking: Image acquisition and processing

Most of the microfluidic devices are made of PDMS or other polymers transparent to visible light. Therefore, they are compatible with epifluorescence microscopy and can be imaged in week-long experiments with a constant frame interval using a camera (Figure 47). *Timelapses* of individual mother cells offer much information compared to microdissection since it is possible to measure the size of the cell, its morphology, its division duration, and many fluorescent reporters. However, this quantity of information, if analyzed manually, makes the whole pipeline low-throughput and comparable to microdissection in terms of tediousness. In other words, microfluidics has displaced the throughput bottleneck from isolating the cells to analyzing the data.

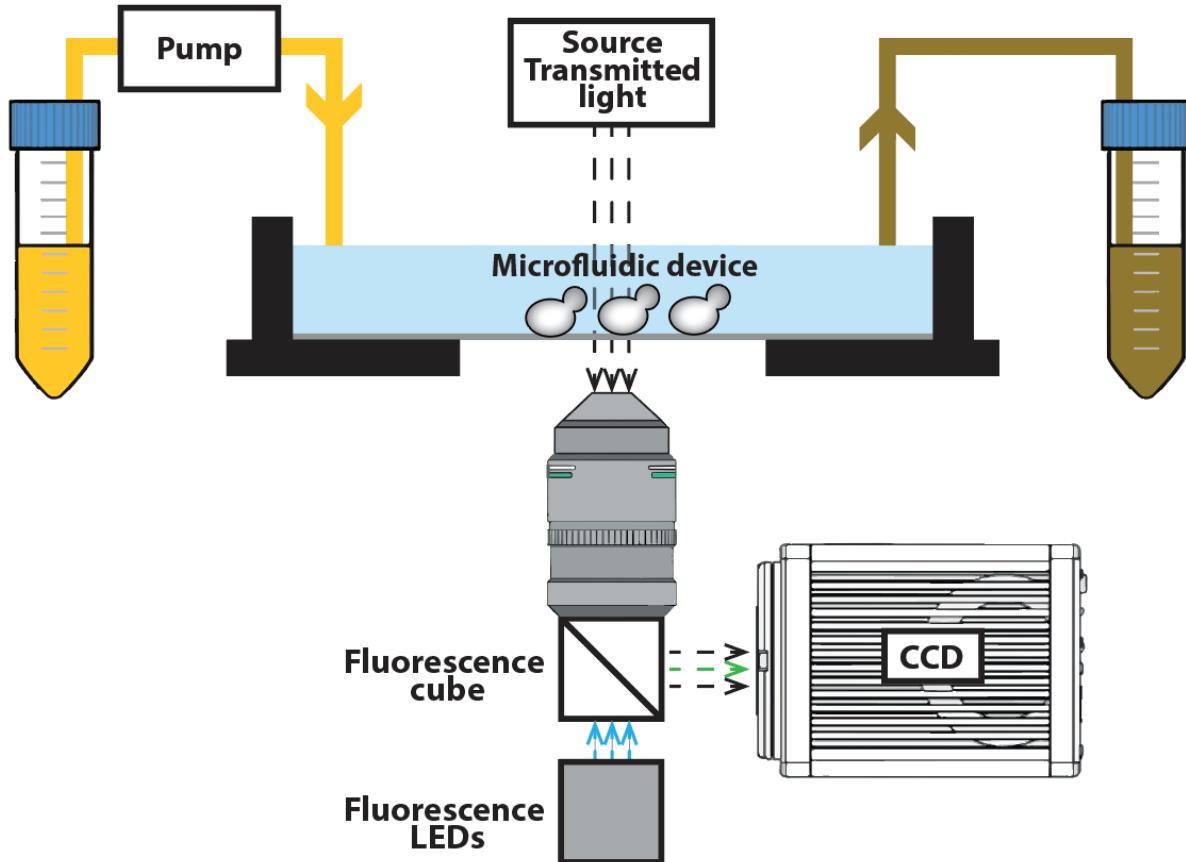
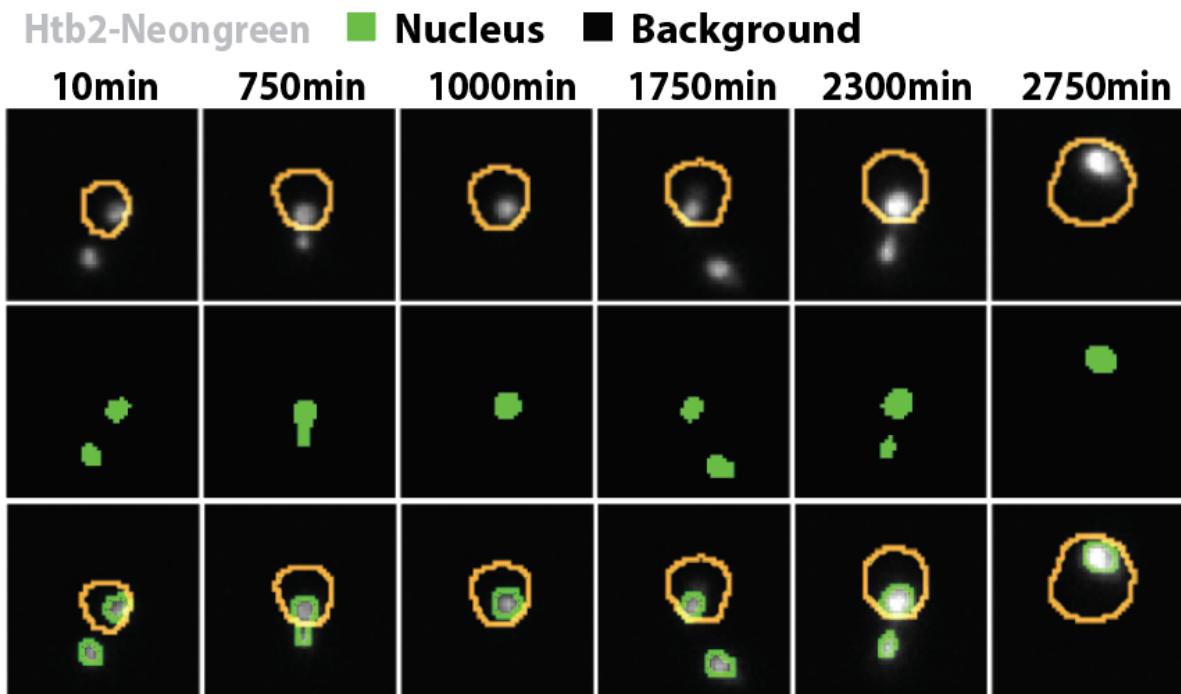


Figure 47: Schematic representation of a typical timelapse microscopy setup with a microfluidic device

It is possible to automate part of the analysis by using classical image processing algorithms. For example, it is very simple to segment (*i.e.*, create a mask of pixels corresponding to the object of interest) an object from an image of a fluorescent reporter (provided the signal-noise ratio is sufficient), using a threshold value on the signal. Similarly, cells can be segmented using watershed from phase-contrast images or using other similar sets of transformations and cost-algorithms from brightfield images [246,451-453,453b]



*Figure 48: Timeseries of images from one cell. (Top): Gray level of the signal from a Htb2-Neongreen nuclear reporter. The orange contour represents the mother cell. (Middle): Pixel mask obtained by thresholding. Each pixel from the input image above a certain value is assigned to a green pixel on the mask. (Bottom): Mother cell contour (orange), mask contour (green) overlaid on the input fluorescence image.*

Nevertheless, such algorithms require fine-tuning to analyze the specific set of images and could fail with a slightly different set. In particular, in the context of aging, cells and their vacuole become bigger, and their aspect can change, especially when the cell is close to death. These changes often trick algorithms tuned with young cells, and manual correction is necessary, which impairs the throughput.

Besides, other complex features can just not be automatically extracted and analyzed using classical approaches, such as detecting when a cell divides. **In other words, classical approaches are ad hoc rigid methods with a narrow range of applications and struggle to extract complex signals from an image.**

For a few years, machine learning (ML) is emerging as a state-of-the-art solution to fit complex models in biology ([454] and see Figure 49). It is used not only for automation of image analysis (*i.e.*, increase the throughput compared to manual work) but also for extracting information from data too complex for a human (for example, denoising with spatial or temporal interpolation [455,456] or detecting organelles from brightfield images).

It is also widely used in omics studies and structural biology with the recent development of AlphaFold to predict protein structures [457].

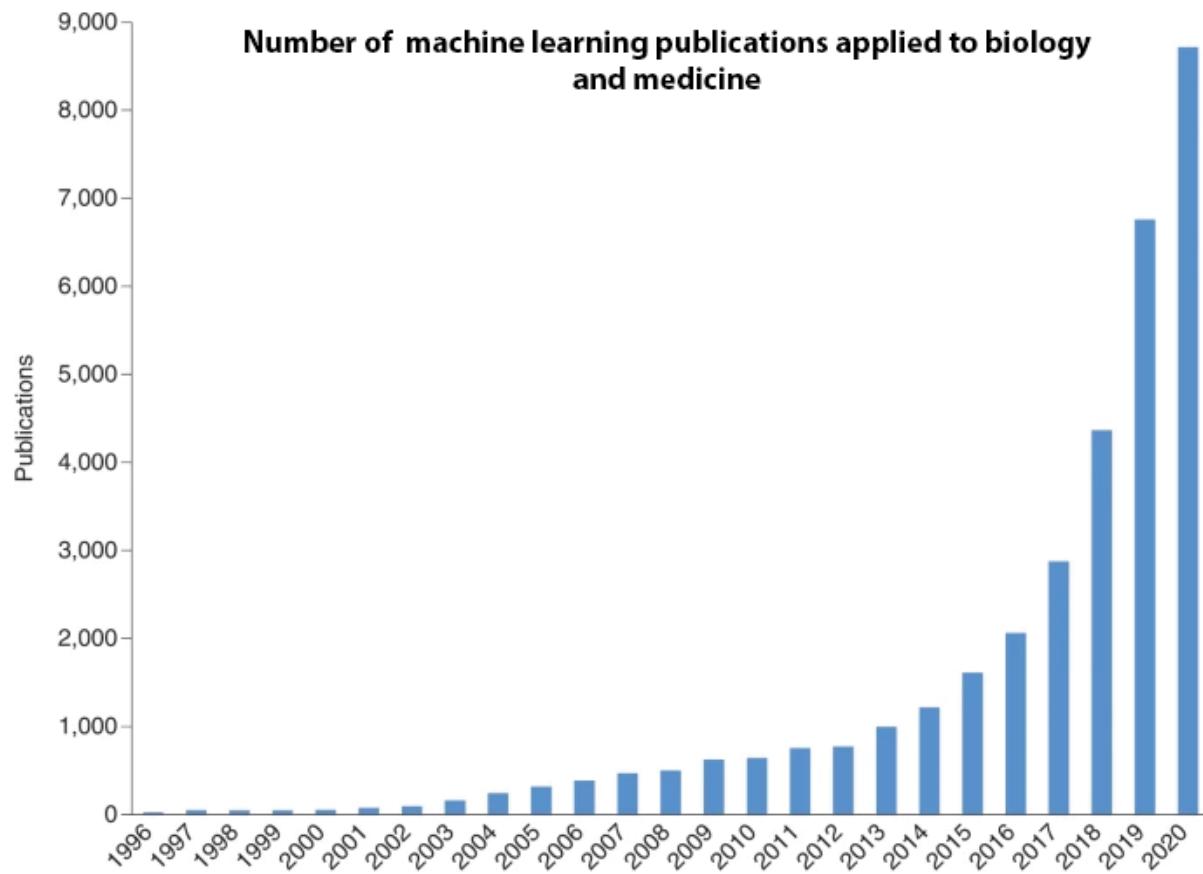


Figure 49: Number of publications in the "machine learning" category in combination with a topic category related to biology or medicine term (From [454]).

The general principle of machine-learning is similar to a fitting process. Indeed, the goal is to create a mathematical model from sample data (called "training data") during a training phase in which the algorithm will tune the parameters of the model to minimize the error (in other words, the algorithm will learn).

Then, the model can output a predicted value from input data (Figure 50).

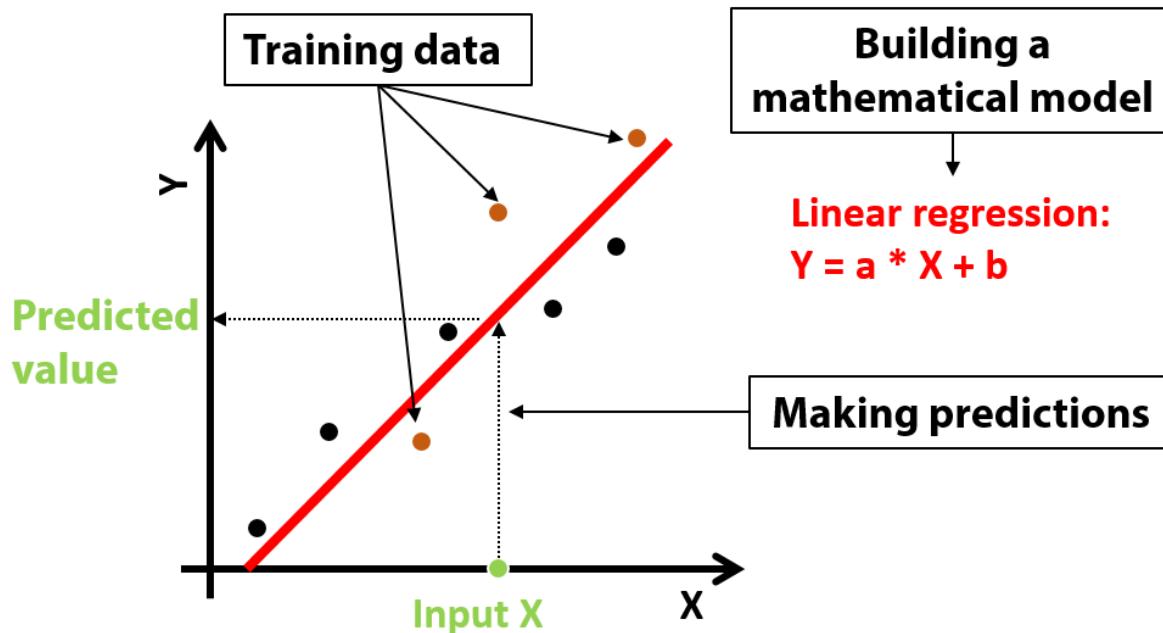
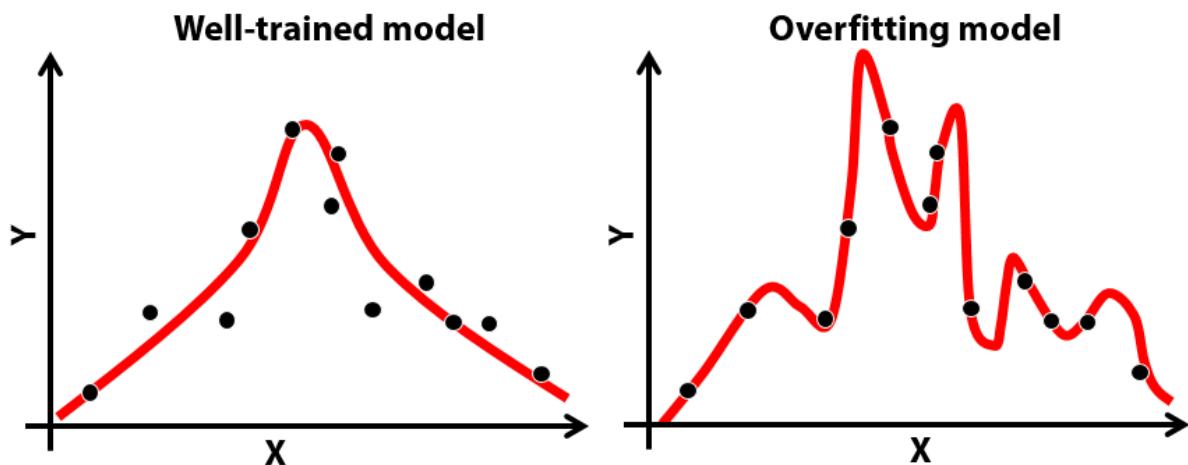


Figure 50: Principle of the creation of a model from a machine learning algorithm

If the model presented in Figure 50 is a simple linear regression that could be obtained with a simple classical algorithm, machine-learning can also fit highly non-linear models and is necessary when the relationship between the data is complex.

It can be used to classify an object between different classes (classification) or return an exact value from a continuum of possibilities (regression). For example, a machine-learning model could try to predict if a patient has a heart disease (classification between "Yes" or "No") based on its weight, age, if it is smoking and if it is exercising (these input data are called *descriptors*). Back to image processing, a ML model could tell if a pixel from an image belongs to a cell or to the background, based on its intensity, neighbors, and value after different image transforms. In this sense, Ilastik has been pioneering the ML approach in bioimage analysis by providing a software to segment cells and biological objects from raw images, based on a given list of descriptors [458].

However, the ability to extract complex relationships from data comes at a price. In fact, the main caveat of ML is that the model can memorize much information from the training data. Therefore, there is a risk of overfitting if the training set is not large enough to extract the general rules from the data. In other words, the algorithm can "learn-by-heart" the data and will have limited predictive power on new data (Figure 51).



*Figure 51: Schematics of the fit (red line) of real data (black dots) from a well-trained model (left) compared to an overfitting model (right).*

Moreover, ML computational time can be longer than classical algorithms (depending on the complexity of both), and must therefore be used only when standard approaches fail or are slower.

From then, different learning algorithms exist such as decision trees and random forests, bayesian networks, and artificial neural networks (ANN). For the rest of this section, we will focus on ANN.

ANN are inspired by the biology of neurons, in which the cell body receives signals from the dendrites and, transduce (or not) the signal to the axon. Artificial neurons perform simple mathematical operations (weighted sum) based on inputs and transduce it into an output (after activation, see function  $f()$  on Figure 52)). ANN are often organized in layers of neurons, and the weights of the different layers are tunable parameters that allow learning the complex relationships between the input and the output. An ANN can have a large number of hidden layers and is then considered as a deep learning (DL) network.

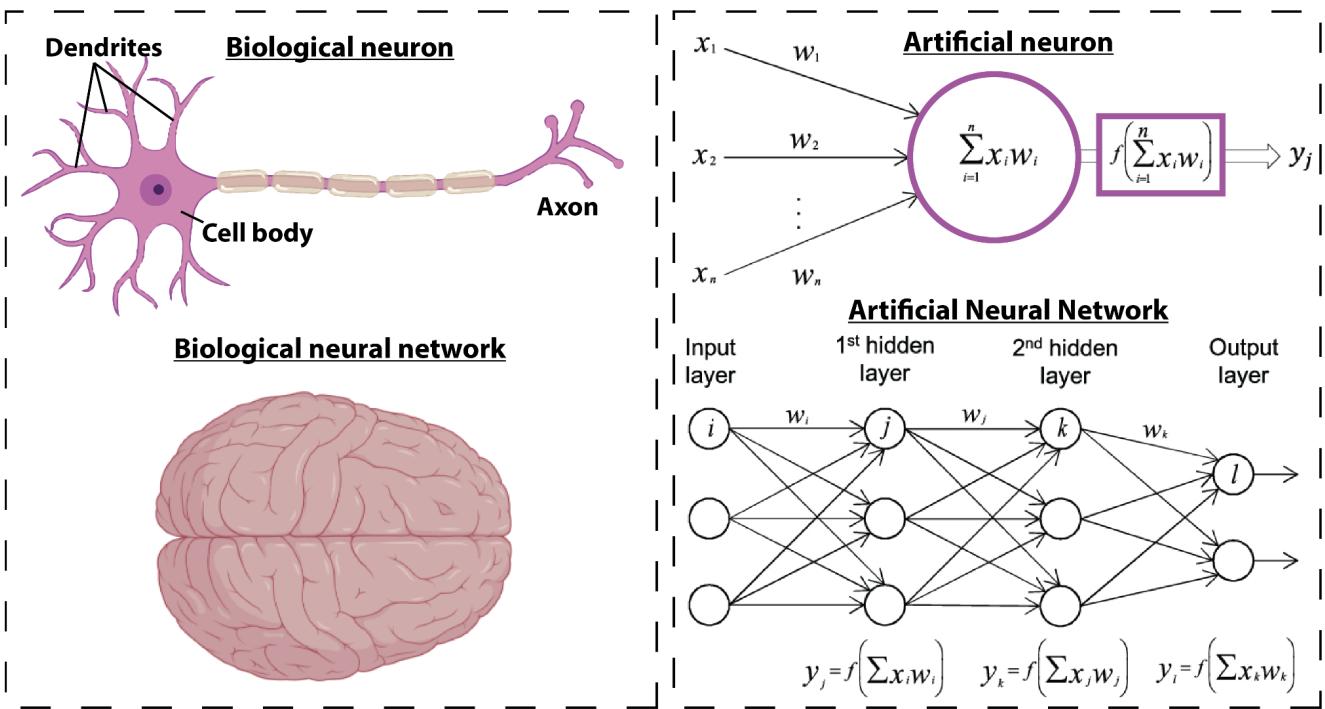
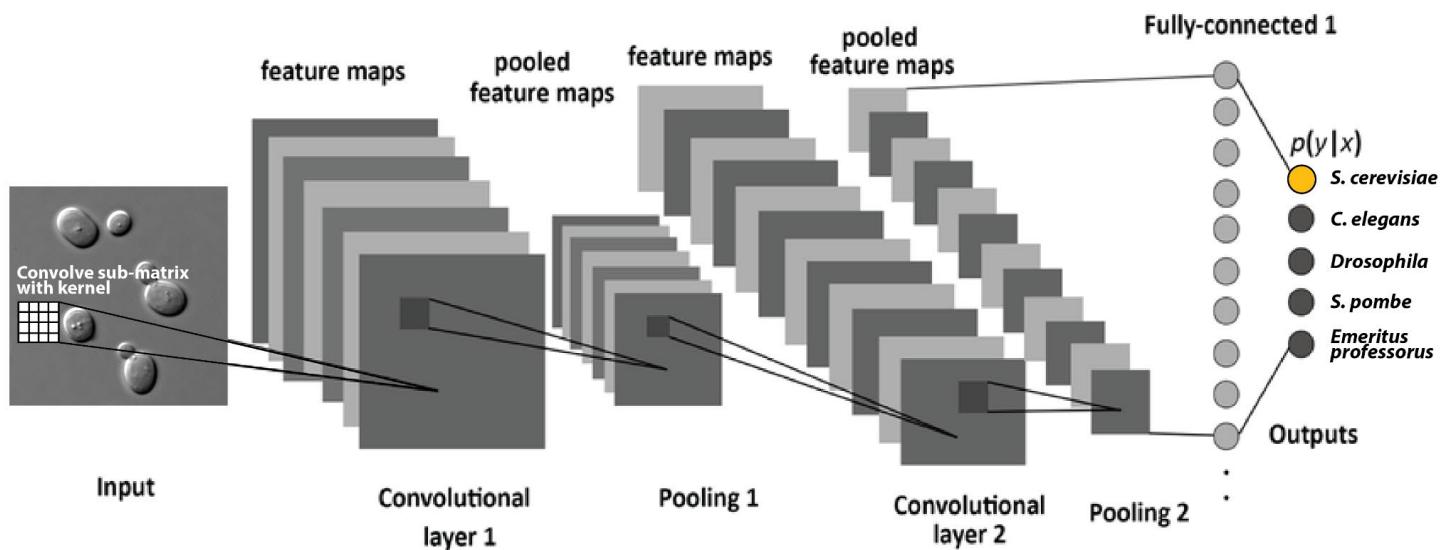


Figure 52: (Left) Simplistic representation of a biological neuron and neural network (brain). The neuron receives information from other neurons through its dendrites and potentialize a response towards its axon accordingly. Putting neurons together can transform complex inputs into complex output (behavior, movements,...). (Right) Representation of an artificial neuron and of a ANN. An artificial neuron receives weighted inputs  $x^*w$  (data or other neurons' output). An activation function transforms the sum of these weighted inputs into an output value  $y$  (connecting to other neurons or to the output layer). The training consists solely in adjusting the different weights  $w$  of the network.

The advantage of ANN (and in particular of DL) compared to other ML approaches is that it does not require the user to select descriptors to make a prediction. Indeed, the nature of the network (*i.e.*, the kind of operations each neuron does and how the neurons are organized) alone is able to extract relevant features from input data. This is very convenient since defining accurate descriptors is difficult. On the other hand, it often requires more training data. Besides, the user does not know which particular feature of the data matters to create the model, which is why DL models are often described as “black boxes”.

Many different types of DL networks exist (with new types emerging every year). For example, Convolutional Neural Networks (CNN) are remarkably efficient with images since they perform convolutions on groups of pixels from the input image. Hence, they can extract the information stored in the value of the pixels themselves, but also of their neighborhood (Figure 53) [459].



*Figure 53: Representation of the architecture of a CNN. At each layer, the input and output are an image (except the last layers), and the operations are convolution with a mask (instead of simple weighted sums). Here, the CNN is used to classify an image into predefined classes. The output of the convolutional layers is connected to a classification layer that transforms images into an array of probabilities. During the training, the weights of the convolution masks and of the classification layer are tuned*

Specific variants of CNN are adapted explicitly for semantic segmentation, such as U-net networks [460], and are so efficient that they are now state-of-the-art for such tasks.

These kinds of networks have been adapted to segmentations of broadscale bioimages [461], [462], but are also being developed to specifically segment yeast cells [463-468] (Figure 54).

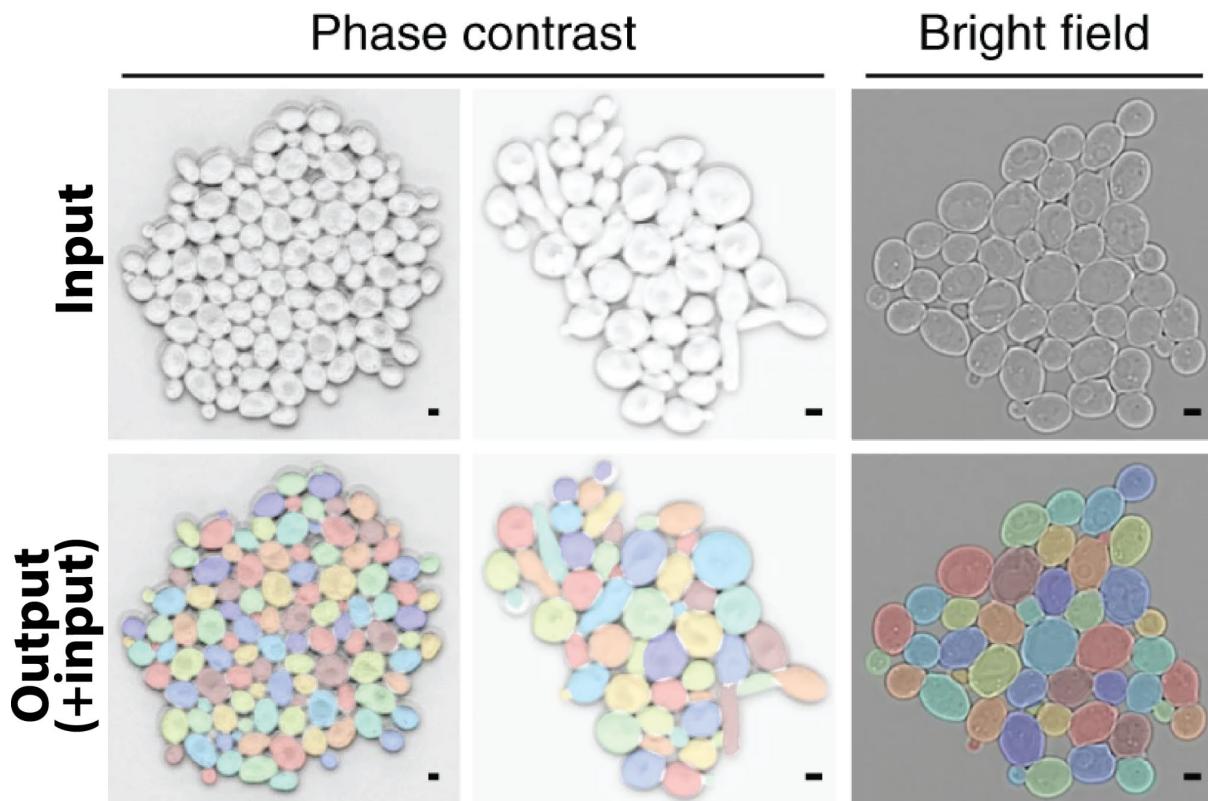
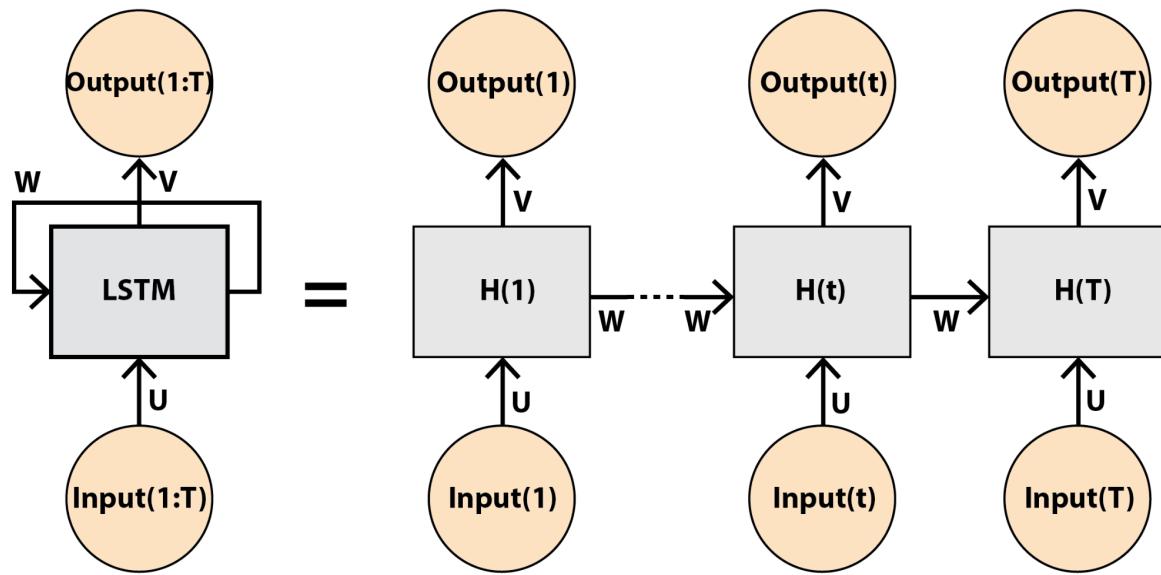


Figure 54: Examples of single-cell yeast segmentation from raw images. (Top): Raw phase contrast or brightfield images. (Bottom): Overlay of the segmentation with the raw image using a U-net. The different colors represent the predicted pixels of a cell. Scalebar: 2 $\mu$ m (From [463])

Deep-learning can also be used to track particle movements (like cells) [469] or detect divisions in very stereotyped organisms such as *E. coli* [470].

Another type of network is specifically efficient in analyzing temporal and repetitive signals. Indeed, Recurrent Neural Networks (RNN) can take into account the time dependencies between the timepoints by including the output of nodes in their input, therefore creating some kind of memory of the previous timepoints (Figure 55).

In particular, Long Short Term Memory (LSTM) networks are particularly suited for analyzing temporal data such as speech [471].

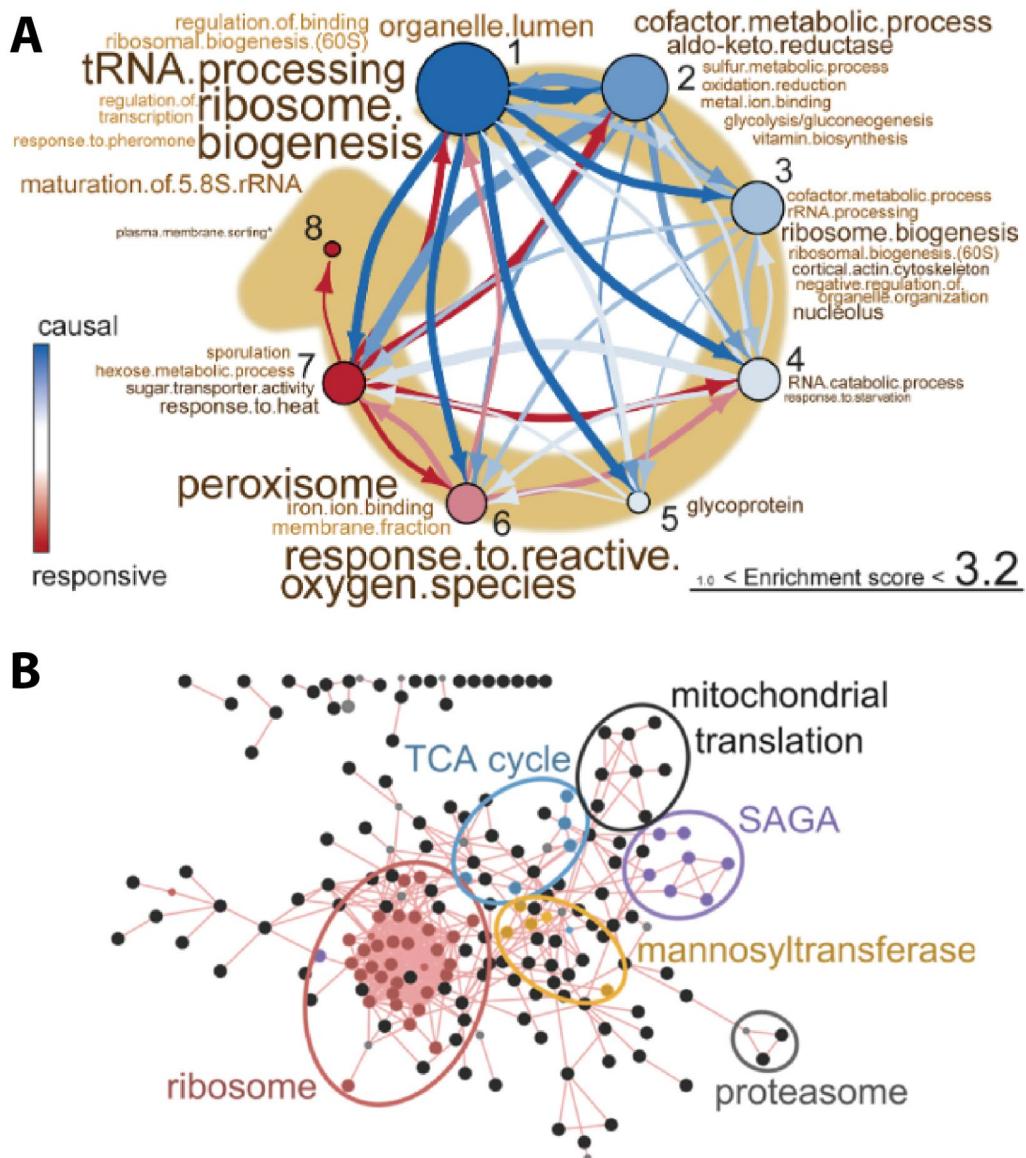


*Figure 55: Representation of the architecture of a unidirectional RNN. Such networks take timeseries as an input, and the output from the function  $H$  of each timepoint is fed to the node treating the next timepoint.  $U$ ,  $V$ , and  $W$  are the weights of the nodes.*

However, even though deep-learning models provide unprecedented capabilities to extract meaningful information from images or temporal signals, its potential to exploit the time-dependencies in temporal sequences of images has been almost completely overlooked. Yet, the analysis of complex temporal behaviors of biological systems - a primary focus in cellular biology - may largely fail when using pipelines that do not exploit the underlying temporal correlations between images in time-lapse datasets. For example, no algorithm is capable of detecting yeast cell divisions efficiently.

## Summary of the introduction and Objectives

As seen all along [Introduction I](#), a large number of biological processes are affected during aging. Yeast is a powerful model to study this phenomenon, and systematic studies, as well as more focused analyses, have identified many hallmarks of aging and genes of longevity.



*Figure 56: The output of two large-scale screening studies. (A): Functional clustering from proteome- and transcriptome-wide data from old cells. A specific data processing was used to infer the degree of causality of each cluster. Arrow thickness, colors, and direction represent the strength and causality of the connections between two nodes. In this model, the protein biogenesis-related genes are the primary causal force during aging (From [294]). (B): Functional clustering of long-lived deletions. The overrepresented categories are colored and circled [236])*

However, understanding how these different hallmarks are linked between one another is necessary for the field to grasp this complex phenomenon. More precisely, it is important to try drawing aging pathways and define which processes are causal for the loss of fitness and

## Chapter I: Summary of the introduction and Objectives

---

which are consequential among these pathways. This task is made difficult by the fact that certain alterations are mechanisms to keep the global homeostasis while others participate in its loss. Several comprehensive studies have tried to link different ontologies (Figure 56) and to bridge several hallmarks of aging.

For example, the loss of protein stoichiometry affects vacuolar proton-pumps, affecting the vacuole's pH in turn. Alkalization of the vacuole in turn was linked with mitochondrial decline, which in turn promotes protein oxidation and genomic instabilities (see p.38).

Besides, some proteins or metabolic pathways seem to be at the core of aging. For example, Tor1 controls many processes described earlier, at many different levels (not only ribosome biogenesis and cell growth, but also rDNA stability in a Sir2-dependent and -independent manner [386], autophagy, proteostasis [472-475], and even cytosolic and vacuolar pH [476]). Similarly, Sir2 seems to be involved in most of the processes described in Introduction I. It controls ERC levels, rDNA stability, and silencing and is required to filter oxidized mitochondria from the daughter by acting on the polarisome and the retrograde actin transport [276]. Besides, having defective mitochondria elevates Sir2 levels [477]. Sir2 is also necessary for the asymmetric segregation of protein aggregates in a similar manner to oxidized mitochondria [308,309,478-480]. Sir2 also interacts with the SAGA complex, known for regulating the tethering of ERCs to the pores [323].

Therefore, while these hub proteins and pathways seem at the center of aging, studying them directly is challenging because of their strong pleiotropy [237,481].

On the other hand, other hallmarks are already known to happen very late in life, such as aneuploidy and DNA mutations. These phenotypes are not likely to be aging factors since they affect the whole progeny and are more likely proximal causes of death than primary causes of aging.

More generally, where all these hallmarks stand in the aging process, whether they form different parallel pathways or rather a network, is almost entirely undefined, and many contradicting studies exist on most of these hallmarks (see introduction). This is in part because traditional methods do not allow proper capture of the dynamics of the events (see p.67), which in turn participate in the idea that aging in yeast is a gradual decline of parallel functions (Figure 44, p.68), might it be true or not.

The relatively recent development of new technologies such as microfluidics and long-term timelapse microscopy enables longitudinal analyses of single-cells, thus circumventing the

population biases. Thanks to this approach, propositions of ordered series of events and pathways of aging are emerging.

Indeed, several longitudinal studies have noted that cells were dying with distinct terminal bud morphology, *i.e.*, with a spherical (or “rounded”) bud or ellipsoidal (or “elongated” bud). [221,223,245,283].

Similarly, our lab described that the progressive lengthening of the cell cycle, previously described at the population level (see p.33 Division time), was actually a very abrupt event when looking at the single-cell level [246], that was termed **Senescence Entry Point (SEP)**.

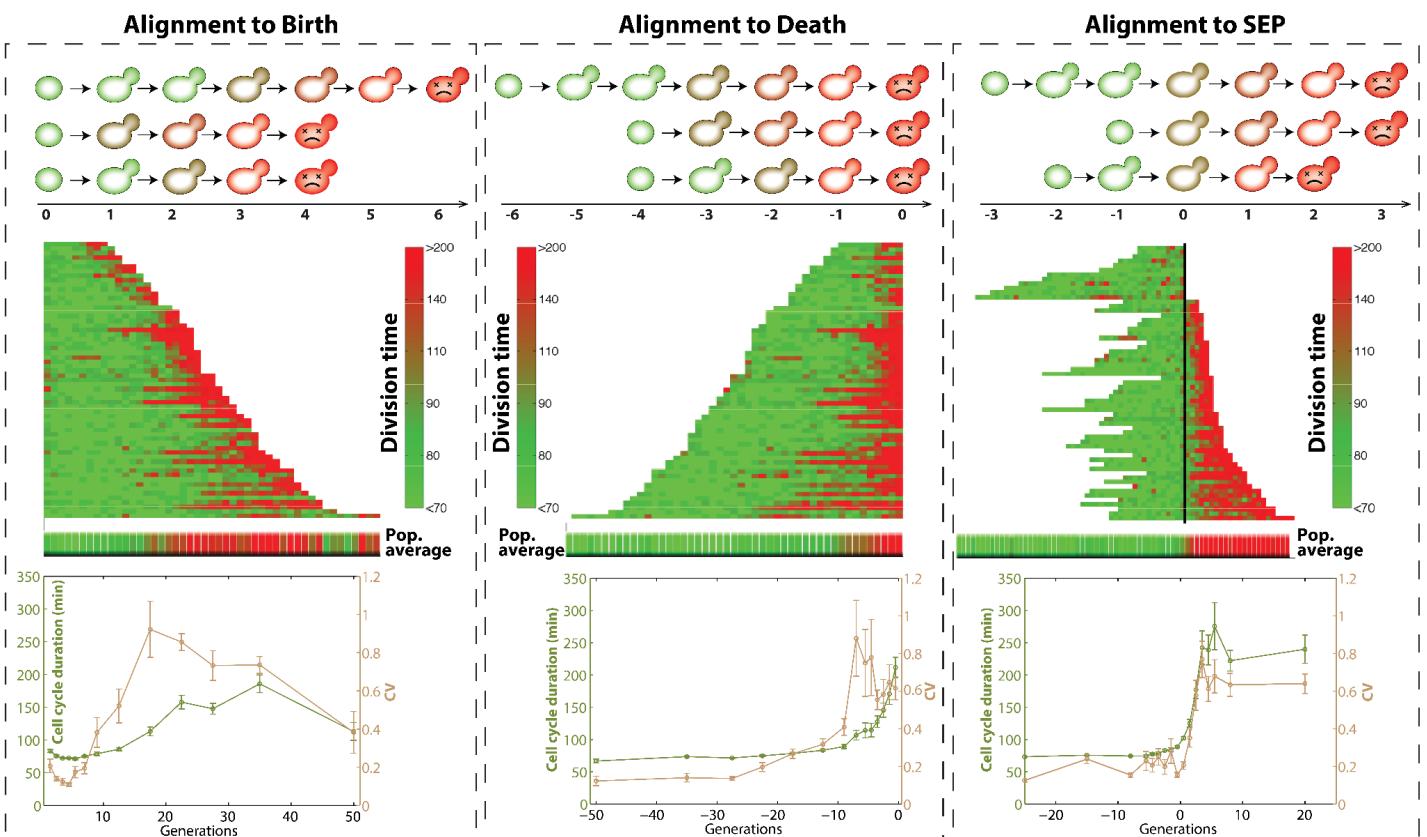
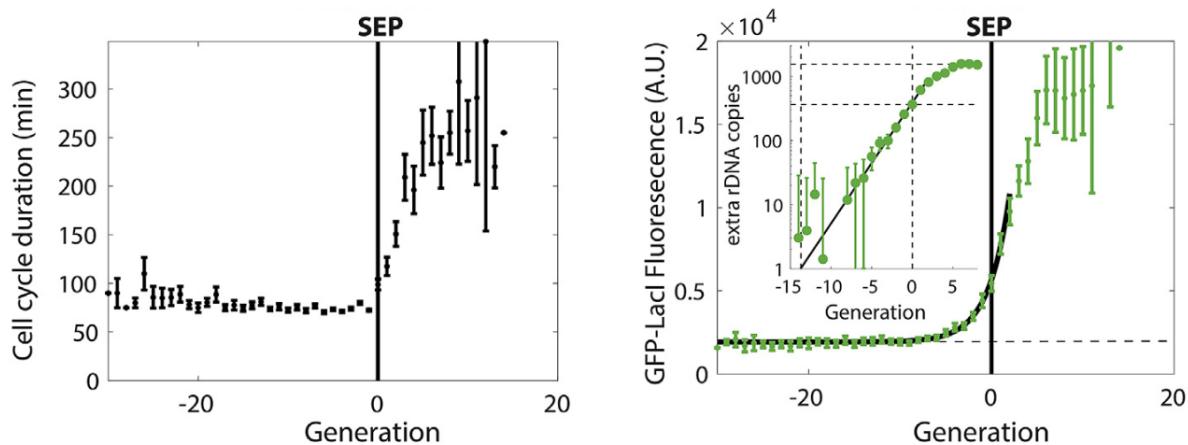


Figure 57: Different alignments (from birth, death, or the SEP) of single-cells division duration along lifespan. (Top): Schematics of the alignment method. (Middle) Each line represents a cell, and the colorcode represents the division duration. (Bottom): Mean division duration and coefficient of variation (CV). (Adapted from [246])

This is a striking illustration of how averaging a heterogeneous signal from a population can lead to misinterpretations if the single-cell trajectories are not correctly aligned. Hence, aligning cells relative to the occurrence of the phenotype of interest (here, the division slowdown) before averaging is necessary to reduce variability to its minimum (Figure 57, right). When this in-silico synchronization is not possible, aligning cells relative to their death is often better than aligning from birth (Figure 57, middle vs. left) since the onset of aging hallmarks is often closer to the death than to the birth of cells (as it is done with population assays).

Besides, the power of longitudinal tracking in this study showed that the loss of mitochondrial potential (*petiteness*) was a stochastic event, independent of age or of the SEP.

Building on that, further work tried to ordinate some of the known hallmarks of aging and proposed a choreography of events followed by most of the wild-type cells [226]. Indeed, nine cells out of ten (after removing *petite* cells from the analysis) are experiencing the same aging scenario (Figure 59), starting with 1/ the excision of an ERC. Then, 2/ this circle self-multiplies throughout the divisions until a threshold number of copies, at which the cell reaches the SEP.



*Figure 58: Average cell-cycle duration (left) and number of presumptive rDNA copies (including ERCs) (right), aligned to the SEP, as a function of age. N=64. Error bar represents the SEP. (From [226]).*

Interestingly, the activity of the rDNA transcription machinery, as well as the pre-rRNA content, are upregulated before the SEP, following the ERCs accumulation dynamics. The fact that the ERC accumulation (followed using a LacO system inserted in each repeat of the rDNA) always precedes the SEP and that the SEP occurs at a threshold number of circles strongly suggests that ERCs are the cause (direct or indirect) of this cell-cycle slowdown (Figure 58). Moreover, that would explain why the SEP is so abrupt (happening in less than three divisions) since the ERC number goes from ~240 copies to ~550 copies around the SEP.

Then, from the SEP, 3/ the N/C ratio starts increasing, and the cells show accumulating defects such as nucleus fragmentation and aberrant nucleolus size, until death.

Supporting this model and the causality of ERCs, two-thirds of the cells lacking FOB1 (known to reduce the excision rate, see p.56) do not experience a SEP nor an ERC accumulation, and those who do have a SEP reach it later but also accumulate ERCs until the same threshold than WT cells.

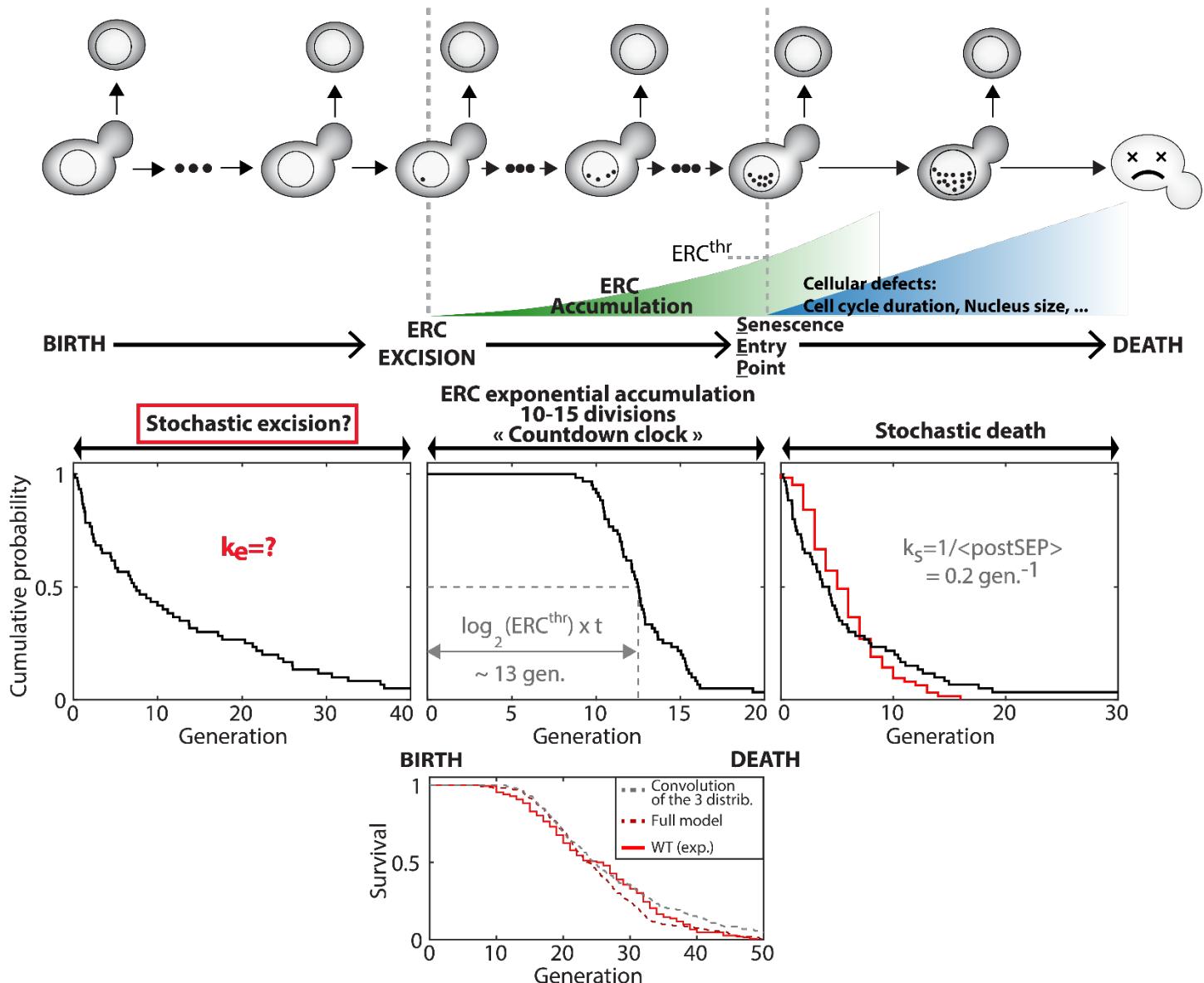


Figure 59: (Top): Schematic describing the three phases involved in the ERC-dependent senescence (ERC excision, self-multiplication, and post-SEP interval). (Middle): Cumulative probability associated with the three phases, numerically computed (black line) or from experimental data (red line). (Bottom): Survival curve obtained experimentally (solid line), by convoluting the three distributions from the middle lane (grey dashed lined), or by adding a mild ERC-independent cause of death to the convolution. (From [226])

This model raises several molecular questions raised by this model (discussed in the general discussion, p.141). Beyond that, we can note several points about the statistics of all the 3 phases:

Indeed, the period 2/ is quasi-deterministic (as modeled mathematically [226] and shown in Figure 59). This is explained by the well-controlled replication machinery which drives the accumulation until the threshold triggering the SEP.

Then, period 3/ between the SEP and death, is age-independent, with a constant 20% probability of dying at each division.

Therefore, none of these processes are age-dependent.

**Thus, what are the statistics of the period 1/ from birth to excision?**

**Is 1/ an age-dependent or a stochastic distribution? In other words, is the probability of ERC excision influenced by other aging factors, or is it the first event of a pathway of aging?**

Bulk assays report that Sir2 protein levels and silencing at the rDNA are decreased in replicatively old mothers while rDNA recombinations are increased [257,321], though unpublished data from our group suggest it is not the case. Similarly, a recent study suggests that the increase of cell size could decrease Sir2 levels (without direct evidence) [240].

Therefore, the few data available in the literature suggest that the probability of excision could increase with age.

On the other hand, numerical modeling shows that it is possible to recapitulate the survival curve of WT cells by convoluting an exponential decay (i.e., age-independent process, representing 1/ the excision phase), with a gaussian decay (pseudo-deterministic process, representing 2/ the accumulation phase) with another exponential decay (representing 3/ the post-SEP phase). In other words, ERC excision can be independent of age in this 3 phases model. This result differs from previous mathematical modeling in which a quadratic increase of the ERC excision probability with age is needed to recapitulate the experimental survival curve [426]. However, this model does not take into account the 3rd phase but only considers death at a threshold number of ERCs, which explains why it needs an age-dependent distribution for the first excision.

The hypothesis that the probability of ERC excision is independent of age raises a broader conceptual implication. In fact, this means that it is theoretically possible to reproduce a Gompertzian distribution (describing aging per definition) solely from age-independent processes. In other words, aging could arise from stochasticity.

To test this hypothesis experimentally but also to try rationalizing the different hallmarks of aging into pathways, a large number of cells must be analyzed in different mutants and conditions. Yet, despite recent progress in microfluidic-based approaches, replicative lifespan assays still suffer from throughput problems. Indeed, the low number of cells per experiment and the manual analysis of the timelapse data currently limit the potential for large-scale longevity screens.

## 4. Objectives

Consequently, my PhD thesis had two objectives:

1. Develop a platform to track and analyze cells throughout their replicative lifespan in a high-throughput manner.
2. Measure the excision rate of ERC during aging: Is it a stochastic or age-dependent process?

# Results

This result section will be split into 2 parts:

**Results I:** Setting up a platform for automated and high-throughput tracking of replicative lifespan

**Results II:** Measuring the ERC excision probability with age

Each part has its own short introduction to remind the main challenges and its own discussion, and will be put into perspective in a later General discussion.

## 5. Results I: Setting up a platform for automated and high-throughput tracking of replicative lifespan

### 1.1. Introduction

The current microfluidic devices and imaging systems have a limited throughput due to the number of cells they can trap and image simultaneously. More importantly, replicative lifespan assays consist of counting divisions, and up to now, this task has to be performed by a human, which significantly impairs the analysis throughput.

Machine-learning could tackle this problem, but existing pipelines can only segment cells and never exploit the underlying temporal correlations between images in time-lapse datasets. In order to increase the throughput power of replicative lifespan experiments, we created a platform to 1. Trap 32000 mother cells distributed in 16 different conditions 2. Track them using timelapse microscopy, and 3. Automatically detect when they divide, measure their size and fluorescence.

### 1.2. Results

(See attached paper along the next pages. Please check the latest version online, since modifications might be done after the publication of this thesis)

# DetecDiv, a deep-learning platform for automated cell division tracking and replicative lifespan analysis

Théo Aspert<sup>1234\*</sup>, Didier Hentsch<sup>1234</sup> and Gilles Charvin<sup>1234\*</sup>

1) Department of Developmental Biology and Stem Cells, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France

2) Centre National de la Recherche Scientifique, UMR7104, Illkirch, France

3) Institut National de la Santé et de la Recherche Médicale, U964, Illkirch, France

4) Université de Strasbourg, Illkirch, France

\*: Corresponding authors: [charvin@igbmc.fr](mailto:charvin@igbmc.fr), [aspertt@igbmc.fr](mailto:aspertt@igbmc.fr)

## Abstract

Automating the extraction of meaningful temporal information from sequences of microscopy images represents a major challenge to characterize dynamical biological processes. So far, strong limitations in the ability to quantitatively analyze single-cell trajectories have prevented large-scale investigations of the mechanisms that drive the entry into replicative senescence in yeast. Here, we have developed DetecDiv, a microfluidic-based image acquisition platform combined with deep learning-based software for high-throughput single-cell division tracking. We show that DetecDiv can reconstruct cellular replicative lifespans with outstanding accuracy. In addition, this methodology provides comprehensive temporal cellular metrics using time-series classification and image semantic segmentation. Hence, this methodology provides an all-in-one toolbox for quantitative division tracking assays that will pave the way for high-throughput phenotyping of single-cell dynamics and cellular aging studies.

## Introduction

Yeast has a limited replicative lifespan (RLS, i.e., 20-30 divisions) before entering senescence and dying<sup>1</sup>. Over the last decades, this simple unicellular has become a reference model for understanding the fundamental mechanisms that control longevity<sup>2,3</sup>. Several independent mechanistic models have been proposed to explain entry into replicative senescence<sup>4-6</sup>. In

this context, whether there are multiple parallel causes responsible for senescence remains highly debated<sup>7,8</sup>.

A crucial difficulty in solving this puzzle lies in the very labor-intensive nature of RLS assays<sup>9</sup> and the limited information derived from them regarding the dynamics of senescence entry. The initial development of microfluidic systems for RLS assays has partially alleviated this problem by allowing continuous observation of individual cell divisions and relevant fluorescent cellular markers under the microscope from birth to death<sup>10-12</sup>. Recent efforts further increased data acquisition throughput<sup>13,14</sup> and attempted to automate data analysis<sup>15,16</sup>. Yet, retrieving individual cellular lifespans from large sets of image sequences so far remained an insurmountable bottleneck to characterize senescence entry quantitatively or to screen large numbers of mutants and environmental conditions.

Here, we report the development of DetecDiv, an integrated platform that combines high-throughput observation of cell divisions using a microfluidic device, a simple benchtop image acquisition system, and a deep learning-based image processing software with several image classification frameworks. Using this methodology, one can accurately track cell division in an automated manner with outstanding accuracy. By combining this pipeline with additional deep learning models for time series classification and semantic segmentation, we provide a comprehensive toolset for an in-depth quantification of single-cell trajectories during entry into senescence.

## Results

To overcome the current limitations inherent to replicative lifespan assays, we have developed an imaging platform that combines a custom benchtop microscope, a microfluidic device, and a set of deep learning-based image processing pipelines.

The microscope was built using a rigid frame with inverted epifluorescence optics, transmission (bright field) illumination, a camera, and a motorized stage (Fig. S1A and B). The motorized frame carries the microfluidic device to trap individual cells and follow their successive divisions from birth to death (Fig. 1A, S1C, and S1D). Even though its principle is similar to previously reported designs<sup>13,14,17</sup>, we have brought significant improvements to the trap geometry to increase cell retention, avoid replacement of a mother cell by its daughter, and prevent device clogging and any source of contamination (see Fig S1E-G and supplementary text for details). The device includes 16 independent chambers (with 2000

## **Chapter I: Results I: Setting up a platform for automated and high-throughput tracking of replicative lifespan**

---

traps per chamber) to image different strains in parallel or to varying environmental conditions (Fig. 1A and 1B). Altogether, this system allows following the successive divisions and the entry into senescence of typically 30000 individual cells in parallel with a 5-min resolution (knowing that there are ~500 traps per field of view using a 20x objective), i.e., about 1 to 2 orders of magnitude above the previously described techniques <sup>10,13</sup>.

This image acquisition system generates a large amount of cell division data (on the Terabytes scale depending on the number of channels, frames, and fields of view), only a tiny part of which can be manually curated in a reasonable time. In particular, the determination of replicative lifespans requires counting successive cell divisions until death, hence, reviewing all images acquired for each cell in each field of view over time. In addition, automating the division counting process is complicated by the heterogeneity in cell fate (i.e. division times, cell shape), especially during the entry into senescence. To overcome this limitation, we have developed an image classification pipeline to count divisions and reconstruct the entire lifespan of individual cells dividing in the traps (Fig. 1C). For this, we have trained a GoogleNet convolutional neural network (CNN) <sup>18</sup> to determine the budding state of the trapped cells by assigning one of six possible classes (unbudded, small-budded, large-budded, dead, empty trap, clogged trap) to each frame, see Fig. S2 and S3A. In this framework, the alternation between the 'large budded' and the 'unbudded'/'small budded' states revealed the successive cell divisions, and the occurrence of the 'dead' class allowed us to reconstruct the cell's lifespan (Fig. 1C)

However, the assignment of the cellular state based on the CNN, which processes the images independently of one another, led to sporadic ambiguities and errors that compromise the identification of division events (Fig. 2A), hence the distribution of division times (Fig. 2B) and *a fortiori* of the lifespans (Fig. 2A and 2C). These problems could be partially alleviated by post-processing the predictions made by the CNN (see "CNN+PP" in Fig. 2A-C and supplementary text for details). Yet, to improve the robustness of the method, we have combined the CNN with a long short-term memory network (LSTM) <sup>19,20</sup>, to take into account the time-dependencies between images (Fig. 1C). Thus, by providing full image sequences rather than individual images, we obtained an outstanding accuracy and recall for both division quantification and lifespan reconstruction (Fig. 2B, 2C, S3D, S3E, and Movie M1). Comparing the predictions by the classifier to the manually annotated data ("ground truth") revealed a non-significant difference in the distributions and an excellent correlation ( $R^2=0.99$  for both divisions and lifespans, Fig 2B and 2C). To estimate the robustness of the classification model which was only trained on images of WT cells, we measured the large-

scale RLS in classical longevity mutants. Remarkably, we recapitulated the increase (resp. decrease) in longevity observed in the *fob1Δ* (resp. *sir2Δ*) mutant<sup>21,22</sup> and we could compute the related death rate with a high-confidence interval using a large-scale dataset (Fig 2D)<sup>23</sup>. Importantly, only 200 manually annotated lifespans (i.e. used during the training procedure) were necessary to achieve robust RLS reconstruction. Thus, rapid user annotation of a small cohort of cells allows the model to be deployed on larger datasets in different genetic contexts and/or environments.

We then sought to apply other classification schemes of DetecDiv to further characterize the trajectories of the cells as they transition to senescence. First, we set up an LSTM sequence-to-sequence classifier to detect the onset of cell-cycle slowdown during entry into senescence (also referred to as the senescence entry point or SEP<sup>12</sup>), see Fig. 3A and S4. We thus trained the classifier to assign a ‘pre-SD’ or ‘post-SD’ label (before and after the cell-cycle slowdown, respectively) to each frame, using the sequence of cellular state probabilities (i.e., the output of the CNN/LSTM image classifier described in Fig. 1C) as input. Using this method, we could successfully identify the transition to a slow division mode (Fig. 3B and 3C) and recapitulate the evolution of average division times after aligning individual trajectories from that transition (Fig. 3D).

Second, we used an encoder/decoder network based on a Resnet50 CNN<sup>24</sup> and the DeepLab v3+ architecture<sup>25</sup>, see Fig. S5, to segment brightfield and fluorescence images of cells carrying a histone-Neongreen fusion (see Fig 4A, Movie M2 and supplementary text)<sup>25</sup>. After training the model on ~1500 manually segmented brightfield images using three output classes (i.e., ‘background,’ ‘mother cell,’ ‘other cell’), we obtained accurate mother cells contours (Fig. 4A-C and S6). This allowed us to quantify the cellular volume increase (4C and 4D), as previously reported<sup>23</sup>. A similar training procedure with ~3000 fluorescence images yielded accurate nuclei contours (see Fig. 4E, 4F, and S7). It successfully recapitulated the sharp burst in histone fluorescence that follows cell-cycle slowdown (Fig. 4G, 4H)<sup>23</sup>, hence further validating our methodology for automated quantitative analyses of division tracking and replicative lifespan assays.

## Discussion

In this study, we report the development of a microscopy pipeline to acquire and track cell division events with high throughput, hence unleashing the potential of microfluidic cell trapping devices to perform fully automated replicative lifespan analyses.

The imaging system was designed to perform heavy-duty image acquisition sequences (i.e., no filter wheel, fixed objective) to generate high-throughput microscopy datasets. The hardware could be easily assembled from simple optical components -for a price of about one-third that of a commercial automated microscope. By processing temporal sequences of images rather than individual ones, our software demonstrated an outstanding accuracy that matches human capabilities for image classification yet with much higher throughput.

The robustness of the imaging pipeline benefited from improvements made in the design of the microfluidic device (see supplementary text). Therefore, our framework now overcomes all intrinsic technical limitations of conventional RLS assays and provides an unprecedented potential to perform large screens for players and environmental perturbations that dynamically control replicative longevity.

More broadly, this work illustrates how temporal dependencies in image sequences can be exploited using a combined CNN and LSTM architecture to accurately reveal and quantify dynamic cellular processes. Despite large efforts to make deep-learning models available to the community of microscopists, very little work has attempted to fully exploit information encoded in image sequences. With its comprehensive set of generic classification schemes that can be fully user-parameterized, DetecDiv may be used well beyond the scope of the present study and applied to any biological context with complex temporal patterns (cellular differentiation, cell division, organelles dynamics, etc).

## **Methods**

### **Strains**

All strains used in this study are congenic to S288C (see Supplementary Table 1 for details). See supplementary methods for detailed protocols for cell culture.

### **Microfabrication and microfluidics**

The designs were created on AutoCAD to produce chrome photomasks (jd-photodata, UK). The microfluidic master molds were then made by standard photolithography processes (see supplementary text for details).

The microfluidic device is composed of geometric microstructures that allow mother cells trapping and flushing of successive daughter cells (see Fig. S2 and supplementary text). The cell retention efficiency of the traps is 99% after the five first divisions. We designed a particle filter with a cutoff size of 5 µm to prevent dust particles or debris from clogging the chip. The microfluidic chips were fabricated with PDMS using standard methods (PDMS, Sylgard 184, Dow Chemical, USA, see supplementary text for detailed protocols). We connected the chip using PTFE tubing (1mm OD), and we used a peristaltic pump to ensure media replenishment (Ismatec, Switzerland). We used standard rich media supplemented with 2% dextrose (YPD). See supplementary methods for additional details.

## **Microscopy**

The microscope was built from a modular microscope system with a motorized stage (ASI, USA, see the supplementary text for the detailed list of components), a 20x objective 0.45 (Nikon, Japan) lens, and an sCMOS camera (ORCA flash 4.0, Hamamatsu, Japan). A dual-band filter (#59022, Chroma Technology, Germany) coupled with a two-channel LED system (DC4104 and LED4D067, Thorlabs, USA). Sample temperature was maintained at 30°C thanks to a heating system based on an Indium Thin Oxide coated glass and an infrared sensor coupled to an Arduino-based regulatory loop. Micromanager v2.0<sup>26</sup> was used to drive all hardware, including the camera, the light sources, and the stage and objective motors. We developed a custom autofocusing routine to minimize the autofocus time ([https://github.com/TAspert/DetecDiv\\_Hardware](https://github.com/TAspert/DetecDiv_Hardware)). The interval between two frames for all the experiments was 5min. We could image approximately 80 fields of view (0.65mmx0.65mm) in brightfield and fluorescence (using a dual-band GFP-mCherry filter) with this interval.

## **Image processing**

We developed Matlab software, DetecDiv, which provides different classification models: image classification, image sequence classification, time series classification, and pixel classification (semantic segmentation), see Fig. S9. DetecDiv was developed using Matlab, and additional toolboxes (TB), such as the Computer Vision TB, the Deep-learning TB, and the Image Processing TB. A graphical user interface was designed to facilitate the generation

## Chapter I: Results I: Setting up a platform for automated and high-throughput tracking of replicative lifespan

---

of the training sets. The DetecDiv software is available for download on GitHub: <https://github.com/gcharvin/DetecDiv>

### Image classification for division tracking and lifespan reconstruction

DetecDiv was used to classify images into six classes after supervised training using a GoogleNet<sup>18</sup> network combined with an LSTM network<sup>20</sup>. See supplementary text for details.

### Image segmentation from brightfield and fluorescent images

DetecDiv was used to segment images using a pixel classification model called Deeplab v3+<sup>25</sup>, after supervised training based on 1000-3000 manually annotated images. See supplementary text for details.

### Cell-cycle slowdown detection

DetecDiv was used to detect cell-cycle slowdown from a temporal sequence of classes obtained using the division tracking network. The training was based on manual annotation of 200 lifespans. See supplementary text for details.

### Statistics

All experiments have been replicated at least twice. Data are presented in Results and Figures as the mean  $\pm$  SEM (curves) or median. Group means were compared using the Two-sample t-test. A P value of  $< 0.05$  was considered significant.

### Computing

Image processing was performed on a computing server with 8 Intel Xeon E5-2620 processors and 8 co-processing GPU units (Nvidia Tesla K80), each of them with 12Go RAM. Under these conditions, the image classification of a single trap (roughly 60x60pixels) with 1000 frames took between 3 and 5s for the CNN/LSTM classifier. For image segmentation, it took about 30s to classify 1000 images.

## Acknowledgments

We thank Audrey Matifas for constant technical support throughout this work, Sophie Quintin and Nacho Molina for carefully reading the manuscript. We are grateful to Olivier Tassy for their insightful discussions. We thank Denis Fumagalli at the IGBMC Mediaprep facility for media preparation. We are grateful to the IT service for efficient support and providing the computing resources. We thank the Charvin lab members, Bertrand Vernay, Jerome Mutterer,

Serge Taubert, and the IGBMC imaging facility for discussions and technical support. This work was supported by the Agence Nationale pour la Recherche (T.A. and G.C.), the grant ANR-10-LABX-0030-INRT, a French State fund managed by the Agence Nationale de la Recherche under the frame program Investissements d'Avenir ANR-10-IDEX-0002-02.

## Data Availability

Annotated datasets and trained classifiers used in this study are available for download as indicated:

- Lifespan analyses:
  - Data: [doi.org/10.5281/zenodo.5552642](https://doi.org/10.5281/zenodo.5552642)
  - Trained Network (CNN+LSTM): [doi.org/10.5281/zenodo.5553862](https://doi.org/10.5281/zenodo.5553862)
- Brightfield image segmentation:
  - Data: [doi.org/10.5281/zenodo.5553771](https://doi.org/10.5281/zenodo.5553771)
  - Trained Network (Encoder-Decoder Deeplabv3+):  
[doi.org/10.5281/zenodo.5553851](https://doi.org/10.5281/zenodo.5553851)
- Cell-cycle slowdown detection:
  - Data: [doi.org/10.5281/zenodo.5553796](https://doi.org/10.5281/zenodo.5553796)
  - Trained Network (LSTM): [doi.org/10.5281/zenodo.5553829](https://doi.org/10.5281/zenodo.5553829)

Information regarding the microfluidic device, the custom imaging system are available on [https://github.com/TAspert/DetecDiv\\_Data](https://github.com/TAspert/DetecDiv_Data).

## Code availability

The custom MATLAB software DetecDiv, used to analyze imaging data with deep-learning algorithms, is available on <https://github.com/gcharvin/DetecDiv>.

This software distribution features a tutorial on how to use the graphical user interface:

[https://github.com/gcharvin/DetecDiv/blob/master/Tutorial/GUI\\_tutorial.md](https://github.com/gcharvin/DetecDiv/blob/master/Tutorial/GUI_tutorial.md)

It also provides the main commands to use the DetecDiv pipeline in custom user-defined scripts:

[https://github.com/gcharvin/DetecDiv/blob/master/Tutorial/commandline\\_tutorial.md](https://github.com/gcharvin/DetecDiv/blob/master/Tutorial/commandline_tutorial.md)

## Chapter I: Results I: Setting up a platform for automated and high-throughput tracking of replicative lifespan

A demo project that contains all the necessary files to learn how to use DetecDiv can be downloaded from zenodo :

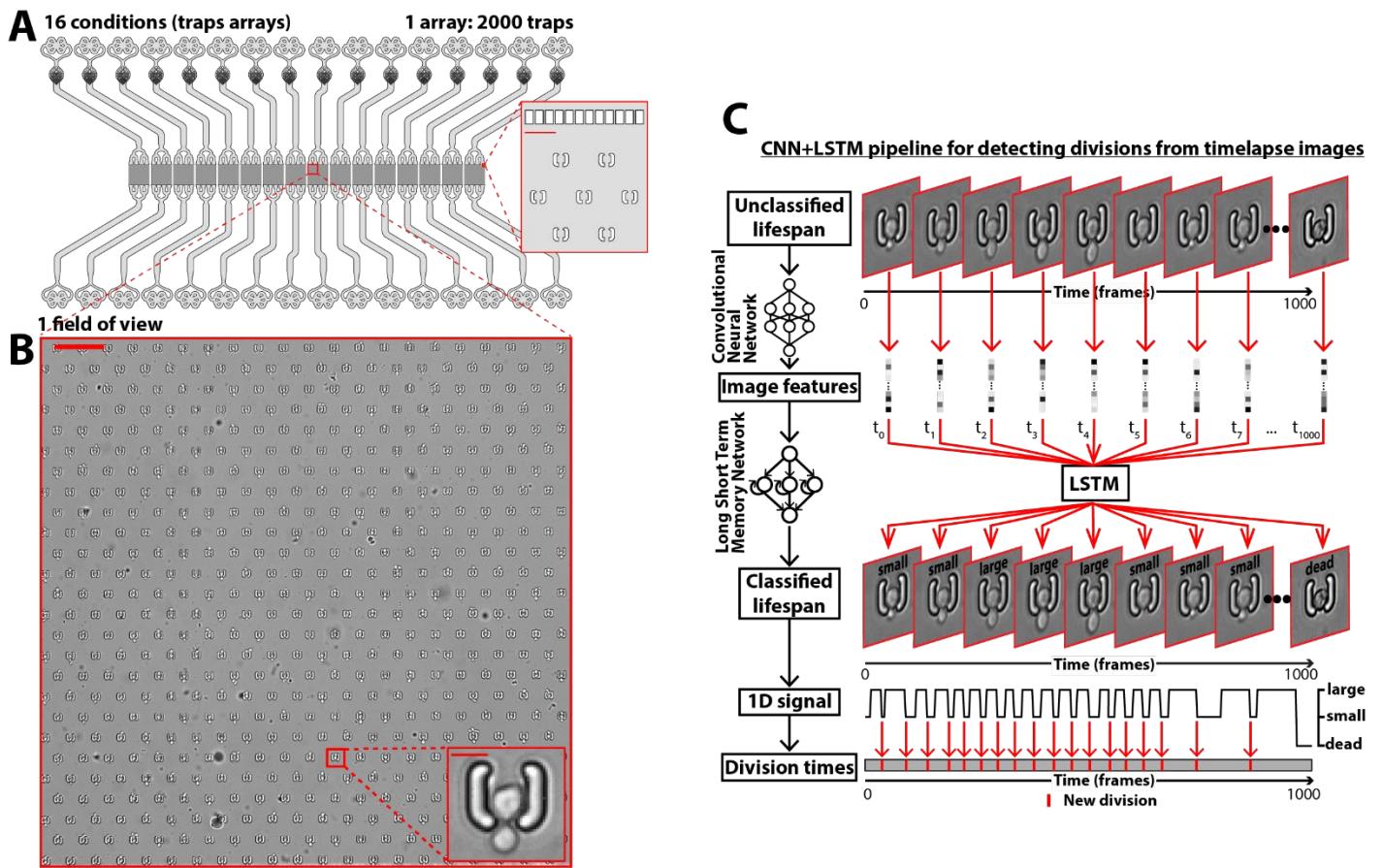
<https://doi.org/10.5281/zenodo.5771536>

## References

1. Mortimer, R. K. & Johnston, J. R. Life span of individual yeast cells. *Nature* 183, 1751–1752 (1959).
2. Denoth-Lippuner, A., Julou, T. & Barral, Y. Budding yeast as a model organism to study the effects of age. *FEMS Microbiol. Rev.* 38, 300–325 (2014).
3. Janssens, G. E. & Veenhoff, L. M. Evidence for the hallmarks of human aging in replicatively aging yeast. *Microb. Cell Fact.* 3, 263–274 (2016).
4. Hughes, A. L. & Gottschling, D. E. An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast. *Nature* 492, 261–265 (2012).
5. Sinclair, D. A. & Guarente, L. Extrachromosomal rDNA circles--a cause of aging in yeast. *Cell* 91, 1033–1042 (1997).
6. Aguilaniu, H., Gustafsson, L., Rigoulet, M. & Nyström, T. Asymmetric inheritance of oxidatively damaged proteins during cytokinesis. *Science* 299, 1751–1753 (2003).
7. Dillin, A., Gottschling, D. E. & Nyström, T. The good and the bad of being connected: the integrins of aging. *Curr. Opin. Cell Biol.* 26, 107–112 (2014).
8. He, C., Zhou, C. & Kennedy, B. K. The yeast replicative aging model. *Biochim. Biophys. Acta Mol. Basis Dis.* 1864, 2690–2696 (2018).
9. McCormick, M. A. et al. A Comprehensive Analysis of Replicative Lifespan in 4,698 Single-Gene Deletion Strains Uncovers Conserved Mechanisms of Aging. *Cell Metab.* 22, 895–906 (2015).
10. Lee, S. S., Vizcarra, I. A., Huberts, D. H. E. W., Lee, L. P. & Heinemann, M. Whole lifespan microscopic observation of budding yeast aging through a microfluidic dissection platform. *Proceedings of the National Academy of Sciences* 109, 4916–4920 (2012).
11. Xie, Z. et al. Molecular phenotyping of aging in single yeast cells using a novel microfluidic device. *Aging Cell* (2012).
12. Fehrman, S. et al. Aging yeast cells undergo a sharp entry into senescence unrelated to the loss of mitochondrial membrane potential. *Cell Rep.* 5, 1589–1599 (2013).
13. Jo, M. C., Liu, W., Gu, L., Dang, W. & Qin, L. High-throughput analysis of yeast replicative aging using a microfluidic system. *Proc. Natl. Acad. Sci. U. S. A.* 112, 9364–9369 (2015).
14. Liu, P., Young, T. Z. & Acar, M. Yeast Replicator: A High-Throughput Multiplexed Microfluidics Platform for Automated Measurements of Single-Cell Aging. *Cell Rep.* 13, 634–644 (2015).

15. Ghafari, M. et al. Complementary performances of convolutional and capsule neural networks on classifying microfluidic images of dividing yeast cells. *PLoS One* 16, e0246988 (2021).
16. Ghafari, M., Mailman, D. & Qin, H. Application Note:  $\mu$ Polar - An Interactive 2D Visualization Tool for Time-Series Microscopic Images. (2021) doi:10.2139/ssrn.3827406.
17. Crane, M. M., Clark, I. B. N., Bakker, E., Smith, S. & Swain, P. S. A Microfluidic System for Studying Ageing and Dynamic Single-Cell Responses in Budding Yeast. 9, e100042 (2014).
18. Szegedy, C. et al. Going deeper with convolutions. in 2015 IEEE Conference on Computer Vision and Pattern Recognition (CVPR) 1–9 (2015).
19. Venugopalan, S. et al. Sequence to sequence -- video to text. in 2015 IEEE International Conference on Computer Vision (ICCV) (IEEE, 2015). doi:10.1109/iccv.2015.515.
20. Hochreiter, S. & Schmidhuber, J. Long Short-Term Memory. *Neural Comput.* 9, 1735–1780 (1997).
21. Defossez, P. A. et al. Elimination of replication block protein Fob1 extends the life span of yeast mother cells. *Mol. Cell* 3, 447–455 (1999).
22. Lin, S. J., Defossez, P. A. & Guarente, L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. *Science* 289, 2126–2128 (2000).
23. Morlot, S. et al. Excessive rDNA Transcription Drives the Disruption in Nuclear Homeostasis during Entry into Senescence in Budding Yeast. *Cell Rep.* 28, 408–422.e4 (2019).
24. He, K., Zhang, X., Ren, S. & Sun, J. Deep Residual Learning for Image Recognition. in 2016 IEEE Conference on Computer Vision and Pattern Recognition (CVPR) 770–778 (2016).
25. Chen, L.-C., Zhu, Y., Papandreou, G., Schroff, F. & Adam, H. Encoder-decoder with atrous separable convolution for semantic image segmentation. *arXiv [cs.CV]* (2018).
26. Edelstein, A. D. et al. Advanced methods of microscope control using  $\mu$ Manager software. *J Biol Methods* 1, (2014).
27. Pokhrel, A., Dyba, T. & Hakulinen, T. A Greenwood formula for standard error of the age-standardised relative survival ratio. *Eur. J. Cancer* 44, 441–447 (2008).

**Figure 1**



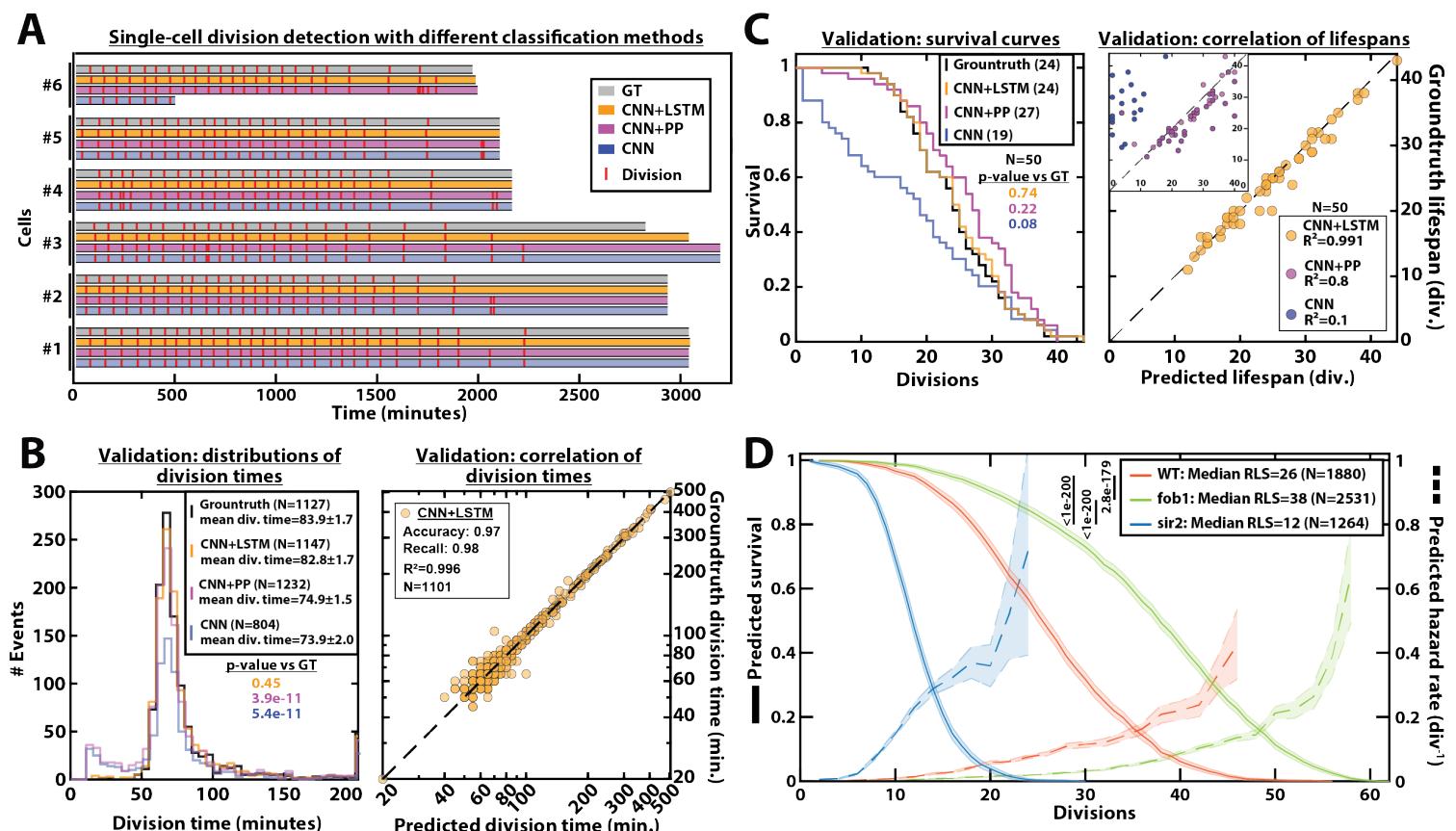
**Figure 1 - DetectDiv division tracking and RLS reconstruction pipeline**

A) Left: Sketch of the microfluidic device, featuring 16 independent channels with 2000 individual cell traps in each; Right: zoom on the cellular traps, scale bar: 20μm.

B) Brightfield image from a typical field of view obtained with the imaging setup, scale bar: 60μm; Inset: Zoom on one trap containing a budding yeast, scale bar: 5μm.

C) Principles of the DetectDiv division tracking and lifespan reconstruction pipeline; Brightfield images are processed by a convolutional neural network (CNN) to extract representative image features. The sequence of image features is then processed by a long short-term memory network (LSTM) that assigns one of the 6 predefined classes ('unbud', 'small', 'large', 'dead', 'clog', 'empty'), taking into account the time dependencies. Temporal oscillations between "large" and "small" or "large" and "unbudded" indicate the completion of the division cycle. The appearance of the "dead" class marks the end of the lifespan.

**Figure 2**



**Figure 2 - Validations of the division tracking and RLS reconstruction**

A) Comparison of the different methods used for 6 sample cells. The gray bars represent the ground truth data made from manually annotated image sequences. Colored lines indicate the corresponding predictions made by CNN/LSTM (orange), the CNN + post-processing (magenta), and the CNN (blue) networks (see Methods and supplementary text for details). The red segments indicate the position of division events.

B) Left: histogram of division times representing ground truth data and predictions using different processing pipelines. The p-value indicates the results of a rank-sum test comparing the predictions to the ground truth for the different pipeline variants. The total number of division events annotated in the ground truth or detected by the networks are indicated in the legend. Right: Scatter plot in log scale representing the correlation between ground-truth-calculated division times and those predicted by the CNN/LSTM network.  $R^2$  represents the coefficient of correlation between the two datasets. Accuracy and recall are defined in the supplementary text.

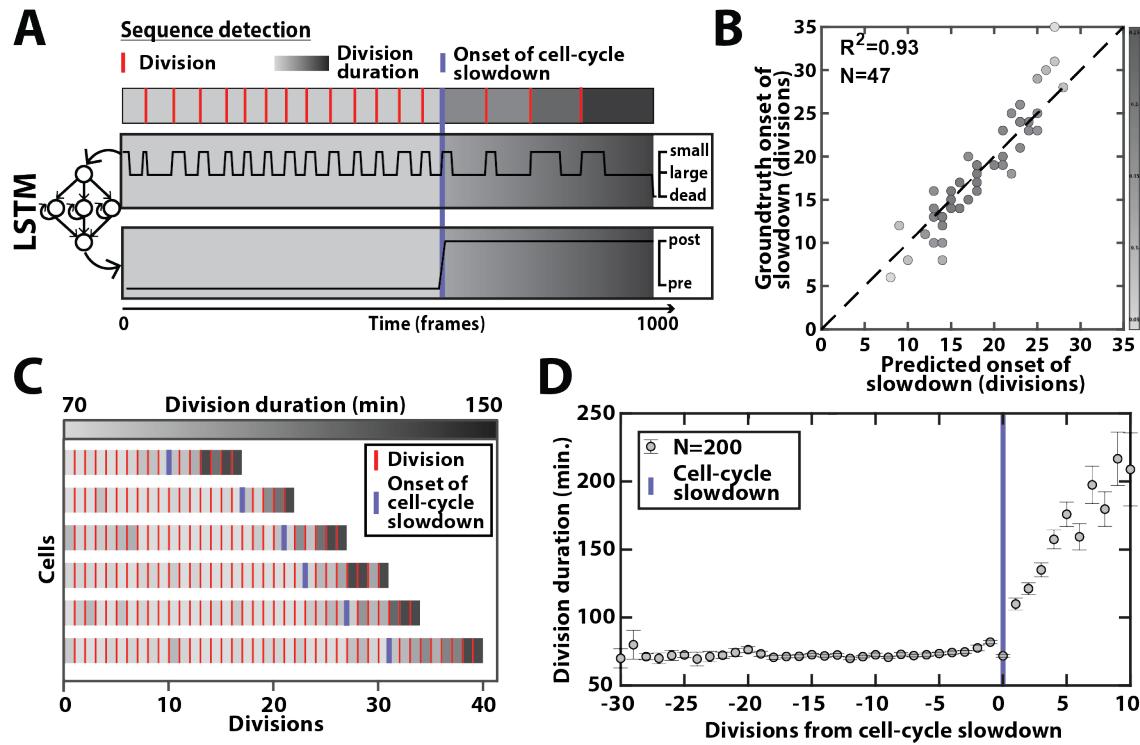
C) Left: cumulative distribution showing the survival of cells as a function of the number of divisions (N=50 cells). The numbers in the legend indicate the median replicative lifespans. The p-value indicates the results from a statistical rank-sum test. Right: Scatter plot showing

## Chapter I: Results I: Setting up a platform for automated and high-throughput tracking of replicative lifespan

the correlation of the replicative lifespans of individual cells obtained from the ground truth with that predicted by the CNN/LSTM architecture (N=50). Inset: same as the main plot, but for the CNN and CNN with post-processing pipelines.  $R^2$  indicates the coefficient of correlation between the two datasets.

D) Replicative lifespans obtained using the CNN/LSTM network for longevity mutants (solid colored lines, genotype indicated). The shading represents the 95% confidence interval calculated using the Greenwood method<sup>27</sup>. The median RLS and the number of cells analyzed are indicated in the legend. The dashed lines with shading represent the hazard rate and its standard deviation estimated with a bootstrap test (N=100). Results from log-rank tests (comparing WT and mutant distributions) are indicated on the left of the legend.

**Figure 3**



**Figure 3 - Deep learning-based measurement of the dynamics of entry into senescence**

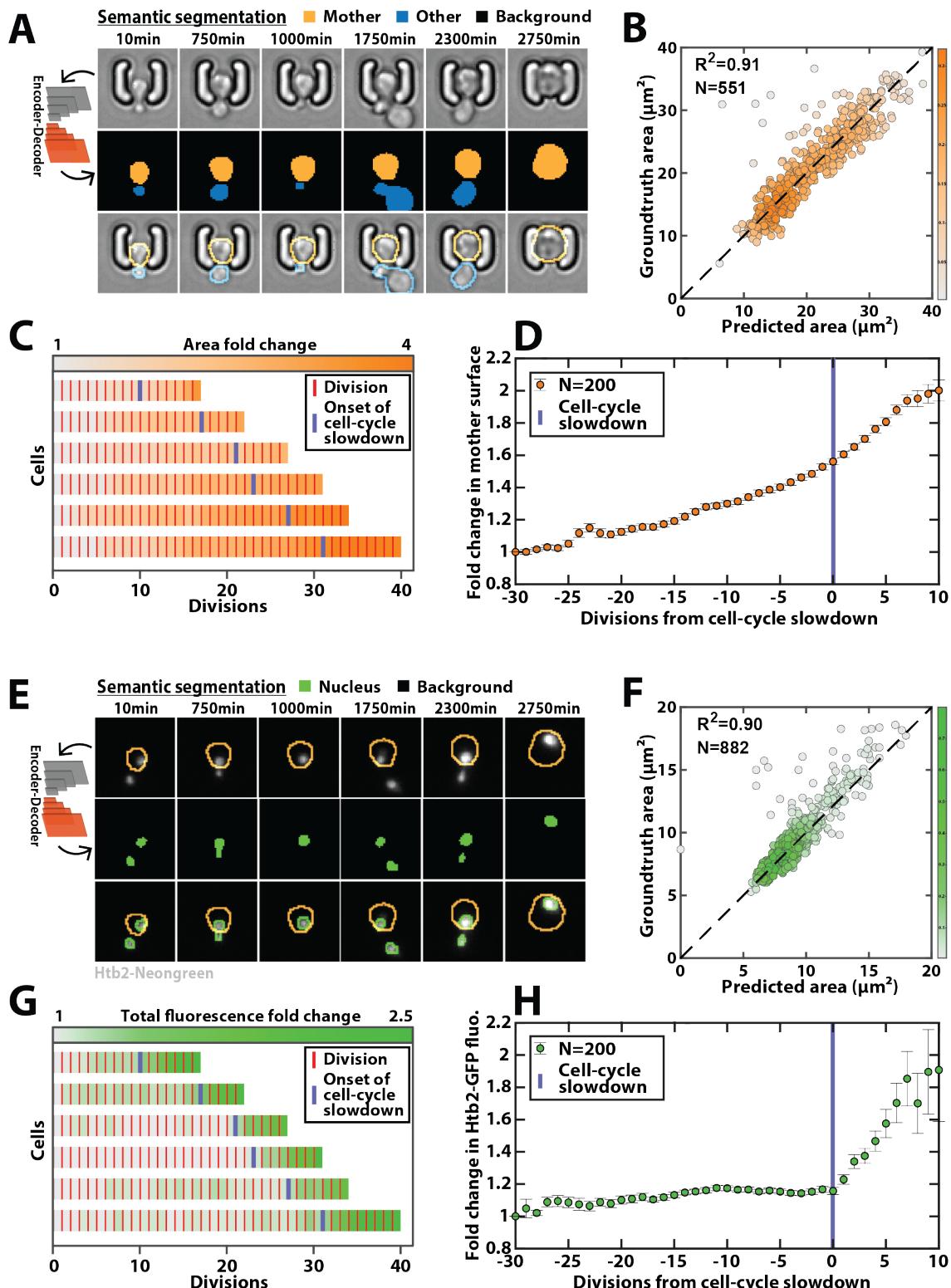
A) Sketch depicting the detection of the time of slow division (SD) state. The temporal sequence of classes probabilities (ie, unbud, small, large, dead) is fed into an LSTM network that predicts the onset of cell-cycle slowdown by assigning one of the two predefined classes pre-SD or post-SD to each frame.

B) Correlogram showing the correlation between the onset of cell-cycle slowdown predicted by the LSTM network and the ground truth data, obtained as previously described <sup>12</sup>. The color code indicates the local density of the points using arbitrary units as indicated by the color bar.

C) Sample trajectories indicating the successive divisions of individual cells (red lines) along with the division times (color-coded as indicated).

D) Average division time versus division index after aligning all individual trajectories from the onset of cell-cycle slowdown <sup>12</sup>. Each point represents an average over up to 200 cell trajectories. The error bar represents the standard error-on-mean.

**Figure 4**



**Figure 4- Deep learning-based semantic segmentation of cells and nuclei**

A) Principles of semantic cell contours segmentation based on brightfield images ; Top and middle row) Individual brightfield images were processed by the DeeplabV3+ network that was trained to perform pixel classification using three predefined classes representing the

## **Chapter I: Results I: Setting up a platform for automated and high-throughput tracking of replicative lifespan**

---

background (black), the mother cell of interest (orange), or any other cell in the image (blue).

B) Bottom row: overlay of brightfield images with segmented cellular contours .

C) Correlogram showing the correlation between individual cell area predicted by the segmentation pipeline and the ground truth data, obtained by manual annotation of the images. The color code indicates the local density of the points using arbitrary units.

Sample trajectories indicating the successive divisions of individual cells (red lines) along with the cell surface (color-coded as indicated).

D) Average mother cell surface versus division index after aligning all individual trajectories from the onset of cell-cycle slowdown<sup>12</sup>. Each point represents an average over up to 200 cell trajectories. The error bar represents the standard error-on-mean.

E) Principles of semantic cell nuclei segmentation based on fluorescent images of cells expressing a histone-Neongreen fusion. The semantic segmentation network was trained to classify pixels between two predefined classes ('background' in black, 'nucleus' in green).

F) Same as B) but for nuclear surface.

G) Same as C) but for nuclear fluorescence.

H) Same as in D) but for nuclear fluorescence.

## Supplementary text

Théo Aspert<sup>1234\*</sup>, Didier Hentsch<sup>1234</sup> and Gilles Charvin<sup>1234\*</sup>

1) Department of Developmental Biology and Stem Cells, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France

2) Centre National de la Recherche Scientifique, UMR7104, Illkirch, France

3) Institut National de la Santé et de la Recherche Médicale, U964, Illkirch, France

4) Université de Strasbourg, Illkirch, France

\*: Corresponding authors: [charvin@igbmc.fr](mailto:charvin@igbmc.fr), [aspertt@igbmc.fr](mailto:aspertt@igbmc.fr)

## Supplementary Methods

### Cell culture

For each experiment, freshly thawed cells were grown overnight, diluted in the morning, and allowed to perform several divisions (~5 hours at 30°C) before injection into the microfluidic device. Yeast extract Peptone Dextrose (YPD) medium was used throughout the experiments. We found that synthetic media (SCD) tend to form small crystals that escape the microfluidic filter and may be detrimental to long-term cell growth. However, this phenomenon was not observed with YPD media.

### Microfluidic mold fabrication

The designs were created on AutoCAD (see [https://github.com/TAspert/DetecDiv\\_Data](https://github.com/TAspert/DetecDiv_Data) to download the design) to produce chrome photomasks (jd-photodata, UK). Then, the microfluidic master molds were made using two rounds of classical photolithography steps. The array of 2000 traps was created from a 5.25µm deposit by spinning (WS650 spin coater, Laurell, France) 3mL of SU8-2005 negative photoresist at 2500rpm for 30sec on a 3" wafer (Neyco, FRANCE). Then, a soft bake of 3min at 95°C on heating plates (VWR) was performed, followed by exposure to 365nm UVs at 120 mJ/cm<sup>2</sup> with a mask aligner (UV-KUB3, Kloé, FRANCE). Finally, a post-exposure bake identical to the soft bake was performed before development using SU-8 developer (Microchem, USA).

The second layer with channel motifs was made of a 30µm deposit of SU8-2025, by spinning it at 2500rpm for 30s. Subsequently, a soft bake of 3min at 65°C and 6min at 95°C was performed. The wafer was then aligned with the mask containing the motif of the second

layer before a 160mJ/cm<sup>2</sup> exposure. A post-exposure bake similar to the soft bake was then performed.

After each layer, we performed a hard bake at 150°C for 15min to anneal potential cracks and stabilize the photoresist. Finally, the master molds were treated with chlorotrimethylsilane to passivate the surface.

### **Microfluidics chip design, fabrication, and handling**

The microfluidic device is composed of an array of 2050 microstructures able to trap a mother cell while removing successive daughter cells, as previously designed (Ryley and Pereira-Smith 2006; Zhang et al. 2012; Lee et al. 2012), (Crane et al. 2014; Jo et al. 2015; Liu, Young, and Acar 2015). The traps are composed of two symmetrical structures separated by 3μm (see Fig. 1 and S1C and S1D), in such a way that only one cell can be trapped and remain in between the structures. We have measured that 99% of the cells that underwent at least 5 divisions in the trap would stay inside until their death. Moreover, a particle filter with a cutoff size of 15μm is present before each array of traps, preventing dust particles or debris from clogging the chip (Fig. S1C).

The microfluidic devices were fabricated using soft-lithography by pouring polydimethylsiloxane (PDMS, Sylgard 184, Dow Chemical, USA) with its curing agent (10:1 mixing ratio) on the different molds. The chips were punched with a 1mm biopsy tool (KAI, Japan) and covalently bound to a 24 × 50 mm coverslip using plasma surface activation (Diener Zepto, Germany). The assembled chips were baked for 1 hour at 60°C to consolidate covalent bonds between glass and PDMS. The chip was then plugged using a 1mm Outer Diameter (O.D.) PTFE tubing (Adtech, UK) and the channels were primed using culture media for 5min. After that, cells were injected through the outlet using a 5mL syringe and a 26G needle for approximately 1 minute per channel by applying very gentle pressure. The cell filter placed upstream of the trapping area prevented the cells from entering the tubing connected to the inlet (see Figure S1E). Then, the inlet of each microfluidic channel was connected to a peristaltic pump (Ismatec, Switzerland) with a 5μL/min rate to ensure a constant replenishment of the media and dissection of the daughter cells (Fig. S1E). This procedure avoids potential contamination by cells forming colonies upstream of the trapping area, which would induce the clogging of the device after 1-2 days of experiment, therefore making long lasting experiments more robust.

## Microscopy

### Microscope

The microscope was built from a modular microscope system (RAMM, ASI, USA) with trans- (Oly-Trans-Illum, ASI, USA) and epi- (Mim-Excite-Cond20N-K, ASI, USA) illumination. This microscope frame provides a cost-effective solution to build a minimal microscopy apparatus to perform robust image acquisition over several days (Fig. S1B).

It is equipped with a motorized XY stage (S551-2201B, ASI, USA), a stage controller (MS200, ASI, USA), and a stepper motor to drive the 20x N.A. 0.45 Plan Fluor objective (Nikon, Japan) and an sCMOS camera (ORCA flash 4.0, Hamamatsu, Japan) with 2048 x 2048 pixels (i.e., 650 microns x 650 microns field of view at 20x magnification). We used a dual-band filter (#59022, Chroma Technology, Germany) coupled with two-channel LED illumination (DC4104 and LED4D067, Thorlabs, USA), which allows fast imaging of GFP and mCherry without any filter switching.

### Sample holder and temperature control

We designed a custom 3D-printed sample holder (by extruding PLA material with a MK3S+ printer, Prusa Research, Czech republic) for the microfluidic device to ensure the mechanical stability of the microfluidic device.

In addition, we developed a custom temperature control system to maintain a constant temperature (30°C) and guarantee optimal cell growth throughout the experiment. Briefly, we used an Indium Tin Oxide (ITO) Coated glass, an electrically conductive and transparent material, in direct contact with the PDMS chip. Therefore, applying a voltage to the ITO glass allowed the Joule effect to heat the glass and the adjacent PDMS chip.

To achieve a temperature control loop, we used an infrared sensor attached to the objective and facing towards the bottom glass coverslip in contact with the cells. The sensor allowed *in situ* temperature measurement and was used in an Arduino-based PID control loop to regulate the heating power to maintain the setpoint temperature. About 1W was sufficient to maintain a 30°C temperature at room temperature. Notably, the temperature profile obtained with this method was homogenous and constant throughout the experiment. Furthermore, the glass is fully transparent to visible light. In addition, it does not interfere with fluorescent light when using an inverted microscope since it is located at the end of the optical path, after the sample.

### Software and time-lapse acquisition parameters

Micromanager v2.0. was used to drive the camera, the light source, the XYZ controller, and the LED light source for fluorescence epi-illumination. We developed a specific program in order to drive the temperature controller from the Arduino (The source code is available on github: [github.com/TAspert/ITO\\_heating\\_device](https://github.com/TAspert/ITO_heating_device)).

Unless specified otherwise, the interval between two brightfield frames for all the experiments was 5minutes, and images recorded over 1000 frames (i.e, ~3 days). We used three z-stack for brightfield imaging (spaced by 1.35 microns) to ease the detection of small buds during the image classification process for cell state determination. Fluorescent images were acquired with a 10min interval using 470nm illumination for 50ms. Up to 80 fields of view were recorded over the 5-min interval.

### Autofocusing

To keep a stable focus through the whole experiment, we developed a custom software-based autofocus routine that finds the sharpest image on the first field of view and then applies the focus correction to the rest of the positions. This method provides faster scanning of all fields of view in a reasonable time. Nevertheless, it is almost as efficient as performing autofocusing on each position since the primary source of defocusing in our setup is the thermal drift, which applies identically to all the positions.

## **Image processing**

### DetectDiv software

We designed custom software DetectDiv as a Matlab backend to provide an expandable interface to perform microscopy data processing, based on existing resources in Matlab's Deep Learning Toolbox and Computer Vision Toolbox. DetectDiv can be used with an arbitrarily large number of classes, image channels, types, and sizes (Figure S9). Therefore, its capabilities go far beyond the scope of this study.

First, DetectDiv offers graphical tools to identify regions of interest in image sequences. In addition, a cross-correlation function can be used to automatically detect similar regions in images, such as the traps in the microfluidic device.

Then, several classification models can be defined to process the images: 1) Image classification using a convolutional network (CNN, see Fig. S2); 2) a combined CNN/LSTM classifier as reported in fig. 1; 3) an LSTM network to perform sequence-to-sequence (as in Fig. 2A) or sequence-to-one classification; 4) An encoder/decoder classifier to perform pixel classification (semantic segmentation) based on the Deeplab v3+ architecture (Chen et al. 2018), see Fig 2A and 2B; 5) Similar routines as in 1-4, but for regression analyses (not used

## Chapter I: Results I: Setting up a platform for automated and high-throughput tracking of replicative lifespan

---

in the present study). DetecDiv allow the user to choose among several CNNs -such as GoogleNet or Resnet50 for all image classifications/segmentations applications.

DetecDiv provides a graphical user interface to generate the groundtruth required for both training and testing the classifiers used in the image classification, pixel classification, and time-series classification pipelines. Furthermore, we paid attention to making this step as user-friendly as possible. For instance, we used keyboard shortcuts to assign labels to individual frames (it takes about 5-10 min to annotate 1000 frames in the case of cell state assignment). Similarly, direct “painting” of objects with a mouse or a graph pad can be used to label images before launching the training procedure for pixel classification.

DetecDiv training and validation procedures are run at the command line, which allows using remote computing resources, such as a CPU/GPU cluster. All the relevant training parameters can be easily defined by the user. We designed generic routines to benchmark the trained classifiers that allow an in-depth evaluation of the classifiers’ performances(Laine et al. 2021). Trained classifiers can be exported to user-defined repositories and classified data can be further processed using custom Matlab scripts, and images sequences can be exported as .mat or .avi video files.

Last, DetecDiv provides additional post-processing routines to extract division and lifespan data for further analysis, as performed in the present study.

### Convolutional Neural Networks (CNN) for image classification

We used an image classifier to assess the state of cells in the cell cycle (small, large-budded, etc.) using brightfield images of individual traps. For each frame, we combined the three z-stack images described above into a single RGB image, which was used as input for the classifier.

We defined six classes, four of which representing the state of the cell, i.e., unbudded (‘unbud’), small-budded (‘small’), large-budded (‘large’), dead (‘dead’), as shown on Fig. S3A. Two additional classes are related to the state of the trap: trap with no a cell (‘empty’) and clogged trap (‘clog’). We trained a pre-trained GoogleNet convolutional neural network (Szegedy et al. 2015) to classify images according to these six classes using a training set of 200000 representative manually annotated brightfield images (i.e., 200 traps monitored during 1000 frames). The training of the classifier was achieved using Adaptive Moment estimation (Adam) optimizer (Kingma and Ba 2015). Specific parameters used can be found in the supplementary table T2.

## **Chapter I: Results I: Setting up a platform for automated and high-throughput tracking of replicative lifespan**

---

After the training procedure, we tested the classifier using a dataset composed of 50 independent cell traps (i.e. 50000 images) that were manually annotated and used for benchmarking (Fig. S3).

### Division time measurement and replicative lifespan (RLS) reconstruction

As the image classifier outputs a label for each frame corresponding to one of the 6 classes defined above, we used the sequence of labels to reveal the successive divisions of the cells: the oscillations between the “large” and “small” or “large” and “unbudded” classes captured the entry into a new cell cycle. The appearance of the “dead” class was used to assess the end of the replicative lifespan. This set of rules was used to compute the division times and the RLS of each individual cell when using either the CNN or the combined CNN/LSTM architecture (see below). However, in order to improve the accuracy of the method based only on the CNN, we implemented an additional “post-processing” step (referred to as PP in Figure 1), namely that two consecutive frames with a “dead” label are necessary to consider a cell as dead.

### Image sequence classification using combined CNN and a long short-term memory network (LSTM)

Based on the rules defined above, and despite very good benchmarks obtained for image classification (See Figure S3B and S3C), the CNN-based classifier was unable to accurately capture the replicative lifespan in the test set (see Fig. 1E). Indeed, the sporadic appearance of the “dead” class led to an underestimate of the actual RLS, and the incorrect classification of some small-budded and large-budded cells provided some abnormally short estimates of cell cycle durations (see the first peak in the distribution on Figure 1D). Additional post-processing steps, as mentioned above, alleviated the problem of short lifespan estimates (see CNN + PP on Figure 1E).

To provide a more accurate classification of the image according to the cellular state, we added a bidirectional long short-term memory (LSTM) network with 150 hidden units to the CNN network (Hochreiter and Schmidhuber 1997). The LSTM network takes the whole sequence of images as input (instead of independent images in the case of the CNN), hence taking the time-dependencies between successive images into account for improved classification. Two hundred image sequences (each with 1000 frames) were used to train the LSTM network independently of the CNN network (see training parameters in Supplementary table T3 and benchmarks on Figure S3D and S3E). The CNN and the LSTM network were

## **Chapter I: Results I: Setting up a platform for automated and high-throughput tracking of replicative lifespan**

---

then assembled as described in Figure 1B in order to output a sequence of labels for each series of images.

Annotated dataset is available at: [doi.org/10.5281/zenodo.5552642](https://doi.org/10.5281/zenodo.5552642)

Trained network is available at: [doi.org/10.5281/zenodo.5553862](https://doi.org/10.5281/zenodo.5553862)

### Assessment of cell-cycle slowdown using a LSTM network

After classifying an image sequence to reconstruct the lifespans of the cells, we further analyzed the data to determine the onset of cell-cycle slowdown. Unlike our previously developed method based on chi-square minimization with a piecewise linear fit (Fehrmann et al. 2013), we designed a time series classification method to identify when the cell cycle starts to slow down. For this, we trained a bidirectional LSTM network with 150 hidden units to classify all the frames in each lifespan between two classes, i.e., ‘pre-slowdown’ and ‘post-slowdown’. We used class probabilities from the previously described CNN/LSTM (unbudded, small, large, dead), as an input for the classifier (see Figure S4 for benchmarking results on a test set with 50 lifespans).

Annotated dataset is available at: [doi.org/10.5281/zenodo.5553796](https://doi.org/10.5281/zenodo.5553796)

Trained network is available at: [doi.org/10.5281/zenodo.5553829](https://doi.org/10.5281/zenodo.5553829)

### Brightfield and fluorescence images semantic segmentation using DeepLab v3+

Cell and nuclei contours were determined based on brightfield and fluorescence images, respectively, using the deep learning-based semantic segmentation architecture DeepLab v3+ (Chen et al. 2018). DeepLab v3+ possesses an encoder/decoder architecture allowing to classify pixels according to a number of user-defined classes. Here, we used 3 classes (i.e., background, mother cell, other cells) for cell segmentation to distinguish between the mother cell and its surrounding buds or daughter cells, see Figure 2 and S6). For nuclei segmentation, only two classes were defined (i.e., background and nucleus, see Fig. 2 and S6). We used the Resnet50 CNN in the encoder part of the network because it provided better segmentation results than Googlenet. We trained the network using a manually curated training set of 1500 and 3000 images for brightfield and fluorescence images, respectively (see Figure S7 and S8 for benchmarking results). Specific parameters used can be found in the supplementary table T4 (cell segmentation) and T5 (nucleus segmentation).

Annotated dataset is available at: [doi.org/10.5281/zenodo.5553771](https://doi.org/10.5281/zenodo.5553771)

Trained network is available at: [doi.org/10.5281/zenodo.5553851](https://doi.org/10.5281/zenodo.5553851)

### Classifier benchmarking

We used standard benchmarking to estimate the efficiency of image and pixel classifiers. For each classifier, we computed the confusion matrix obtained by comparing the “groundtruth” manually annotated images (or timeseries) to the predictions made by the classifier. We computed the accuracy, the recall, and F-score for each class, which balances accuracy and recall. In the specific case of pixel classification (semantic segmentation), we computed these benchmarks for different values of prediction thresholds used to assign the “mother” and “nucleus” classes, as reported in Figure S7 and S8. Then, we performed the segmentation of images using the threshold value that maximizes the F-score (0.9 and 0.35 for brightfield and fluorescence image classification, respectively).

To benchmark the detection of divisions, we used a custom pairing algorithm to detect false positive and false negative divisions. Using this, we could compute the accuracy and recall of the division detection procedure, and plot the correlation between paired divisions (Fig. 1D).

### Statistics

All experiments have been replicated at least twice. Error bars represent the standard error-on-mean, unless specified otherwise. Results of specific statistical tests are indicated in the figure legends.

## **Supplementary movies legends**

**Supplementary movie 1: Comparison of groundtruth versus classifier predictions for the CNN/LSTM classification of the cellular state**  
[\(<https://www.biorxiv.org/content/10.1101/2021.10.05.463175v2.supplementary-material>\)](https://www.biorxiv.org/content/10.1101/2021.10.05.463175v2.supplementary-material)

The left column represents the class predictions made by the CNN/LSTM classifier, while the right column represents the groundtruth (determined by manual annotation). The two numbers represent the number of buds generated by the cells according to the classifier predictions and manual annotation, respectively.

**Supplementary movie 2: Sample movies of individual cells following cellular state classification, cell and nuclear contour segmentation**

(<https://www.biorxiv.org/content/10.1101/2021.10.05.463175v2.supplementary-material>)

The left column represents the cellular state according to the prediction made by the CNN/LSTM classifier. The middle column shows the brightfield image along with mother cell contours obtained by a semantic segmentation classifier. The right column displays the Htb2-NeonGreen fluorescence channel, along with cells contours and nuclear contours obtained by a semantic segmentation classifier.

## Additional references

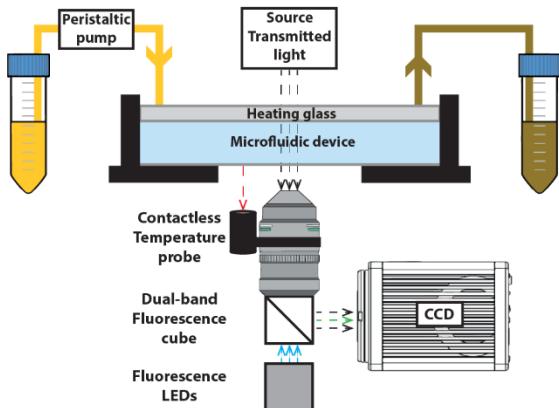
- Chen, Liang-Chieh, Yukun Zhu, George Papandreou, Florian Schroff, and Hartwig Adam. 2018. "Encoder-Decoder with Atrous Separable Convolution for Semantic Image Segmentation." *arXiv [cs.CV]*. arXiv. [https://openaccess.thecvf.com/content\\_ECCV\\_2018/papers/Liang-Chieh\\_Chen\\_Encoder-Decoder\\_with\\_Atrous\\_ECCV\\_2018\\_paper.pdf](https://openaccess.thecvf.com/content_ECCV_2018/papers/Liang-Chieh_Chen_Encoder-Decoder_with_Atrous_ECCV_2018_paper.pdf).
- Crane, Matthew M., Ivan B. N. Clark, Elco Bakker, Stewart Smith, and Peter S. Swain. 2014. "A Microfluidic System for Studying Ageing and Dynamic Single-Cell Responses in Budding Yeast" 9 (6): e100042.
- Fehrman, Steffen, Camille Paoletti, Youlian Goulev, Andrei Ungureanu, Hugo Aguilaniu, and Gilles Charvin. 2013. "Aging Yeast Cells Undergo a Sharp Entry into Senescence Unrelated to the Loss of Mitochondrial Membrane Potential." *Cell Reports* 5 (6): 1589–99.
- Hochreiter, Sepp, and Jürgen Schmidhuber. 1997. "Long Short-Term Memory." *Neural Computation* 9 (8): 1735–80.
- Jo, Myeong Chan, Wei Liu, Liang Gu, Weiwei Dang, and Lidong Qin. 2015. "High-Throughput Analysis of Yeast Replicative Aging Using a Microfluidic System." *Proceedings of the National Academy of Sciences of the United States of America* 112 (30): 9364–69.
- Kingma, Diederik P., and Jimmy Ba. 2015. "Adam: A Method for Stochastic Optimization." <https://www.semanticscholar.org/paper/a6cb366736791bcccc5c8639de5a8f9636bf87e8>.
- Laine, Romain F., Ignacio Arganda-Carreras, Ricardo Henriques, and Guillaume Jacquemet. 2021. "Avoiding a Replication Crisis in Deep-Learning-Based Bioimage Analysis." *Nature Methods* 18 (10): 1136–44.
- Lee, Sung Sik, Ima Avalos Vizcarra, Daphne H. E. W. Huberts, Luke P. Lee, and Matthias Heinemann. 2012. "Whole Lifespan Microscopic Observation of Budding Yeast Aging through a Microfluidic Dissection Platform." *Proceedings of the National Academy of Sciences* 109 (13): 4916–20.
- Liu, Ping, Thomas Z. Young, and Murat Acar. 2015. "Yeast Replicator: A High-Throughput Multiplexed Microfluidics Platform for Automated Measurements of Single-Cell Aging." *Cell Reports* 13 (3): 634–44.
- Ryley, J., and O. M. Pereira-Smith. 2006. "Microfluidics Device for Single Cell Gene Expression Analysis in *Saccharomyces Cerevisiae*." *Yeast* 23 (14-15): 1065–73.
- Szegedy, Christian, Wei Liu, Yangqing Jia, Pierre Sermanet, Scott Reed, Dragomir Anguelov, Dumitru Erhan, Vincent Vanhoucke, and Andrew Rabinovich. 2015. "Going Deeper with Convolutions." In *2015 IEEE Conference on Computer Vision and Pattern Recognition*

(CVPR), 1–9.

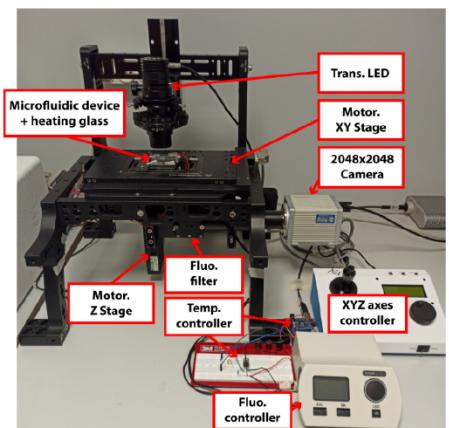
Zhang, Yi, Chunxiong Luo, Ke Zou, Zhengwei Xie, Onn Brandman, Qi Ouyang, and Hao Li. 2012. "Single Cell Analysis of Yeast Replicative Aging Using a New Generation of Microfluidic Device" 7 (11): e48275.

**Figure S1**

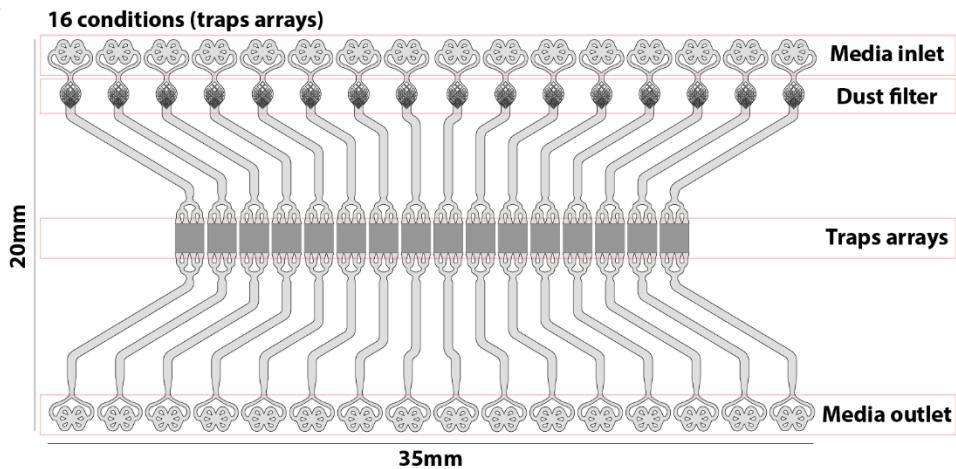
**A**



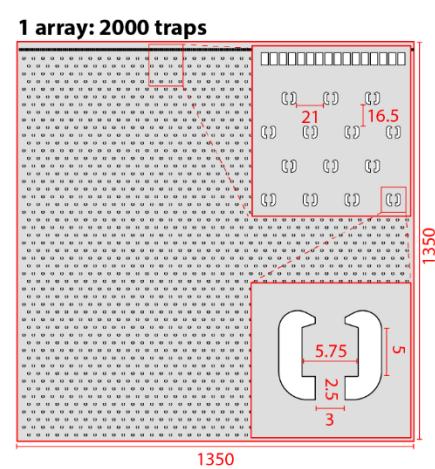
**B**



**C**

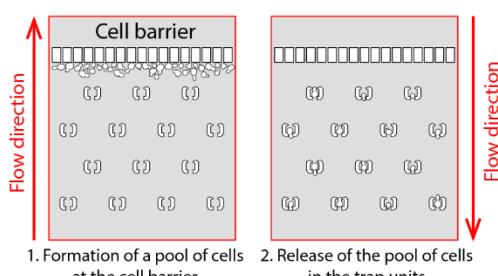


**D**



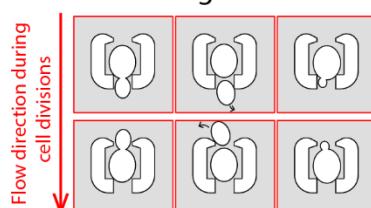
**E**

Trapping of original mother cells



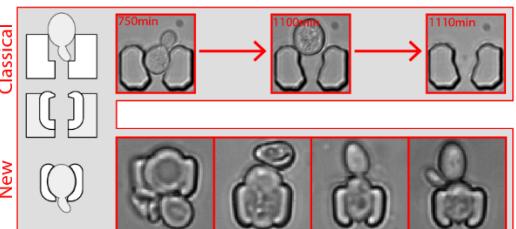
**F**

Automatic dissection of daughter cells



**G**

Optimized retention of big cells



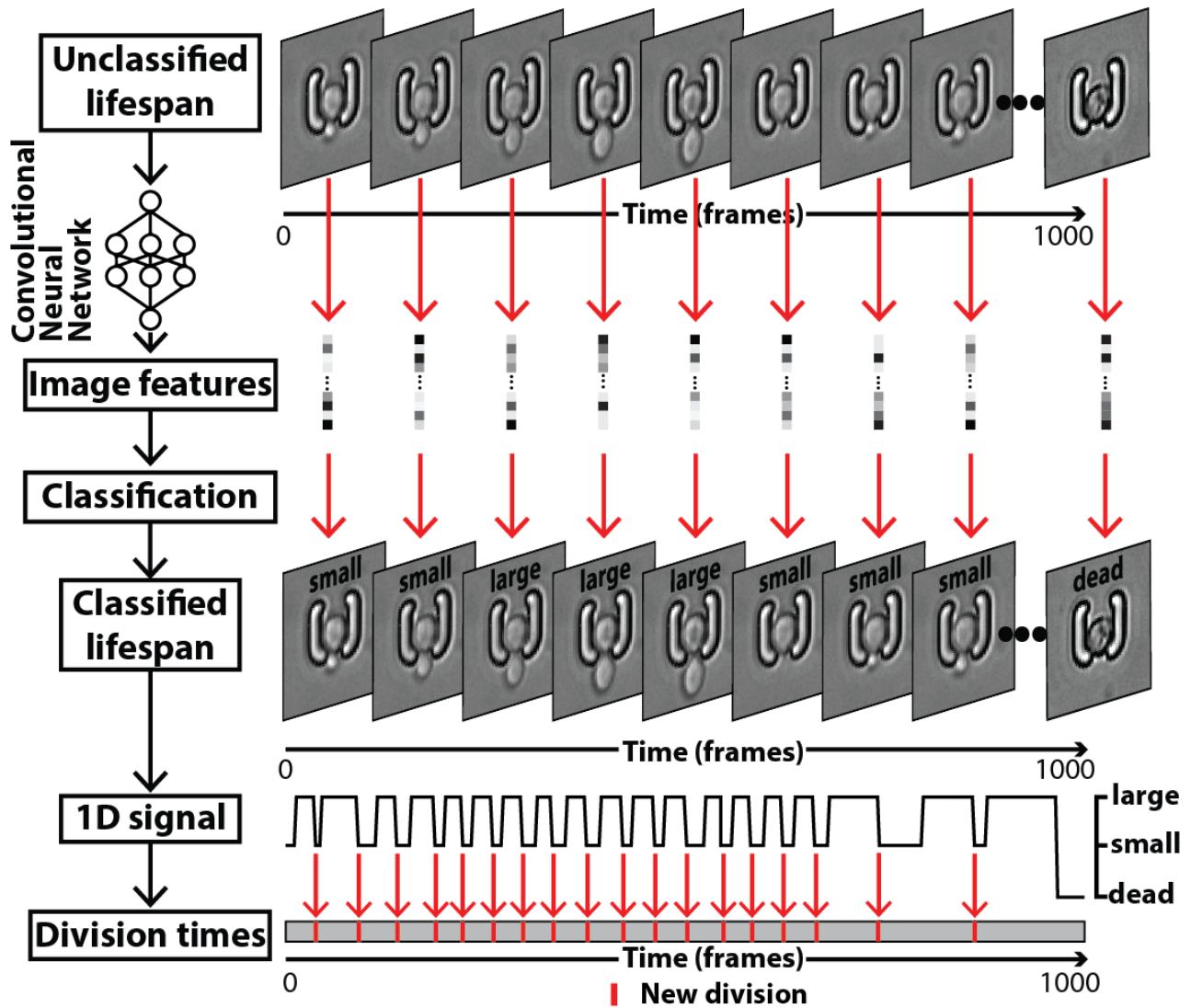
**Supplementary figure 1: Experimental setup and microfluidic device**

- Schematics of the custom imaging setup built for DetecDiv (see supplementary text for details).
- Picture of the imaging setup
- Schematics of the microfluidic device with 16 independent channels. Each channel has one inlet, a dust filter, and one outlet.

- D) Schematics of the array of cell traps. Dimensions are in microns. Inset represents close-ups on indicated areas.
- E) Principle of the cell barrier used to prevent the cells from moving towards the inlet when loading the cells from the outlet. Any cell upstream of the cell array may lead to the formation of colonies hence clog the device over time.
- F) Principle of the automated dissection of daughter cells: the mother is retained within the trap but their successive daughters are flushed away due to constant medium flow. Daughters may either exit the trap from the top or bottom opening.
- G) Unlike previous cell trap geometries ("classical"), the current design ("new") features shallow PDMS walls that can be deformed by large cells, hence ensuring the long term retention of the cells. Two small claws on each side of the trap entrance further enhances retention.

Figure S2

## Classification of images using the GoogleNet CNN (processing images separately)

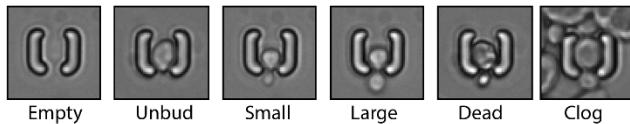


Supplementary figure 2: Principles of division tracking and lifespan reconstruction using a CNN-based image classification

In this framework, the sequence of images is processed by a GoogleNet CNN that processes each image separately. The CNN extracts image features that are used to assign a label to each image among six possible classes (see supplementary methods for details). As with the CNN/LSTM architecture described in Figure 1, the sequence of labels is used to assign division events and the occurrence of cell death.

### Figure S3

#### A Definition of classes



Empty

Unbud

Small

Large

Dead

Clog

#### B GoogleNet (CNN) classification

Groundtruth	clog	3851	68	32	21		
	dead	365	11896		156	15	70
	empty	4		276		2	
	large	12	30		16590	1494	2
	small		1		327	6134	4
	unbud	6	2		38	88	2721

97.0%	3.0%
95.2%	4.8%
97.9%	2.1%
91.5%	8.5%
94.9%	5.1%
95.3%	4.7%

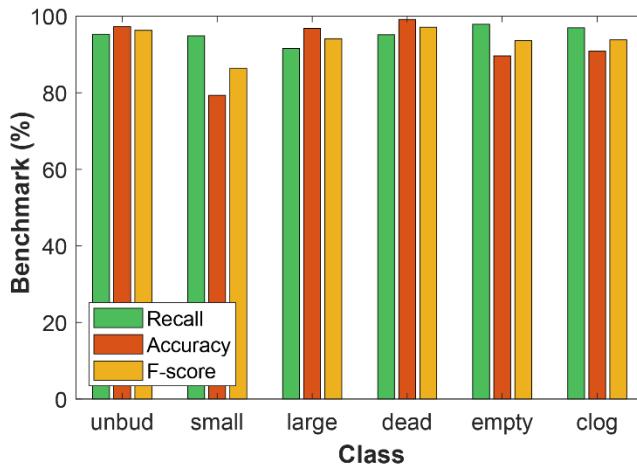
Recall

#### D GoogleNet (CNN) + LSTM classification

Groundtruth	clog	3832	100	35	4		1	96.5%	3.5%
	dead	122	12069		125		186	96.5%	3.5%
	empty			279		3		98.9%	1.1%
	large	1	5		17293	823	6	95.4%	4.6%
	small		3		368	6054	41	93.6%	6.4%
	unbud			4	31	2820		98.8%	1.2%

Recall

#### C



Benchmark (%)

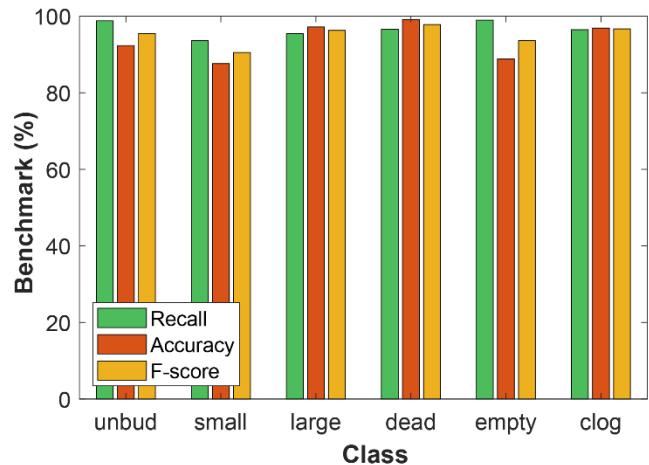
Class

Recall

Accuracy

F-score

#### E



Benchmark (%)

Class

#### Supplementary figure 3: Class definition and image classification benchmarks

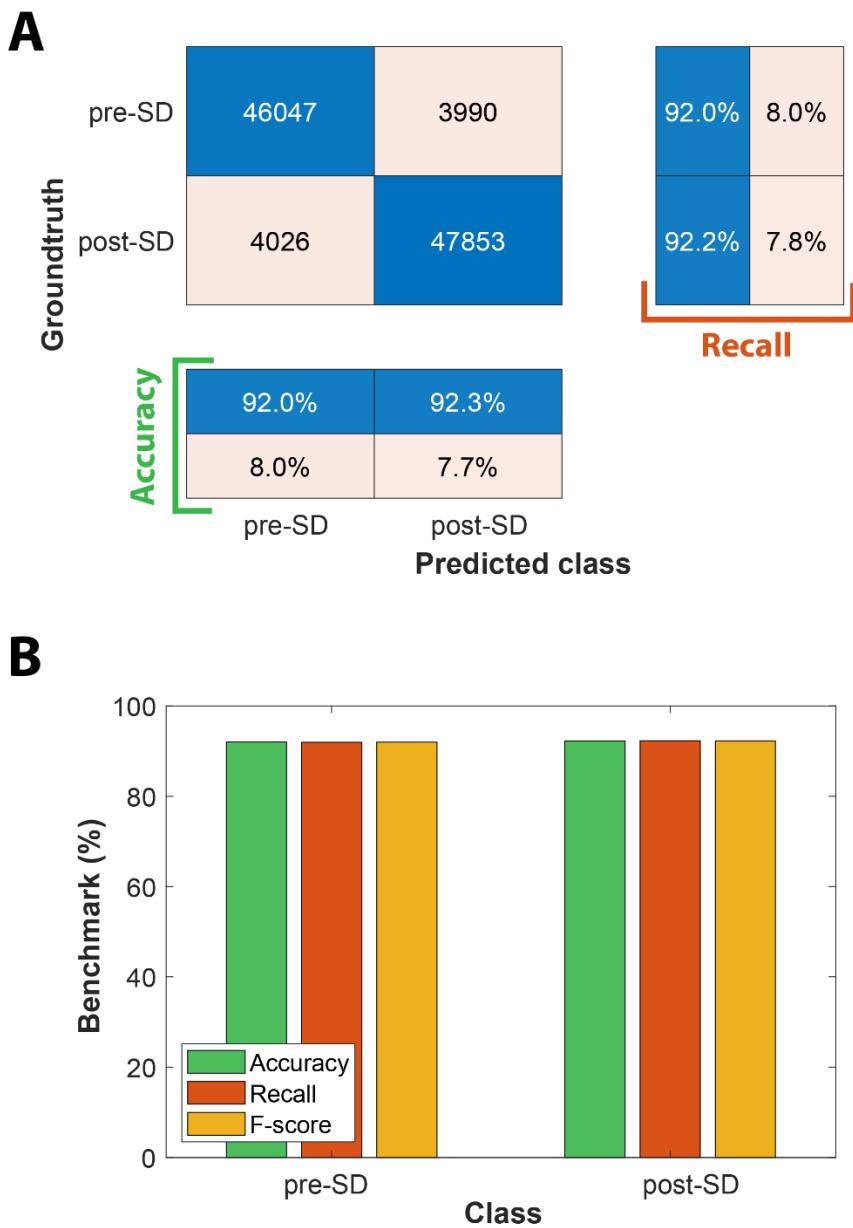
- A) Sample images indicating how each class was defined. "Empty": empty trap, i.e., no cell; "Unbud": unbudded cell; "Small": small-budded cell; "Large": large-budded cell; "Dead": dead cell; "Clogged": clogged trap.
- B) Confusion matrix obtained with a test dataset (50 trapped cells followed over 1000 frames) using the CNN image classifier. Each number in the matrix represents the number of detected events.

**Chapter I: Results I: Setting up a platform for automated and high-throughput tracking of replicative lifespan**

---

- C) Bar plot showing the recall, accuracy and F-score metrics obtained on each class for the CNN image classifier on the test dataset.
- D) Same as B), but for the combined CNN/LSTM architecture
- E) Same as C), but for the combined CNN/LSTM architecture

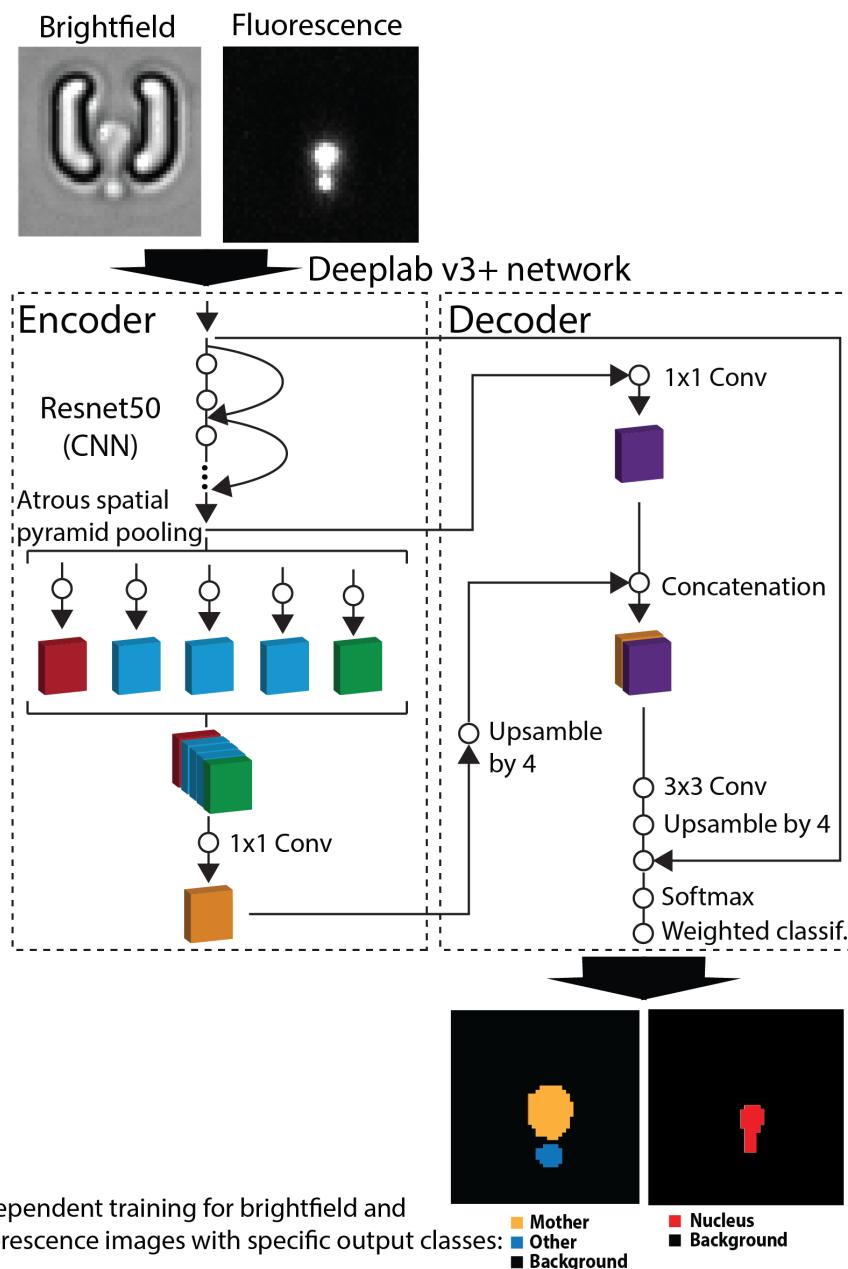
Figure S4



Supplementary figure 4: Classification benchmarks for the detection of the onset of slow division using an LSTM sequence-to-sequence classification

- Confusion matrix obtained with a test dataset (50 timeseries based on the cellular state probabilities output by the CNN/LSTM classifier) using a trained LSTM classifier. Each number in the matrix represents the number of detected events.
- Bar plot showing the recall, accuracy and F-score metrics obtained on each class for the LSTM classifier on the test dataset.

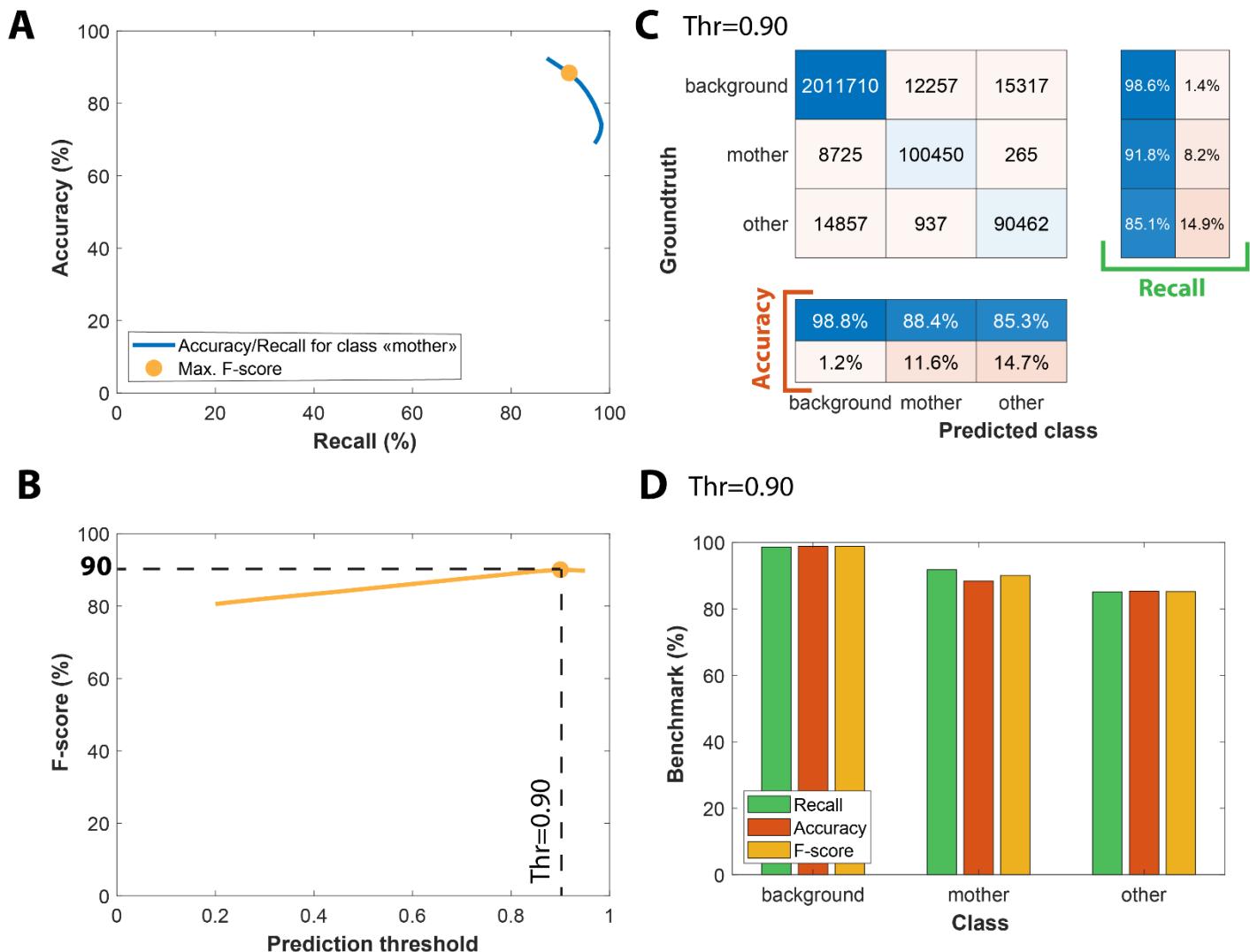
**Figure S5**



**Supplementary figure 5: Principles of the pipeline used for semantic segmentation with DeepLab V3+**

Brightfield or Fluorescence images are separately processed by the DeepLab V3+ encoder/decoder network (Chen et al. 2018) that has been modified to classify image pixels according to user-defined classes (mother /other /background & nucleus / background for brightfield and fluorescence images, respectively). A weighted classification layer is used to deal with class imbalance.

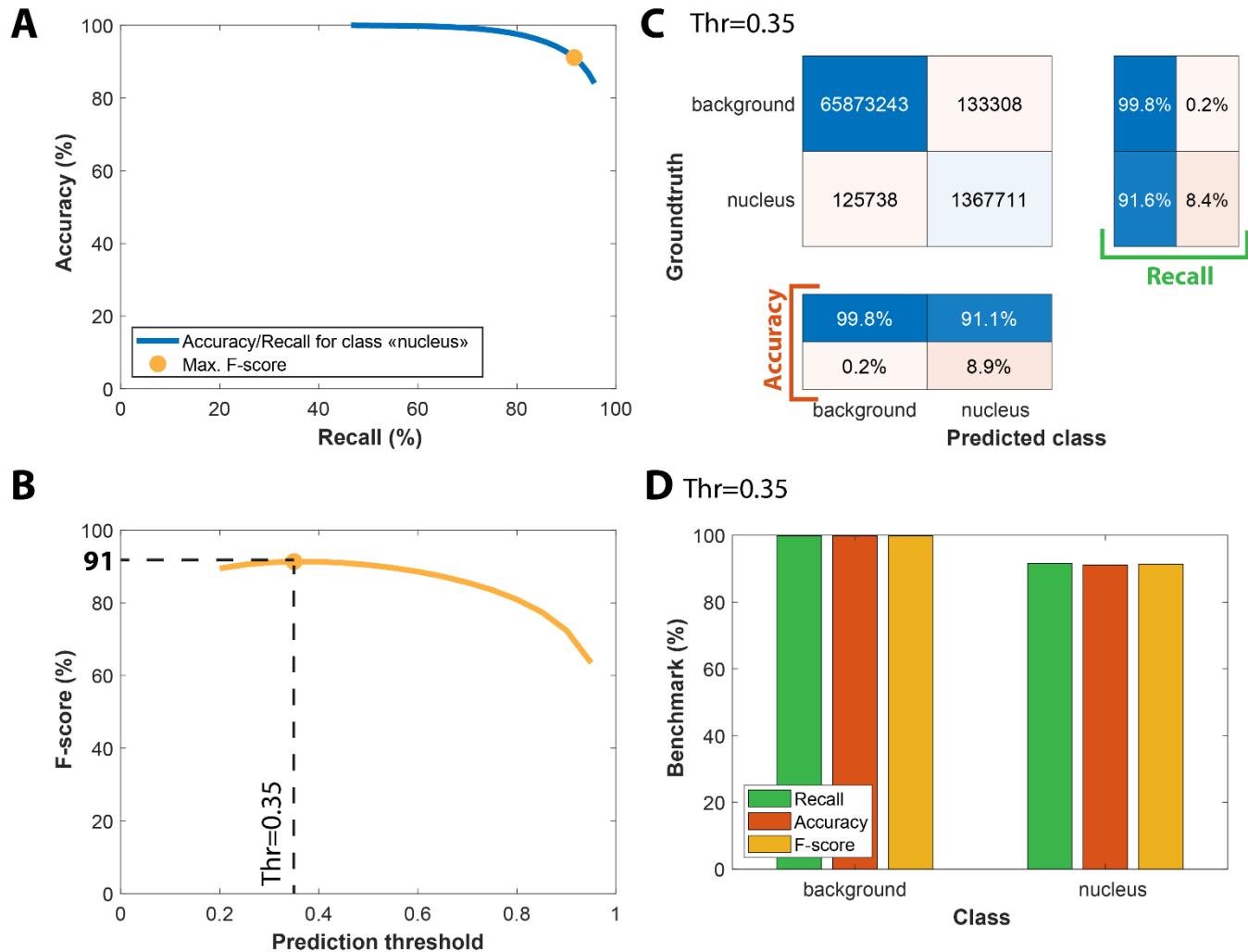
**Figure S6**



**Supplementary figure 6: Benchmarks for the semantic segmentation of brightfield images**

- A) Accuracy/Recall tradeoff plot obtained by varying the output prediction threshold for the class "mother" using a test dataset that contains 50 image sequences with 1000 frames. The orange dot indicates the point that maximizes the F-score.
- B) Evolution of F-score as a function of the output prediction threshold (computed on the [0.2 : 0.95] interval). A threshold value of 0.9 maximizes the F-score (90%).
- C) Confusion matrix obtained with the test dataset using a 0.9 prediction threshold.
- D) Bar plot showing the recall, accuracy and F-score metrics obtained on each class for the pixel classifier on the test dataset.

**Figure S7**



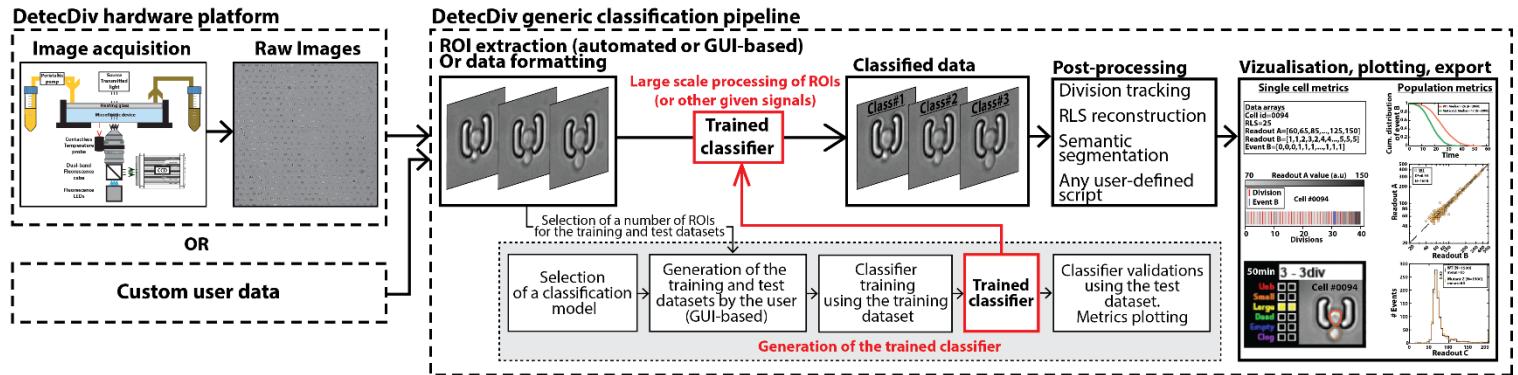
**Supplementary figure 7: Benchmarks for the semantic segmentation of fluorescence images**

- Accuracy/Recall tradeoff plot obtained by varying the output prediction threshold for the class "nucleus" using a test dataset that contains 25 image sequences with 1000 frames. The orange dot indicates the point that maximizes the F-score.
- Evolution of F-score as a function of the output prediction threshold (computed on the [0.2:0.95] interval). A threshold value of 0.35 maximizes the F-score (91%).
- Confusion matrix obtained with the test dataset using a 0.35 prediction threshold.
- Bar plot showing the recall, accuracy and F-score metrics obtained on each class for the pixel classifier on the test dataset.

**Figure S8**



### DetectDiv workflow



### Available classification models

Type	Description	Input	Output	Reference figures in article
<b>Models used in the present article</b>				
Image classification	CNN : GoogleNet, Resnet50, etc.	Individual images	M user-defined classes	Fig. 1 ("CNN"), S2 and S3. Table T2
Image sequence classification	Combined CNN/LSTM network for sequence-to-sequence classification	Sequence of N images	N (frames) x M (user-defined classes)	Fig. 1 ("CNN/LSTM"), S2 and S3. Table T2
Timeseries classification	LSTM network for sequence-to-sequence classification	N (frames) x L vector	N (frames) x M (user-defined classes)	Fig. 2A and S4. Table T3
Pixel classification (semantic segmentation)	Deeplab v3+ (with GoogleNet, Resnet50, etc. encoder)	Individual images	Labeled images (M user-defined classes)	Fig. 2B, 2C, S6-8. Table T4 and T5
<b>Additional models available</b>				
Image sequence classification	Combined CNN/LSTM network for sequence-to-one classification	Sequence of N images	M (user-defined classes)	N/A
Image regression	CNN : GoogleNet, Resnet50, etc.	Individual images	1 number	N/A
Image sequence regression	Combined CNN/LSTM network for sequence-to-sequence regression	Sequence of N images	N (frames) x 1 number	N/A
Timeseries classification	LSTM network for sequence-to-one classification	N (frames) x L vector	M (user-defined classes)	N/A
Timeseries regression	LSTM network for sequence-to-sequence regression	N (frames) x L vector	N (frames) x 1 number	N/A
Timeseries regression	LSTM network for sequence-to-one regression	N (frames) x L vector	1 number	N/A

**Supplementary figure 8: DetectDiv software workflow**

- A) Schematics showing the image processing steps performed for image classification with the DetectDiv software
- B) List of all available classification models available in DetectDiv.

**Table 1**

Strain Table				
Name	Mat	Background	Genotype	Origin
SF1.39	alpha	S288C	BY4742 his3delta1 leu2delta0 lys2delta0 TRP1 ura3delta0	Euroscarf
SF1.46	alpha	S288C	YDL042c::KanMX4 his3delta1 leu2delta0 lys2delta0 ura3delta0	Euroscarf
SF1.47	alpha	S288C	YDR110w::KanMX4 his3delta1 leu2delta0 lys2delta0 ura3delta0	Euroscarf
YAM4.38	alpha	S288C	HTB2-NeonGreen-CaURA his3-1 leu2-3 URA3 met15.0	Charvin lab

**Table 2**

CNN	Value	LSTM bidirectional	Value
ROIs	200	ROIs	200
Total images	200 000	Total data points	200 000
classes	unbudded, small, large dead, empty, clogged	classes	unbudded, small, large dead, empty, clogged
Network	Googlenet	method	adam
method	adam	split	0,7
split	0,7	freeze	n
freeze	n	ExecutionEnvironment	multi-gpu
ExecutionEnvironment	multi-gpu	GradientDecayFactor	0.9
GradientDecayFactor	0.9	SquaredGradientDecayFactor	0.999
SquaredGradientDecayFactor	0.999	Epsilon	1,00E-08
Epsilon	1,00E-08	InitialLearnRate	0.0003
InitialLearnRate	0.0003	LearnRateScheduleSettings	
LearnRateScheduleSettings		Method	piecewise
		DropRateFactor	0.9
		DropPeriod	10
L2Regularization	1,00E-05	L2Regularization	1,00E-05
GradientThresholdMethod	l2norm	GradientThresholdMethod	l2norm
MaxEpochs	6	MaxEpochs	50
MiniBatchSize	128	MiniBatchSize	8
ValidationPatience	Inf	ValidationPatience	Inf
Shuffle	every-epoch	Shuffle	every-epoch
TranslateAugmentation	-5 5		
RotateAugmentation	-30 30		

**Table 3**

LSTM	Value
ROIs	200
Total data points	90092
classes	unbudded, small, large dead, empty, clogged
method	adam
split	0,7
freeze	n
ExecutionEnvironment	multi-gpu
GradientDecayFactor	0.9
SquaredGradientDecayFactor	0.999
Epsilon	1,00E-08
InitialLearnRate	0.0003
LearnRateScheduleSettings	
	Method
	piecewise
	DropRateFactor
	0.9
	DropPeriod
	10
L2Regularization	1,00E-05
GradientThresholdMethod	l2norm
MaxEpochs	50
MiniBatchSize	8
ValidationPatience	Inf
Shuffle	every-epoch

**Table 4**

CNN	Value
Images	1404
classes	Background, Mother, Other
Network	resnet50
method	adam
split	0,9
freeze	n
ExecutionEnvironment	multi-gpu
GradientDecayFactor	0.9
SquaredGradientDecayFactor	0.999
Epsilon	1,00E-08
InitialLearnRate	0.00001
LearnRateScheduleSettings	
	Method
	piecewise
	DropRateFactor
	0.7
	DropPeriod
	2
L2Regularization	0,00E+00
GradientThresholdMethod	l2norm
MaxEpochs	10
MiniBatchSize	8
ValidationPatience	500
Shuffle	every-epoch
TranslateAugmentation	-7 7
RotateAugmentation	-20 20

**Table 5**

CNN	Value
Images	3000
classes	background, fluo
Network	resnet50
method	adam
split	0,9
freeze	n
ExecutionEnvironment	multi-gpu
GradientDecayFactor	0.9
SquaredGradientDecayFactor	0.999
Epsilon	1,00E-08
InitialLearnRate	0.0003
LearnRateScheduleSettings	
	Method
	piecewise
	DropRateFactor
	0.7
	DropPeriod
	2
L2Regularization	1,00E-03
GradientThresholdMethod	l2norm
MaxEpochs	10
MiniBatchSize	128
ValidationPatience	20
Shuffle	every-epoch
TranslateAugmentation	-5 5
RotateAugmentation	-20 20

### 1.3. Perspectives

Though developed for general use (and not only replicative lifespan), this platform can accurately detect the division of cells and quantify many different readouts throughout their lifespan. The monitoring throughput is unprecedented, thanks to a new microfluidic device and a specific imaging system. Besides, analysis is no longer the throughput bottleneck since everything is automated using artificial intelligence with an error rate close to 1%, thanks to its architecture to exploit the time-dependencies in temporal sequences of images.

With that, 16x2000 cells can be screened and analyzed every 4 days, with only 6 hours of active human work. Therefore, this framework increases the screening throughput of 1 to 2 orders of magnitude, which opens new perspectives for understanding the system dynamics of aging.

As a perspective, it would be interesting to train a network to detect petite cells based on their morphology and division time (using mitochondrial markers to generate the groundtruth). Indeed, this artifact from the lab strains has been described as an aging path by many different studies [280,283], and it would be interesting to systematically separate these cells from the others. Similarly, we could classify the cells between the two morphologies often described during aging, "elongated bud" and "round bud" [223], to try to understand if they relate to different aging paths (which is probably also linked to mitochondria [283]).

Another way of understanding aging, aging factors, and rejuvenation is to observe the progeny of old mothers. To study that, I have developed a microfluidic device that allows the tracking of a mother cell until a given timepoint before inverting the budding pattern and replacing the mother cell with its daughter.

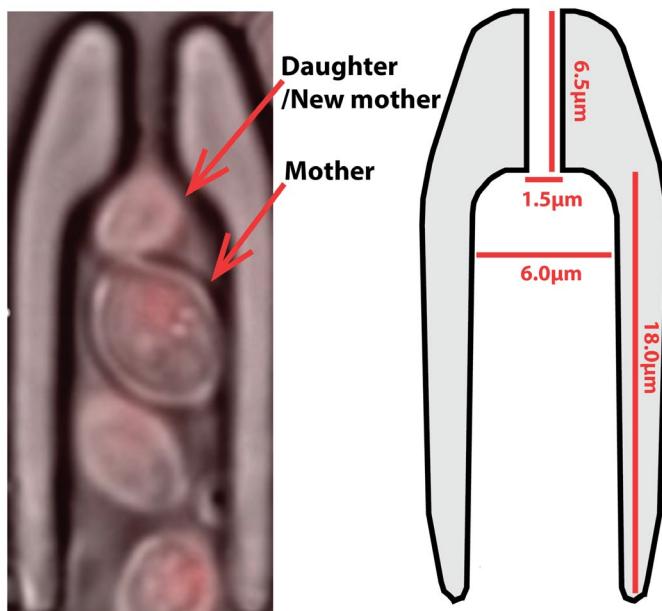


Figure 60: Image (left) and design (right) of a trap used to observe the RLS of a daughter after the replacement of its mother

Indeed, S288C cells have a unidirectional budding pattern and can therefore age in a unidirectional cavity. However, deleting the BUD4 or intermittently degrading Bud4 proteins with an Auxin Inducible Degron system during the experiment would cause a fraction of the mothers to be replaced by their daughter by budding against the tip of the trap instead of towards its exit. Consequently, we could track the partial lifespan of daughters born at different mother ages, in the same automated manner as with mother cells, using the system presented previously. It is indeed straightforward to detect such switch with image classification by using a class for the presence of a small bud at the tip of the cavity (Figure 60, left)

Beyond the replicative lifespan application, our software is designed to be used as a generic interface to set up custom classification pipelines to extract temporal features from any sequences of timelapse images, thanks to its architecture. It is proposed as a stand-alone software that includes all the steps to produce and use custom networks (pre-processing, classifier training and validation, raw data classification, post-processing, and data visualization).

## 6. Results II: Measuring the probability of ERC excision with age

### 1.1. Introduction

In order to determine if the excision of the first ERC - which is the starting point of a major cascade of events leading to death - is more probable in late ages than in early life or not, we need to capture excision events and link them with the replicative age of the cell.

One way of assaying the excision rate of a population is by inserting an ADE2 gene in one rDNA repeat in an ade2 $\Delta$  strain. The absence of Ade2 promotes the accumulation of a red pigment due to a rupture in the adenine metabolic pathway [482]. Therefore, after the excision of the repeat containing the Ade2 gene in a cell, its progeny becomes red. Hence, it is possible to estimate the excision rate of a population by counting the ratio of red/white colonies [356,368]. However, this colony sectoring assay does not inform about the probability of excision with age at the single-cell level, but rather gives the average excision rate of the whole population in exponential phase.

Therefore, we thought to tag rDNA repeats with fluorescence. With this strategy, once an rDNA repeat is excised, it will be replicated at each division and retained in the mother, and the signal from the tag should increase exponentially.

A recent study from our group using a LacI-GFP expressing strain in which each of the ~150 rDNA repeats includes a LacO sequence showed that this exponential accumulation occurs. With that, they could measure the doubling time of the signal and determine that two-thirds of the circles are replicated at each division. However, since the basal fluorescence signal from this strain is 150 units, it is difficult to capture the exact excision time at which the signal goes from 150 to 151 units.

### 1.2. Results

To precisely capture the excision of a repeat with age at the single-cell level, I therefore used a strain on which only one repeat among the 150 has a GFP sequence under a TDH3 promoter, inserted in the intergenic region of the repeat (IGS1 or IGS2). This strain was used in a previous study [328] to study the loss of Pol II silencing at the rDNA with age. Indeed, as seen in the introduction (see p.49), the 35S and 5S genes are transcribed by Pol I and Pol III (respectively), and the IGS1/2 loci are transcribed by Pol II. Using this strain, they could observe an increase of fluorescence in the late ages (an increase of a factor ~4), which implies

that cells experience a sustained loss of silencing of the rDNA. They also validated that this loss of silencing depended on Sir2 (Sir2 is known to be the main silencer of the IGS1/2 [392]) since blocking Sir2 increased fluorescence. Interestingly, they reported that a tiny fraction of cells was displaying a massive increase of fluorescence (an increase of 2 to 3 orders of magnitude) and attributed that to ERC accumulation from the tagged repeat.

Therefore, this strain can act as a transcriptional reporter of the ERC accumulation, and the exponential dynamics of the signal can allow us to determine the time of excision, at which the exponential starts (Figure 61).

However, since the estimated average excision rate is about 10% per generation [226,356] the caveat of this approach is that only ~2% of the wildtype cells will excise the tagged repeat during their lifespan. Thus, having a high-throughput system is necessary in order to capture enough events.

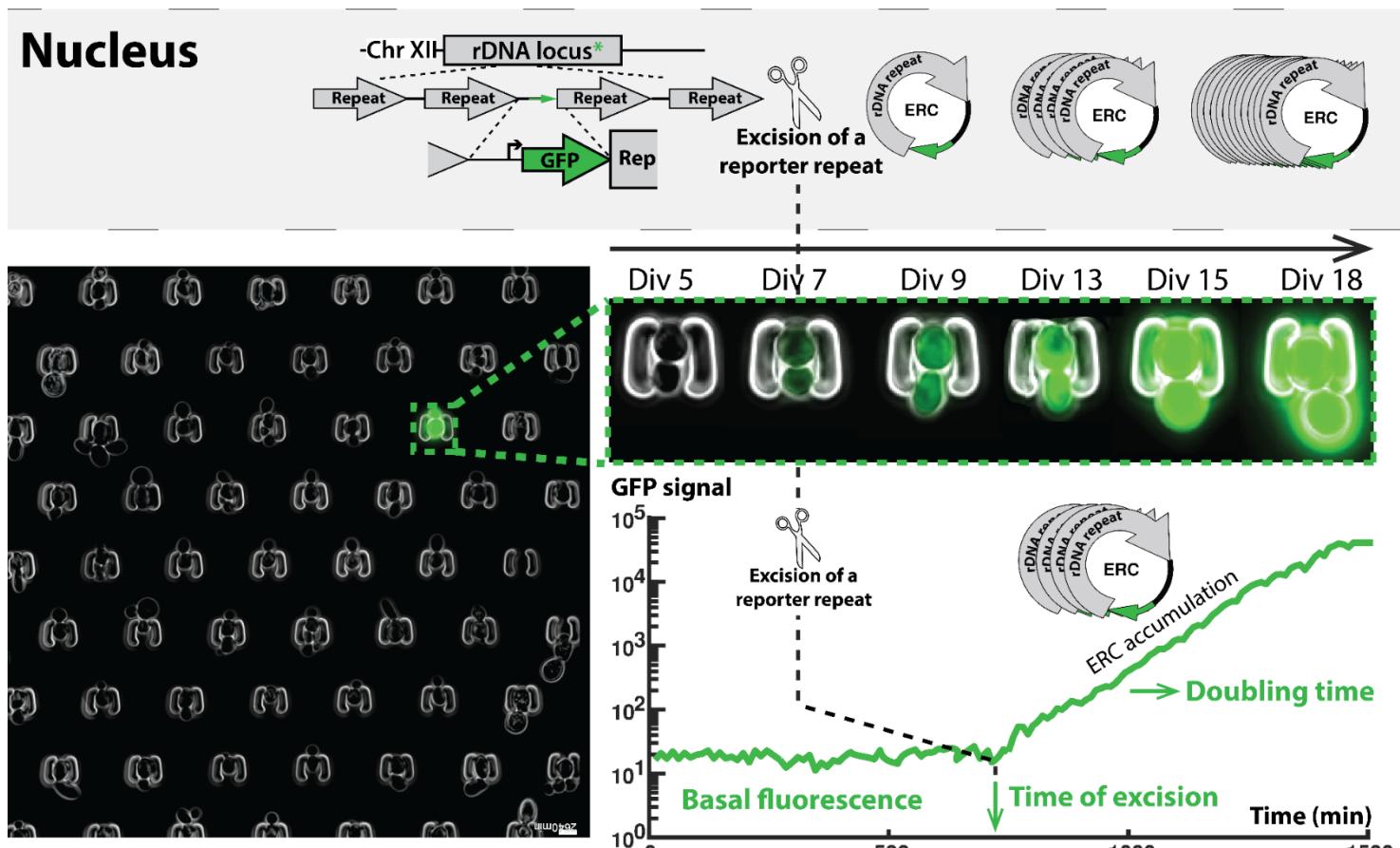
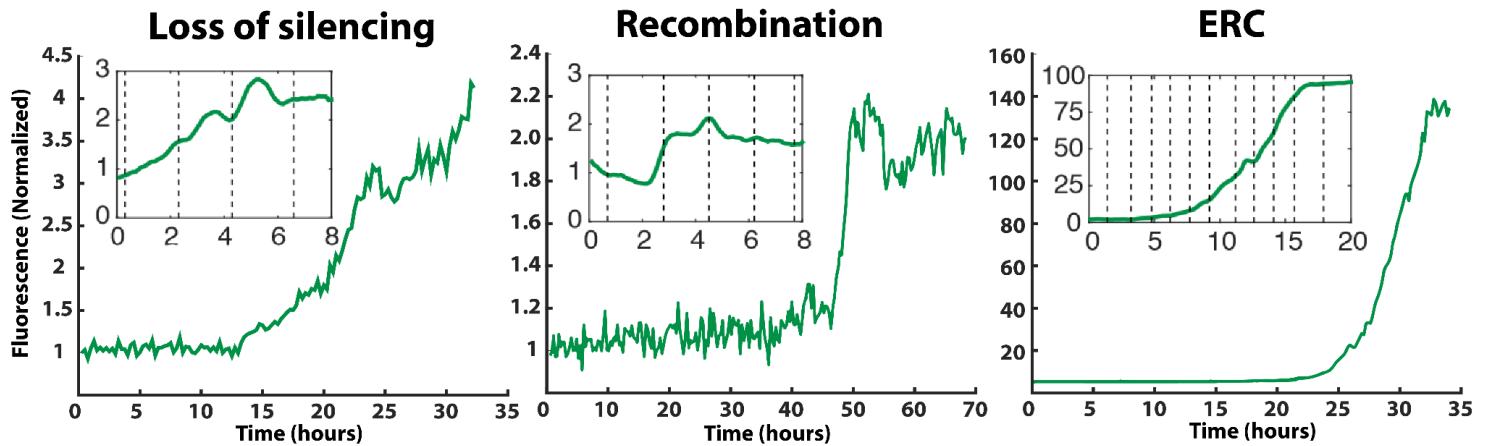


Figure 61: Experimental procedure to measure ERC excisions. An rDNA repeat is tagged with a transcriptional reporter of GFP in the IGS1 or 2 regions. When this repeat is excised, the number of DNA template of this gene increase and the cell emits a proportional amount of fluorescence. (Left): Phase contrast + GFP signal of a typical field of view after 10h showing the rarity of the event.

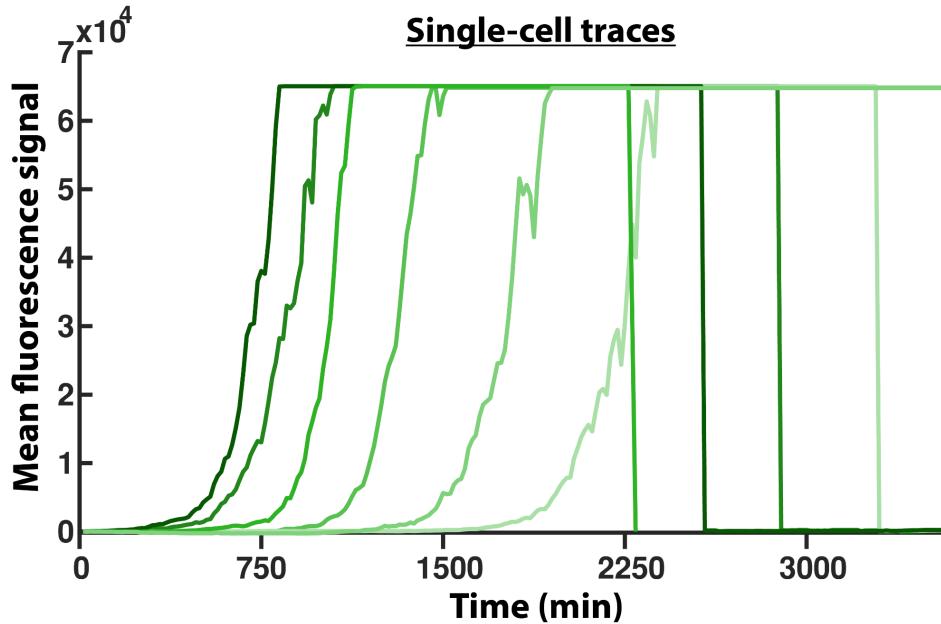
Using this strain with the previously described microfluidic chip, I was able to recapitulate the different signals observed in the original study, namely a loss of silencing (Figure 62, left), a recombination of the tagged repeat (leading to a doubling of the fluorescence, see Figure

33B, p.53 and Figure 62, middle), and the excision and accumulation of the tagged ERC (Figure 62, right).



*Figure 62: Different types of signals obtained with the transcriptional reporter of ERCs. The main graphs were obtained by us, while inset graphs represent what is reported in the original paper [328].*

As expected, 2.4% of the cells ( $N=930$ ) showed an exponential increase of the fluorescence over more than one order of magnitude (condition to consider the signal as the accumulation of a tagged repeat). Besides, these cells displayed the increase at very different moments, suggesting that the excision time was also very variable (Figure 63).



*Figure 63: Mean GFP fluorescence of six different cells as a function of time*

From such a signal, it is possible to extract many parameters, such as the time of excision or the doubling time, using a piecewise and an exponential fit, respectively (Figure 64).

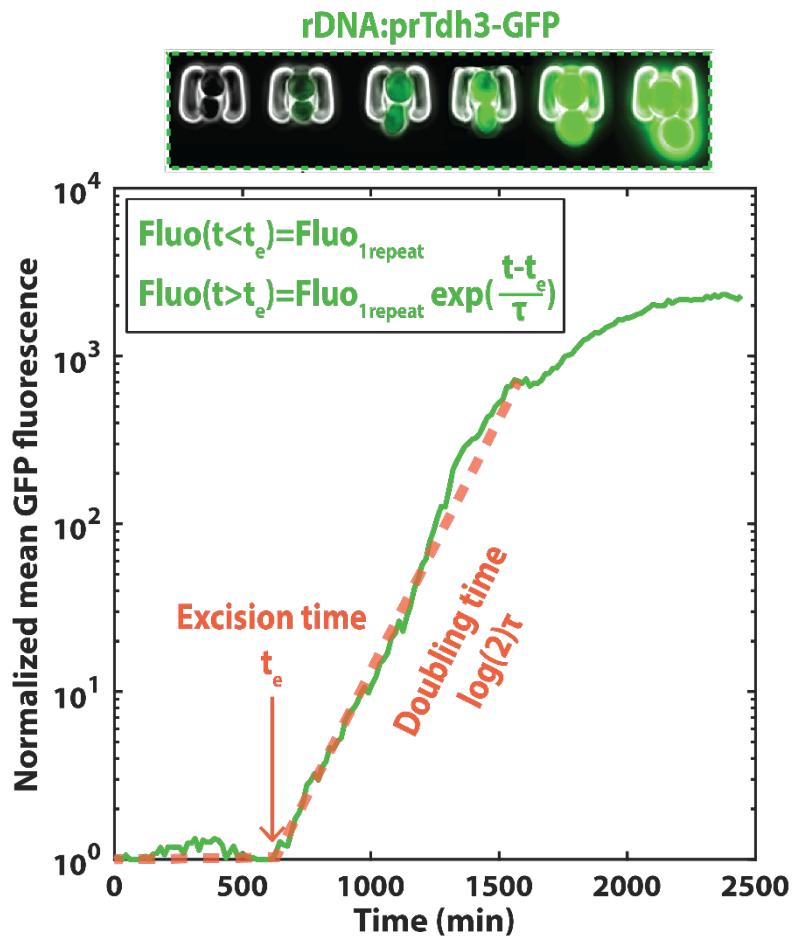
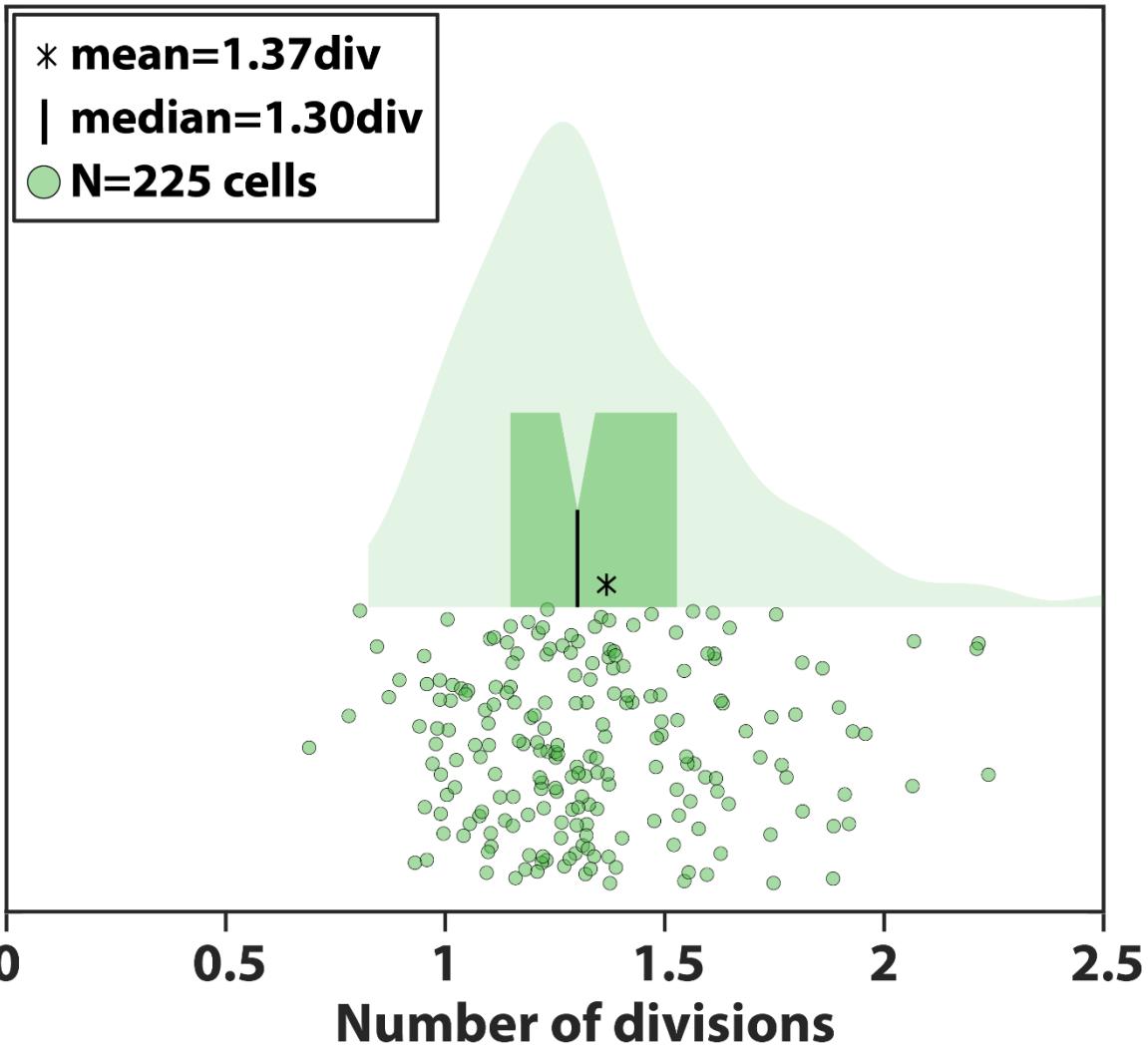


Figure 64: GFP fluorescence of one rDNA::Tdh3pr-GFP cell displaying a strong increase of fluorescence, as a function of time. The fitting of the data with a delayed exponential allows to decipher the time of excision and the doubling time of the fluorescence.

The doubling time of such a signal was found to be slightly higher than the division (1.36 divisions Figure 65), in agreement with the previous study [226], reinforcing the fact that this signal is linked to ERC accumulation and that not all ERCs are replicated at each division.



*Figure 65: Distribution and boxplot of the fluorescence doubling time in rDNA::Tdh3pr-GFP cells displaying an exponential increase of fluorescence*

To have access to the statistics of excision, one can plot the cumulative distribution of excision time (just like plotting a survival curve, see p.4). From that, the best fit is obtained with an exponential decay  $F(t) = \exp\left(-\frac{t}{p_{exc}}\right)$ , with  $p_{exc}$  a constant representing the probability of excision per unit of time (Figure 66). Therefore, the probability of excision is independent of age (from these preliminary results) and of about 10% per division, in agreement with previous numerical models [226].

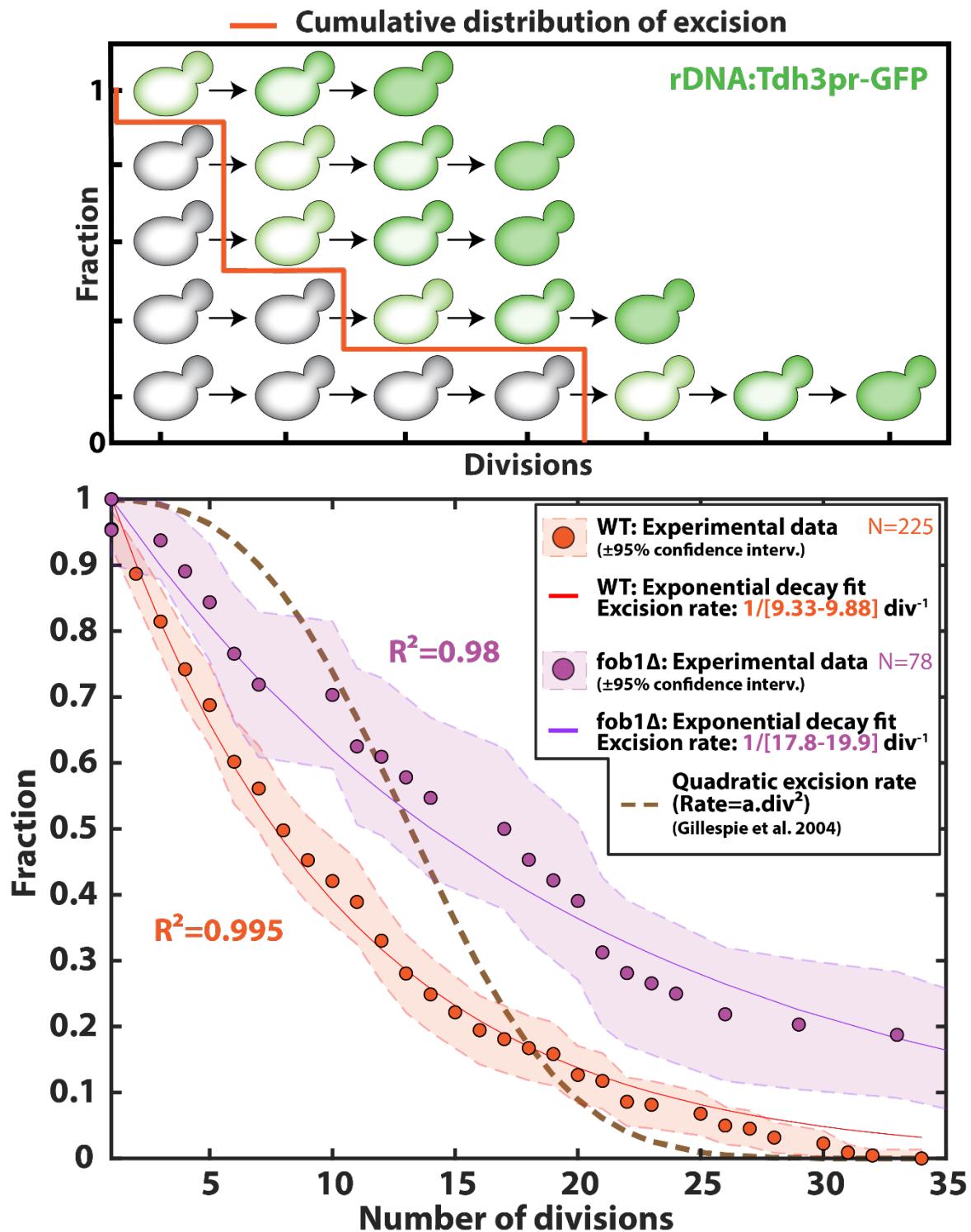


Figure 66: Cumulative fraction of cells in the birth to excision period, among all the cells that displayed an exponential fluorescence increase. Top: explanatory scheme of how the cumulative fraction is computed. Bottom: Data (dots) and fit (solid lines) (Wildtype in orange, *fob1Δ* in purple). The excision rate can be deciphered from the fit and is displayed in the inset with a 5% confidence interval.

Interestingly, the *fob1Δ* mutant (a mutant known to experience less ERC excision, see p.56) has a halved probability of excision per division compared to the WT, which is higher than what was predicted with a previous model [226].

### 1.3. Perspectives

Altogether, these preliminary results suggest that our marker can detect the excision of ERCs and measure the dynamics of their accumulation. In particular, it seems that **the probability of excision is constant with age and that cells can experience it even during their first S phase.** However, several controls must be done in order to validate the approach.

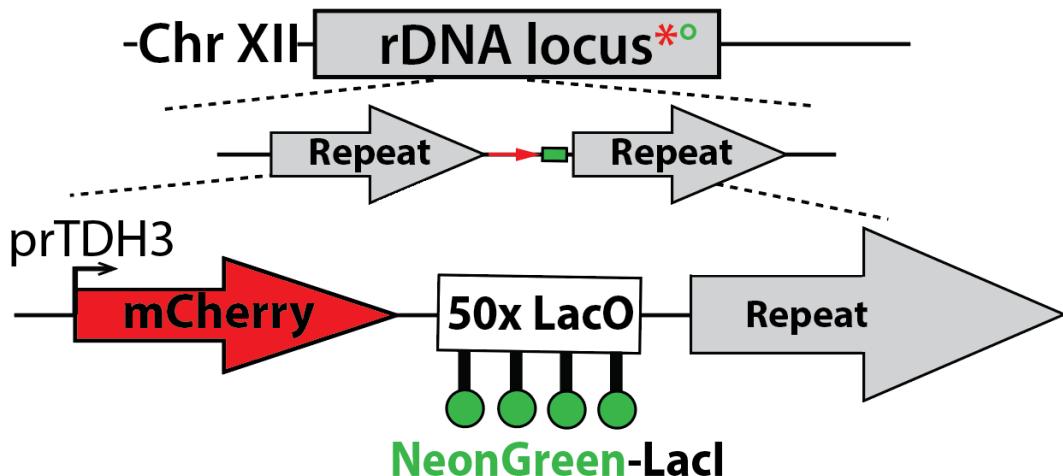
First, it is unclear in the literature whether ERCs are actively transcribed or not. On the one hand, non-coding RNA are enriched in old WT cells and not in *fob1Δ* cells [330]. Moreover, an exponential increase of Pol I, Pol I target transcripts are detected with the same dynamics as ERCs' during aging [226]. On the other hand, half of the rDNA is not transcribed, and adding more repeats to the rDNA array does not change the amount of Pol I transcription [381] (though Pol II transcription has not been looked at in these strains). Even though ERCs are probably not submitted to the same regulations as intrachromosomal repeats, whether or not they are transcribed and which polymerase transcribe them is not evident in literature. Nevertheless, our data provide direct evidence that ERCs are actively transcribed by Pol II, since the dynamics of the transcriptional reporter match with the increase of ERCs numbers [226].

To verify that, it would be interesting to isolate unmodified ERCs from old mothers [483] after enrichment and sorting (Appendix 2), and observe them with an electron microscope to see potential nascent transcription from Pol I [381], or perform ChIP targeting for Pol I.

Even if we could observe an exponential accumulation of fluorescence across several orders of magnitudes, it could be that the *bona fide* excision of the circle happens earlier than the increase of fluorescence. Indeed, it could be that part of the ERCs is silenced and that the observed increase occurs only later in life when the silencing is lost. This is not likely to be the case since we observe increases in fluorescence even after one division. Yet, we could verify that by several means. It is indeed possible to modulate rDNA silencing with drugs such as nicotinamide (an inhibitor of Sir2) or nicotinic acid (an activator of Sir2). Therefore, perfusing this drug in the media in the middle of the experiment and observing if cells with tagged ERCs dramatically change their fluorescence or not would indicate if ERCs are silenced.

To further verify this hypothesis and, more generally, assess if the transcriptional reporter faithfully reports the number of ERCs, we engineered a strain harboring a direct reporter of

ERC (LacO array in a strain expressing LacI-Neongreen) at the same locus as the transcriptional reporter (Figure 67).



*Figure 67: Genetical construction of a double ERC reporter strain. A transcriptional reporter (*prTDH3-mCherry*) is inserted in the *IGS1* and is followed by a 50xLacO sequence. The cell is also expressing Neongreen-Laci from another locus.*

The LacO-Laci-Neongreen system is therefore a more direct reporter of ERCs but emits less fluorescent signal than the transcriptional reporter. Consequently, longer exposures are required to obtain a signal, which affects the temporal resolution (to avoid phototoxicity) and is the reason also why we opted for a transcriptional tag for routine experiments. However, for the control, comparing the dynamics of the two reporters (even at a low framerate) will show if there is a lag between the excision and the transcription of the reporter or not.

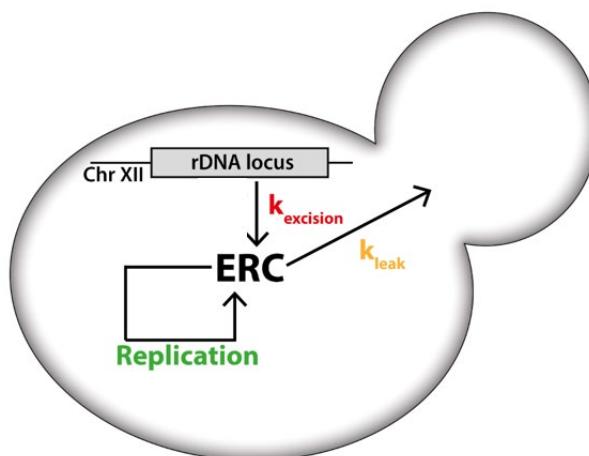
It is also possible that transcription of the GFP under the strong TDH3 promoter influences the probability of excision since it has been shown in different mutants that the number of ERC is linked with the number of ncRNA transcripts [423]. Nevertheless, in the light of our data and of recent studies [330], it is most likely that most of the increase of ncRNA transcripts numbers from population assays comes from ERCs. In other words, the accumulation of ERCs driving the accumulation of ncRNA is more probable than an upregulation of ncRNA transcription driving more ERC generations.

Besides, the RLS of our tagged strain and of the cells displaying the excision of a tagged repeat is identical to that of WT, suggesting that the excision rate is the same between the untagged WT and the tagged WT. However, though the measured excision rate fits with the mathematical model for the WT, that of *fob1Δ* is higher than that of the model. To understand if the transcriptional reporter affects excision rate, we engineered a strain with just a 50x LacO inserted in one repeat (Figure 67 but without transcriptional reporter). Comparing the

excision rate in this strain with that of the transcriptional reporter strain will tell if adding a Pol II target in the intergenic spacer regions promotes excision or not.

Another caveat of having one repeat tagged among 150 untagged is that we cannot be sure that the observed excision is the first one in the life of the cell. If the excision of a repeat does not change the probability of further excisions, then the measured probability is valid. If the excision of a repeat impacts the probabilities of excising another repeat, we would expect even less late events, which would even less favor the age-dependent hypothesis. However, simulating the two hypotheses and generating the subsequent cumulative distribution of excision times needs to be done as an additional control.

This ERC marker would be interesting to be used in different context known to modify the excision rate (like I did with *fob1Δ*), or the accumulation rate (diffusion barrier mutants like *bud6Δ* [354] or perturbations [484], NPC tethering with *sgf73Δ* [356] or rDNA-ARS mutants [202,417]), to further validate the approach (Figure 68).



*Figure 68: Framework of mutants and perturbations to further validate the ERC reporter. Acting on the excision rate, the replication, or the leaking should change the different dynamics of the signal.*

Interestingly, the strain used for measuring ERC excision has another fluorescent reporter (promoter TDH3-mCherry) placed in another repeat [328]. The first advantage of that is that it doubles the probability of seeing an excision event.

In addition, it is possible to measure the loss of Pol II silencing in the rDNA relative to the excision and accumulation of ERCs (Figure 69). Is the loss of silencing causing ERC excision as suggested by the literature? Conversely, is the ERC accumulation causing the loss of silencing by titrating Sir2 proteins? Are these two events unlinked? A deeper analysis of the data will be required to conclude but would bring interesting data to the debate [240,257,321].

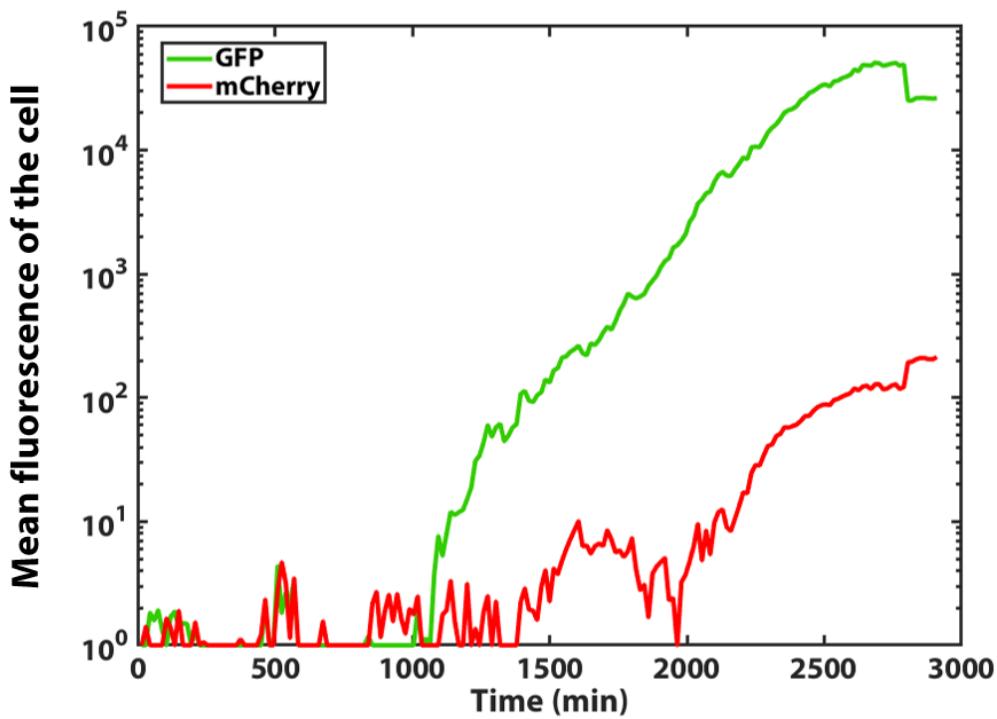


Figure 69: Mean fluorescence from a single cell with two rDNA transcriptional reporters. In this case, an exponential increase of the GFP (ERC accumulation) is followed by a mild increase of mCherry (loss of silencing of second ERC excision).

Moreover, these data were obtained before the development of the high-throughput methodology presented earlier, and more events and experimental replicates would be necessary to properly conclude about the time-dependency of the excision.

Finally, using this pipeline, it will also be interesting to verify that the period between the excision and the SEP is deterministic, as suggested by previous numerical modeling [226].

## General discussion

Aging is a phenomenon affecting many different biophysical processes. In multicellular organisms such as humans, it is associated with an increased death rate and a progressive failure of the different organs due to the aging of the cells of their tissues. However, due to the different scales at play (organism - organs - tissue - cell - organelles - molecules) and the interplay between them, it is very complex to understand why organs fail, how these failures are linked together, or how the cells from these organs age. For this reason, unicellular eukaryotes can be relevant - to a certain extent - to study aging because they are both a cell and an organism while having no cell-cell interaction (in most contexts). In particular, *S. cerevisiae* is an attractive model because of its asymmetric division pattern and the rejuvenation of the progeny of a given cell.

Besides, it has exhaustive mutant collections (deletion and GFP [199]); it is easily manipulated thanks to the new microfluidic technologies and ages fast. Thus, it is possible to screen many different genes with immense statistical power and observe their effect and dynamics at the single-cell level throughout the lifespan. These are unique features of budding yeast and make it a still invaluable model in geroscience. It is true that the molecular mechanisms at play (such as the ERCs) might not all be entirely conserved in "higher" eukaryotes. Nevertheless, understanding how different processes are destabilized, driving to a general loss of homeostasis of the cell and leading to death, would **set a framework to address the system dynamics of aging in organisms in general**.

Indeed, among the many processes identified as losing their homeostasis during aging (see introduction), it is often difficult to separate the causes from the consequences and to differentiate what is a compensatory mechanism from what is a deleterious change [126]. This challenge is made even harder by the heterogeneity (in kinetics and in quality) between isogenic cells in an identical environment.

Double deletion mutants can help distinguish if two genes are from the same pathway or not, but this kind of epistasis analysis is often complex to interpret due to the pleiotropy of many genes.

Therefore, adopting a single-cell and longitudinal approach is almost necessary to tackle this question. For that, microfluidic coupled with timelapse is the state-of-the-art method and has allowed sorting certain known hallmarks of aging in ordered pathways.

## 1. The ERCs pathway of aging: A countdown to death started by a stochastic trigger

Doing that, our lab and others have identified a stereotyped pathway of aging, implying the rDNA followed by most of the wild-type cells. This pathway is triggered by the excision of an rDNA circle (ERC) that self-replicates exponentially throughout the divisions (almost ¾ of the ERCs being replicated at each division) in the mother cell, until a threshold number at which the cell enters a pathological state (Senescent Entry Point). This SEP is preceded by an increase of the rDNA transcription and is defined as a cell-cycle slowdown and an increase of the karyoplasmic ratio. Once passed the SEP, the cell dies with a constant probability at each division (see p.86).

Here, preliminary data suggest that the ERC excision is a stochastic (that is, age-independent) process. This indicates that **this event is most likely not caused by another age-dependent degradation and is instead an upstream event of the aging choreography.**

Interestingly, the cell-cell variability (due to the stochasticity of the process), the fact that ~13 divisions (amplifications of the circles) are needed before reaching the SEP, and the stochastic death once the SEP is reached, hide the fact that the aging curve is composed of age-independent processes (see p.86). In other words, **aging can arise from age-independent processes.**

Although, many questions about the links between the events of this pathway remain unanswered:

Why is the rDNA transcription increased around the SEP? Under normal conditions, the rate of rDNA transcription is buffered against variation in copy numbers [379,381,409]. Therefore, increasing the number of copies (like with ERCs) beyond basal numbers should not lead to an increased transcription (though this model has been recently debated [240,485]). If ERC accumulation is the cause of the whole nucleolus activity [226] is not sure, but the data presented previously using transcriptional reporters seems to prove that **ERCs are transcribed**, which was not directly established so far.

Why is the N/C ratio increasing at the SEP? Indeed and as discussed in the introduction (see p.45), the N/C ratio is very tightly regulated under normal conditions and conserved in many contexts, not only in budding yeast but also in *S. pombe*, mammal cells, and *Xenopus* [337-340]. The nucleocytoplasmic transport has been proposed as a regulator of the nucleus size

## Chapter I: General discussion

---

and modifying import and export rates, and in decreasing, mRNA export [339,340] is known to increase the N/C ratio.

In the context of aging, the NPC homeostasis is disrupted [341,356-358], and in particular old cells show an increased compartmentalization of proteins in the nucleus [226,341].

How to explain these changes? Since they happen every time at an approximate given number of ERCs, these DNA circles are a potential suspect. In line with that, ERCs are thought to attach to the NPC via the SAGA complex, leading to acetylation of several components of the NPCs, in particular, their nuclear basket (see Figure 37 p.60)[356-358]. Another possible explanation would be that the increase of nucleolus size (driven by ERCs) drives this massive specialization of the pores since NPCs adjacent to the nucleolus, even in young cells, are devoid of baskets [347,436,486,487].

Such remodeling of the NPCs could modify their nucleocytoplasmic transport. In fact, basket-less NPCs tethered with plasmids have enriched importins (in particular Kap123 that mediates the nuclear import of ribosomal proteins and of histones), and depleted exportins (in particular exportins involved in mRNA export). Altogether, the increased N/C ratio could be explained by a disruption of the nucleocytoplasmic exchanges, in part altered by ERCs tethering to the NPCs.

Another possibility would be that the excess DNA from the ERC would signal to the nucleus. In fact, the quantity of ERC at the end of the lifespan is similar to the quantity of chromosomal DNA, and early studies proposed that the quantity of DNA controls the nuclear size (called the nucleoskeletal theory [488-490]. However, a more recent study established that it might not be the case [337], so this theory is less likely to explain the increase of nucleus size.

What is causing the SEP? Like the N/C ratio, the cell-cycle duration is tightly regulated under normal conditions, and homeostasis is lost at the SEP. Are ERCs directly causal for that? Is it simply because they increase the total amount of DNA in the cell, or because they are rDNA sequences? Is it linked to the massive upregulation of the rRNA transcription?

First, increased nucleus size and content could disrupt the homeostasis of cyclins, CDKs, and other proteins involved in cell-cycle duration. Indeed, some of these proteins are controlled in concentration [248,491], and the global increase of the concentration of nuclear proteins could affect this equilibrium. Hence, the SEP could be an indirect cause of ERCs, via nuclear deregulation.

On the other hand, the number of ERCs saturates in the post-SEP period, suggesting that the replication machinery is deregulated, potentially because too much DNA is to be replicated.

This is supported by the observation that yeast mutants over-amplifying a  $2\mu$  DNA circle possess a relatively longer S-phase [434]. Moreover, and as enunciated in the introduction, the DNA sequence of these circles contains binding sites for the SBF and MBF transcription factors, which control the G1/S transition (Wittenberg and Reed 2005). Hence, the SEP could be directly caused by an accumulation of DNA titrating the replication machinery and transcription factors of the cell cycle.

If the massive upregulation of rRNA transcription is part of the SEP phenotype is currently assessed in our group. Unpublished data show that deleting Rrn3 (an activator of Pol I) or degrading Pol I in the post-SEP phase do not prolong lifespan nor rescue the SEP phenotype. Therefore, the upregulation of the rRNA transcription is likely to be a simple byproduct of ERCs and not a cause of the SEP.

An interesting corollary of all these questions is that aging can also be used as a perturbation to study fundamental processes that are very well regulated under physiological conditions.

### 1.1. An integrative model of the ERC pathway of aging

One possible integrative model of the ERC pathway of aging starts with a stochastic excision of a circle that multiplies by self-replication with the cell divisions. These circles are tethered to the NPCs so they are not transmitted to the progeny, with the caveat that the affected NPCs get remodeled by losing their basket and disrupting their nucleoplasmic transport. In particular, they would import more matter (notably ribosomal proteins and histones) and export less (in particular, mRNA). Altogether, this overproduction of matter and modification of the import/export homeostasis would lead to an increase in nuclear size and content, which in addition to the DNA burden from these circles, would in turn affect the cell-cycle homeostasis. Then, many other processes, often described as hallmarks of aging, would appear (sharp loss of pH, loss of protein stoichiometry and ribosome biogenesis decline, multinucleation, genomic alterations, etc). Potentially, feedforward and feedback loops exist between these post-SEP processes (such as the loss of protein stoichiometry, affecting the NPC and the translation machinery [294,341], in turn worsening the loss of protein stoichiometry), and participate in the death of the cell.

Why has evolution selected ERCs? As seen in the introduction, the evolutionary theory of aging proposes that the toxicity of aging factors is the consequence of an antagonistic pleiotropy which gives a fitness advantage at early ages. In line with that, ERCs are a

byproduct of rDNA recombinations which is a rapid evolutive mechanism to modulate the size of the rDNA array. Besides, ERCs can reintegrate the rDNA locus and participate in replenishing the number of copies [378]. To validate this hypothesis, it would be interesting to perform competitive assays between WT and *fob1Δ* in different changing environments, and show that the WT outcompete the *fob1Δ* mutant that is unable to adapt its rDNA size.

Altogether, we provide a framework to explain one path of aging by separating three distinct phases: excision, replications, and post-SEP survival. This ordering seems crucial to understanding different mutants' role since the confusion between excision and accumulation is often made in the literature. For instance, a fairly recent study [202] used different rDNA-ARS (which impact the replication efficiency of ERC) and found that weaker ARS leads to increased lifespan. However, they found an additive effect with a SIR2 or a FOB1 deletion and thus concluded that modulating the ARS affects lifespan in an ERC-independent manner. In reality, Sir2 and Fob1 affect the excision rate, while the ARS affect the replication rate, which both affect the ERC quantity in a population.

More generally, to challenge this model, it would be necessary to screen known mutants of longevity and see if they are long-lived because of a decreased excision probability, a slower accumulation, if they trigger the SEP at a higher dose of ERCs, or if they survive better in the post-SEP period (Figure 70).

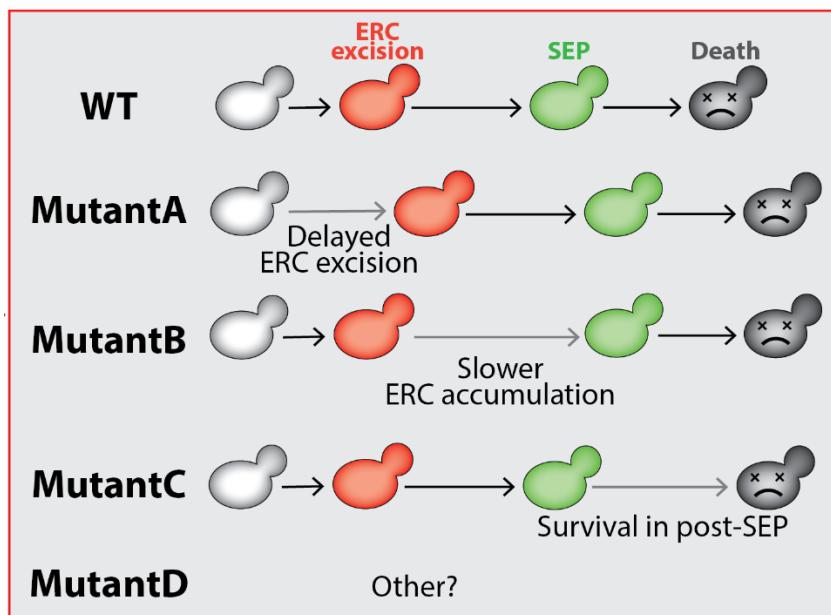


Figure 70: Scheme describing the potential effect of longevity mutants on the ERC-pathway of aging

## 2. Relevance of the ERC/rDNA pathway in other organisms

The molecular relevance of such a pathway in other organisms is debatable. Indeed, though ERCs exist in other organisms, they have not been found as enriched during aging. However, extrachromosomal circles (eccDNA) are a hallmark of cancer cells and have raised much attention in the past few years [492-495], thanks to the adaptive potential they can confer to a cell by increasing gene copy number [496,497]. Though in the case of cancer, eccDNA provides a fitness advantage, they have also been found implicated in the aging of the central nervous system and in neurodegeneration [498].

If ERCs are absent from aging tissues in mammals, the rDNA homeostasis is a recurrent hallmark of aging and cancer. Indeed, since all the cellular functions are dependent on the ribosome biogenesis across the living, the fact that the rDNA is organized in repetitive repeats is very conserved so that cells can crank out many ribosomes simultaneously. Consequently, rDNA sequences are also prone to genomic damage in these organisms and are strongly rearranged during aging and in cancer cells [499-501]. Besides, misregulated ribosome biogenesis and increased rRNA transcription is known to be linked with senescence in mouse and human fibroblasts [502,503]. Along the same line, small nucleoli and reduced ribosome biogenesis are also correlated with longevity in *C. elegans*, *Drosophila*, mice, and human muscle tissues [234,369,370,504]. In line with that, caloric restriction or TORC1 inhibition, two of the most conserved longevity interventions, decreases the nucleolus size [505]. Altogether, this points out that the nucleolus homeostasis and the rDNA pathway(s) of aging that we described have a crucial and conserved role in cancer and aging.

## 3. Other pathways of aging

At least one other pathway (or “mode”) of aging exists and has been described as mutually exclusive with an “rDNA mode” (loss of rDNA silencing, increase of nucleolus size [283]). This rDNA mode is most probably what we described as the ERC/rDNA pathway, while this second mode seems to implicate mitochondria homeostasis [283]. Recently, another independent but concomitant study also reported two divergent populations, this time regarding the vacuolar pH and the iron-sulfur cluster maintenance [280]. It would be interesting to see if these populations also represent the two modes, “rDNA” and “Mitochondria” (which is likely, knowing the link between mitochondria and vacuolar pH and ISC, see p. 38). Nonetheless, if this “mitochondrial pathway” is really aging or simply the age-independent and transmissible loss of mitochondrial potential that can occur in the life of

cells, as described previously [246], has not been carefully analyzed by the authors (though the phenotype seems to be inherited to the daughters, according to the raw data provided). In any way, the fact that two-thirds of the *fob1 $\Delta$*  cells die without ERCs suggests that another path of aging exists. This idea is reinforced by the fact that mathematical modeling fails to perfectly recapitulate the aging curve by simply taking into account the ERC model, and a competing ERC-independent cause dictated by a Gompertzian law (age-dependent mortality, see introduction) needs to be added to the model to better fit the survival curve (Figure 59, p.86). Understanding what causes this age-dependent part, and how ERC-less cells die, is of particular interest.

In a more general manner, we could wonder where all the described hallmarks of aging from the introduction (and many more not presented here) stand on the aging picture.

Altogether, it is very likely that several pathways of aging with different kinetics compete together (potentially interconnected at some nodes) (Figure 71, left), or alternatively, that one path is exclusive to the others [223,283](Figure 71, right) [126,506,507].

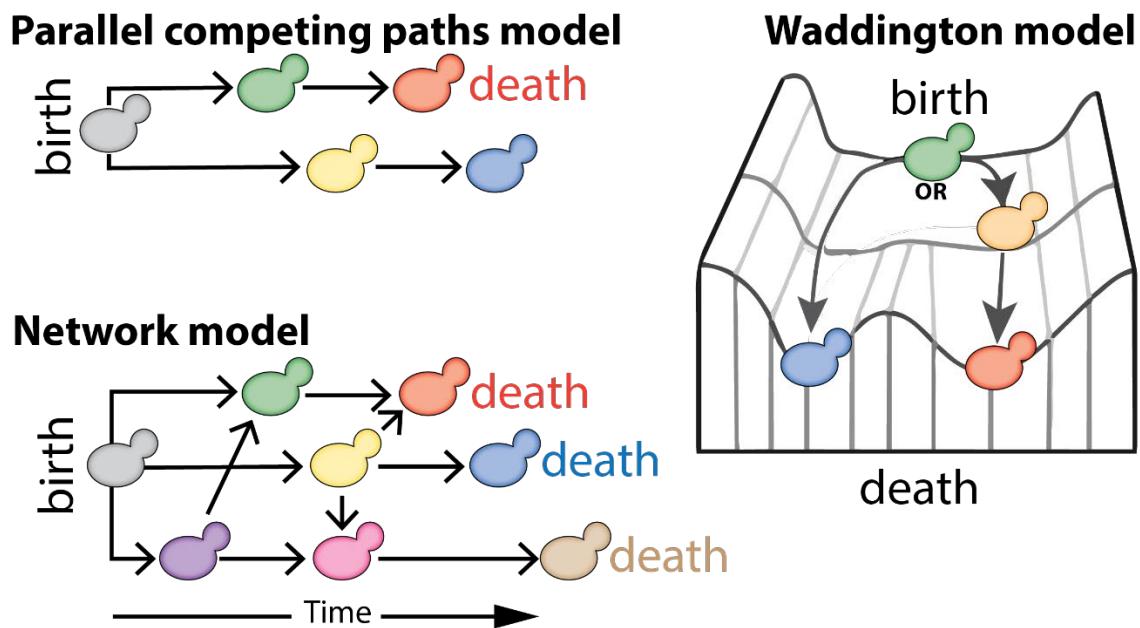


Figure 71: Three different frameworks can model aging. Parallel and independent (top left) or interconnected (bottom left) processes can occur simultaneously, and both lead to death. Conversely, mutually exclusive pathways (cell-fate like) could explain aging (right)

Yet, the current tools have a limited power to test so many hypotheses. Even if microfluidics lifted a manipulation burden, the throughput and, in particular, the speed of analysis (mainly manual), remains an obstacle for systematic and comprehensive screens. For that, **we developed a high-throughput platform for automated and standardized aging assays,**

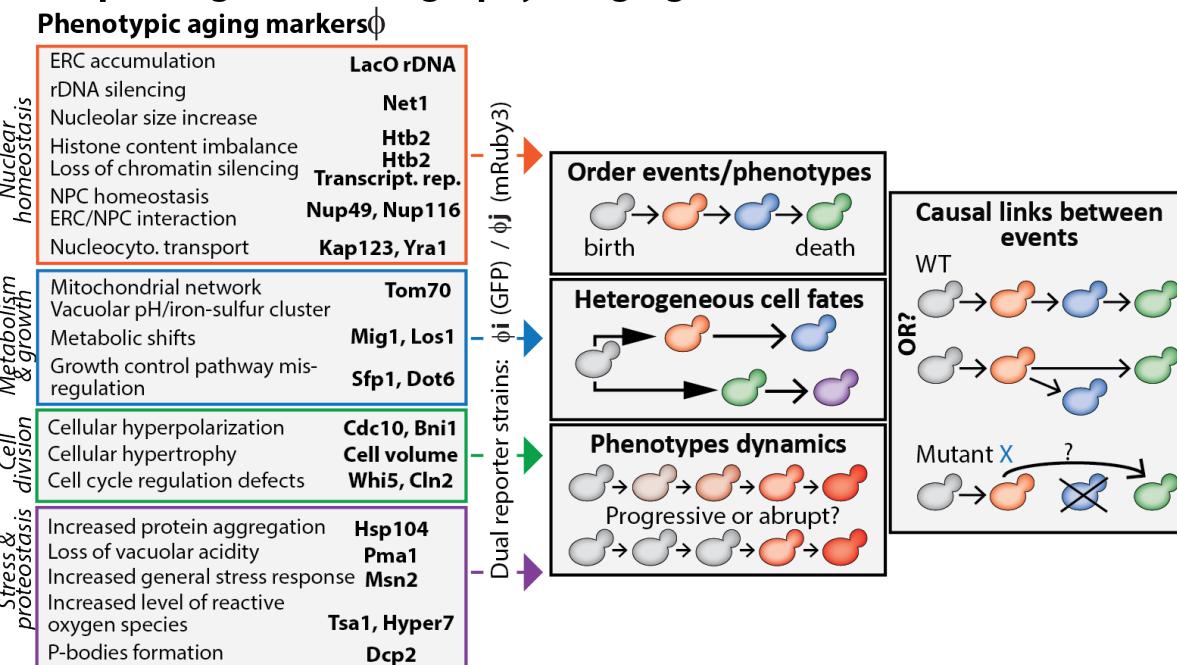
which will allow us to analyze all the known markers of aging at the single-cell level and classify them into aging pathways.

## 4. A general framework to classify hallmarks of aging into pathways

### 4.1. Deciphering the choreography of aging

Indeed, since neither monitoring nor analysis represents a throughput problem (16x2000 cells can be screened and analyzed in 4 days) anymore, it is possible to screen 4 or 5 markers (division duration, growth, and 2-3 fluorescent tags) simultaneously in order to decipher their dynamics and where they stand one towards the others. With that, we will be able to temporally order the phenotypes to know if their occurrence is abrupt or progressive and classify them into one or another aging pathway (Figure 72).

### Deciphering the choreography of aging



*Figure 72: Experimental plan to decipher the choreography of aging. (Left): List of phenotypic aging markers, grouped in functional ontologies, that will be tagged fluorescently. (Right): Types of dynamics that can be assessed with these markers. The color of the cell represents a phenotype of aging, tagged using a fluorescent marker. It is possible to order them temporally (top left tile), to know if they are abrupt or progressive (bottom left tile), to know if they are independent of each other's (mid left tile), or to know if a marker is a link of a chain of causality or a pure byproduct (right tile)*

For instance, we could systematically use a Net1-mRuby3 as a marker of the rDNA/ERC pathway (or simply look at the existence of a SEP, which always correlates with an ERC accumulation [226]), with another marker X. Does X belong to the ERC mode of aging? If so, does it occur before or after the nucleolar misregulation and the SEP? With this approach, we could, for example, assess if the NPC remodeling and the impairment of the nucleocytoplasmic exchange [341,358] are ERC-dependent or not (using tagged Nup116 and

other nucleoporins from different parts of the NPCs, as well as karyopherins), and if yes, whether they occur progressively with the ERC accumulation, abruptly at the SEP, or only in late the post-SEP phase.

Another example application could be to determine if any of the described aging phenotypes (such as Whi5 accumulation [248] or proteostasis collapse [316]) *bona fide* paths of aging or only post-SEP and proximal causes of cell death.

Along the same line, a recent report described P-bodies formation in late ages and linked it to a change in cytoplasmic pH [292]. Could we show that the pH drop and the P-bodies formation are post-SEP events (as suggested in [238] for the pH)?

Beyond allowing the classification of hallmarks in aging pathways, looking at temporal sequences of events can be very informative on the causality. If event A systematically occurs before event B, then B is probably causally linked to A (directly or indirectly). However, this observation remains correlative, and deletion mutants are necessary to reinforce such conclusions and determine if an event is a cause or just a byproduct. Do we still have the rest of the chain of events when deleting a gene causing a marker? If so, then this marker is probably a byproduct of the pathway (Figure 72, top right).

Finally, without even looking at several markers at the same time, measuring the statistics of the occurrence of a given event can inform us about its causes. For example, if an event is not dependent on age or is observed at any age (like the loss of mitochondrial DNA [246] or the excision of an ERC, as presented in this dissertation), then it is not likely to be the result of a pathway of aging, but rather the genesis of a pathway (or a standalone event).

### 4.2. Classifying longevity genes

Similarly, screening the longevity mutants will also allow distinguishing the different paths of aging and the kinetics of each component of the paths.

For example, if two different paths of aging have different kinetics (Figure 73B "WT"), we could determine if longevity mutants are long-lived because they affect the kinetics of one path or the other (Figure 73B "Mutant1"), and which element of the path has its kinetics modified (Figure 73C, and example with the ERC pathway p.138). Along the same line, slowing down the kinetics of the fastest aging path should reveal the other (Figure 73B "Mutant2").

For example, it could be that ERC accumulation (and all its downstream effects) is the first replicative limit encountered by the cell (Scenario "WT" in Figure 73B), but if the kinetics of this pathway is decreased (for example, in a *fob1Δ* mutant), then another competing pathway of aging becomes the new replicative limit, later than in the WT (scenario "Mutant 1" and

"Mutant 2" on Figure 73B). In line with that, one-third of the *fob1Δ* cells die with a SEP and an ERC accumulation, but later than the WT cells (scenario "Mutant 1"), while two-thirds die without a SEP nor ERC accumulation (scenario "Mutant 2").

## Classifying known pro-longevity genes

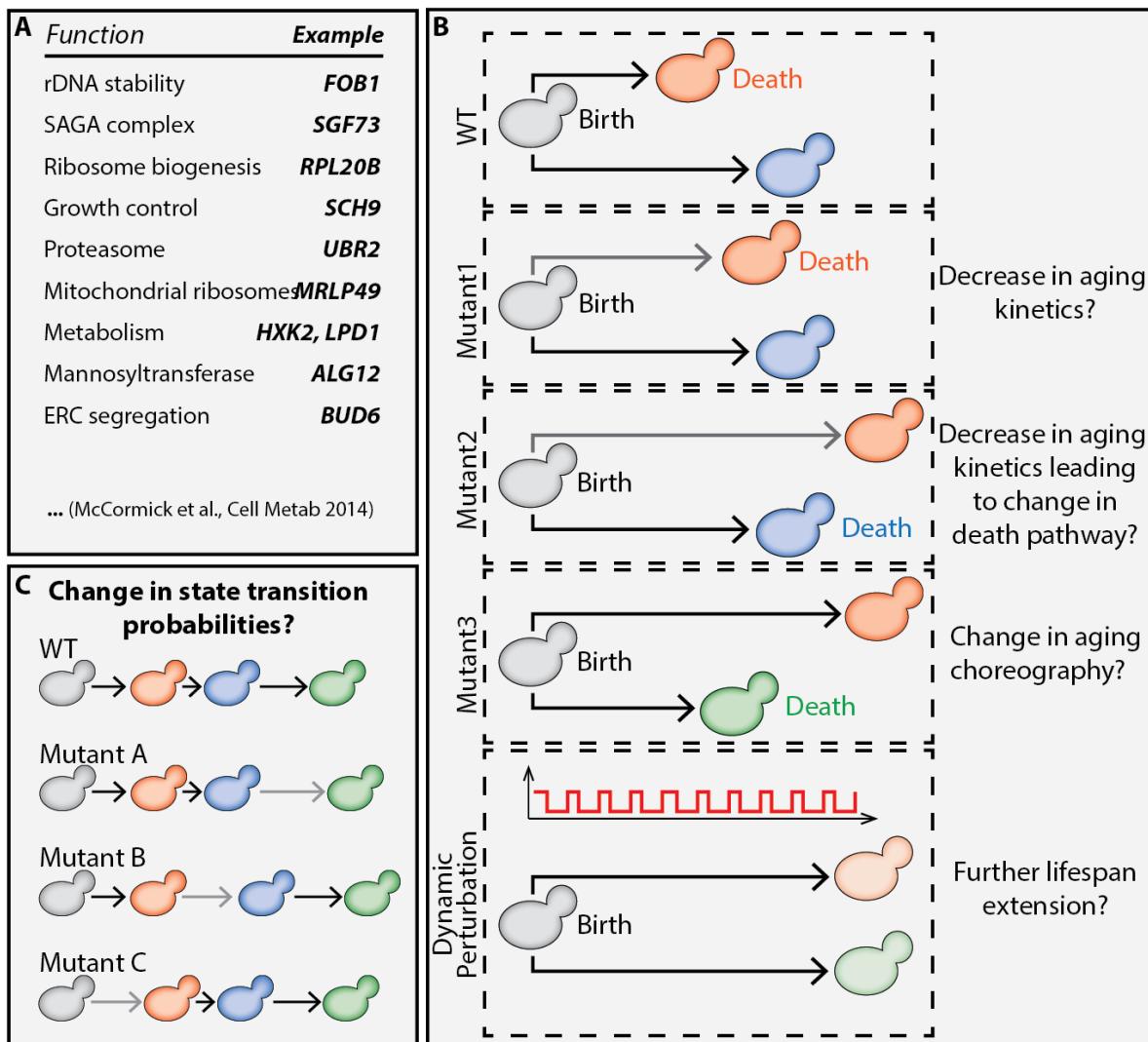


Figure 73: Experimental plan to classify longevity genes. (A): Non-exhaustive list of deletion mutant to screen using markers from Figure 72. (B): With the hypothesis of two concomitant aging pathways (WT scenario), we could observe mutants that are long-lived because they delay one path or the other, therefore changing the proportion of cells dying from one or the other and reveal new paths. (C): Among one pathway that is delayed, we could identify which transition between two nodes of the pathway is slowed down (grey arrow).

Instead of deletion mutants, dynamic genetic perturbations (with an AID- system, for instance [508]) are also a way to decipher the chains of causality. If a gene is thought to have an effect early in the chain of causality, then deleting it at later stages should not affect aging. For instance, deleting *FOB1* after the cells have excised an ERC should have no impact on the rest of the events, (namely the accumulation of ERC, the entry into senescence, and the post-SEP declines), nor on the RLS. Conversely, some deletions could have a deleterious effect

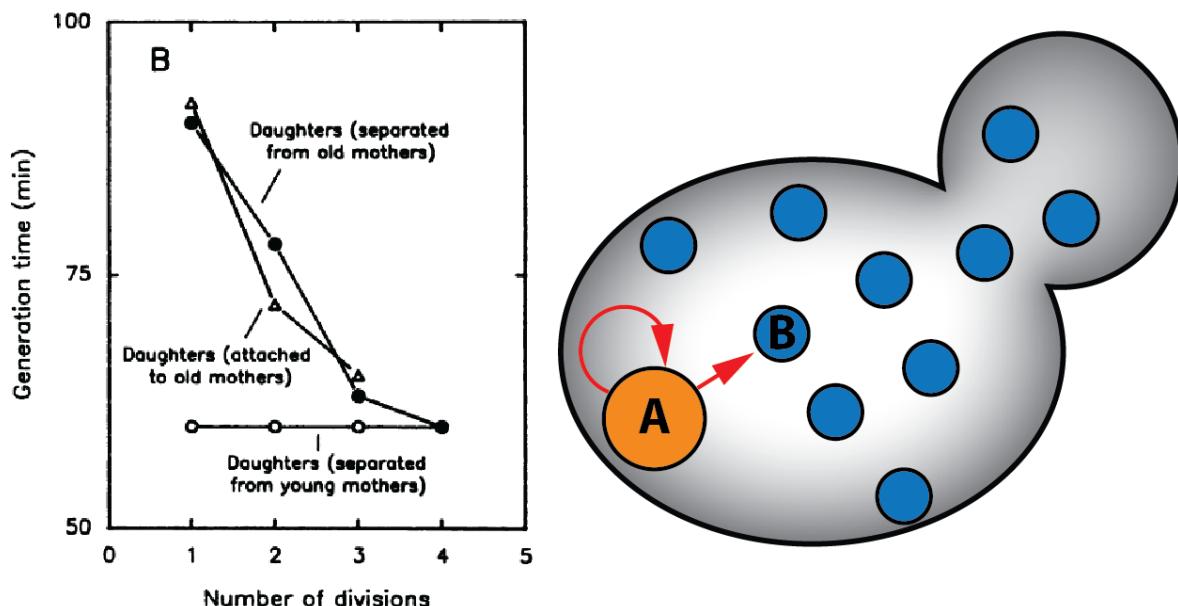
early in life but be beneficial once advanced in the path of aging (such as a GCN5 deletion, which is thought to have a higher excision probability, but a slower ERC accumulation [356]). Similarly, definitive deletion can cause fitness defects, while an intermittent reduction of one protein quantity could increase lifespan. More generally, with such an approach, we could also test the antagonistic pleiotropy theory of aging by suppressing genes early or late in life and measuring the impacts on fitness (growth) and survival.

Along the same line, environmental perturbations such as caloric restriction, amino acid depletion, inhibition of TOR with rapamycin, or growth in respirative media are known to affect lifespan positively [157,158,295]. Understanding how these effects impact longevity is key to grasping aging because of their high degree of conservation and the fact that they act on different hallmarks of aging simultaneously. However, this pleiotropic nature makes the results difficult to interpret, but using our pipeline could help to determine precisely and systematically which aging markers are affected. Besides, no study exists about the lifespan of cells in fluctuating environments such as periodic pulses of caloric restrictions and if it could be possible to further extend the lifespan with such dynamic switches.

This ordering and classification of events in pathways and networks seem basic and obvious but are in reality rarely applied due to the cumbersome task of tracking cells manually throughout aging. We think that the new tool we developed will help realize such comprehensive and systematic analyses and that budding yeast remains the most suitable model for this approach.

## 5. Observing the daughters to understand aging factors

To better identify and understand the aging factors, one could also observe the progeny of old cells. So far, careful examination of daughters has been almost absent from the literature, except from early studies [211,212,224]. Nevertheless, this approach can be very informative about what drives aging in the mother, and help tell which hallmark is a *bona fide* aging factor (that is, not transmitted to the daughter).



*Figure 74: Examining the lifespan of daughters from old mothers can be informative. (Left) Division time as a function of the divisions, of daughters from old mothers (black circle and triangle) compared to daughters from young mothers (white circles) (From [211]). (Right): Hypothetic new model of aging factor. A factor A, non-necessarily toxic, asymmetrically segregated and accumulating in the mother, drives the accumulation of a factor B, toxic but not actively segregated and thus being diluted throughout the divisions in the daughter.*

For example, daughters from old mothers divide slower than those from young mothers, but it is back to normal after a few divisions [211] and Figure 74). This suggests that these daughters inherit a factor that slows down the division time, which is not asymmetrically segregated and not self-amplifying (since it is diluted at each division).

In other words, it is possible that a toxic factor is evenly segregated to the daughter but does not accumulate there because its production relies on another factor retained in the mother (Figure 74). Such concepts question the validity of the aging factor definition and framework (accumulation in the mother, toxicity, and non-transmission to the daughter [213]).

Yet, these experiments are in practice difficult to realize with the current tools, since the only way to study daughters from old mothers so far is to use microdissection or to harvest them from enrichment methods. Either way, it is impossible to correlate the mother's lifespan with that of the daughters.

Using the device and experimental setup presented in the discussion of the first project (p.129), we could capture the entire lifespan of a daughter cell born at any mother age.

Beyond understanding aging factors, we could also determine which damages are irreversibly passed to the progeny, such as -among the obvious ones- loss of heterozygosity, nuclear

## Chapter I: General discussion

---

fragmentation, and loss of mtDNA. In this sense, we could adequately characterize mitochondrial damages that have often (and still recently) been described as aging factors, though they are inherited by the whole progeny in some cases [246,262,264].

However, though they cannot be described as an aging factor per se, traits that decrease the lifespan while being inherited (DNA mutations, nuclear fragmentation, loss of mtDNA, etc) contribute to aging and should not be ignored. Therefore, we could question the replicative aging model and consider the rejuvenation loss as a hallmark of aging, since genomic instabilities and some epigenetic alterations are part of human aging and are transmitted to the daughter cells as well.

Altogether, this suggests that studying how the daughters of old mothers age and rejuvenate has been overlooked so far but could become a powerful angle to tackle replicative aging thanks to newly available technologies.

## References Chapter I

- [1] Z. A. Medvedev, "An attempt at a rational classification of theories of ageing," *Biol Rev*, vol. 65, no. 3, pp. 375–398, 1990, doi: 10.1111/j.1469-185x.1990.tb01428.x.
- [2] E. J. Williamson *et al.*, "Factors associated with COVID-19-related death using OpenSAFELY," *Nature*, vol. 584, no. 7821, pp. 430–436, 2020, doi: 10.1038/s41586-020-2521-4.
- [3] M. Roser, H. Ritchie, and E. Ortiz-Ospina, "World Population Growth," *Our World in Data*, 2013, [Online]. Available: <https://ourworldindata.org/world-population-growth>
- [4] M. R. Rose, *Evolutionary biology of aging*. 1991.
- [5] Wikipedia, "List of world records in masters athletics," n.d. [https://en.wikipedia.org/wiki/List\\_of\\_world\\_records\\_in\\_masters\\_athletics](https://en.wikipedia.org/wiki/List_of_world_records_in_masters_athletics) (accessed Nov. 14, 2021).
- [6] B. Gompertz, "On the nature of the function expressive of the law of human mortality, and on a new mode of determining the value of life contingencies. In a letter to Francis Baily, Esq. F. R. S. &c," *Philosophical Transactions Royal Soc Lond*, vol. 115, no. 115, pp. 513–583, 1825, doi: 10.1098/rstl.1825.0026.
- [7] C. E. Finch, M. C. Pike, and M. Witten, "Slow Mortality Rate Accelerations During Aging in Some Animals Approximate That of Humans," *Science*, vol. 249, no. 4971, pp. 902–905, 1990, doi: 10.1126/science.2392680.
- [8] J. W. Vaupel, T. E. Johnson, and G. J. Lithgow, "Rates of Mortality in Populations of *Caenorhabditis elegans*," *Science*, vol. 266, no. 5186, pp. 826–826, 1994, doi: 10.1126/science.7973641.
- [9] W. Mair, P. Goyer, S. D. Pletcher, and L. Partridge, "Demography of Dietary Restriction and Death in *Drosophila*," *Science*, vol. 301, no. 5640, pp. 1731–1733, 2003, doi: 10.1126/science.1086016.
- [10] H. Qin and M. Lu, "Natural variation in replicative and chronological life spans of *Saccharomyces cerevisiae*," *Exp Gerontol*, vol. 41, no. 4, pp. 448–456, 2006, doi: 10.1016/j.exger.2006.01.007.
- [11] O. R. Jones *et al.*, "Diversity of ageing across the tree of life," *Nature*, vol. 505, no. 7482, pp. 169–173, 2014, doi: 10.1038/nature12789.
- [12] G. C. Williams, "Pleiotropy, natural selection, and the evolution of senescence," *Evolution*, vol. 11, no. 4, pp. 398–411, 1957, doi: 10.1111/j.1558-5646.1957.tb02911.x.
- [13] A. Weismann, "Essays upon heredity and kindred biological problems," Vol. 1. 2d ed. Oxford: Clarendon., 1891.
- [14] L. Hayflick and P. S. Moorhead, "The serial cultivation of human diploid cell strains," *Exp Cell Res*, vol. 25, no. 3, pp. 585–621, 1961, doi: 10.1016/0014-4827(61)90192-6.
- [15] A. M. Olovnikov, "A theory of marginotomy The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon," *J Theor Biol*, vol. 41, no. 1, pp. 181–190, 1973, doi: 10.1016/0022-5193(73)90198-7.
- [16] C. B. Harley, A. B. Futcher, and C. W. Greider, "Telomeres shorten during ageing of human fibroblasts," *Nature*, vol. 345, no. 6274, pp. 458–460, 1990, doi: 10.1038/345458a0.
- [17] B. Lakowski and S. Hekimi, "Determination of Life-Span in *Caenorhabditis elegans* by Four Clock Genes," *Science*, vol. 272, no. 5264, pp. 1010–1013, 1996, doi: 10.1126/science.272.5264.1010.
- [18] C. A. Wolkow, K. D. Kimura, M.-S. Lee, and G. Ruvkun, "Regulation of *C. elegans* Life-Span by Insulinlike Signaling in the Nervous System," *Science*, vol. 290, no. 5489, pp. 147–150, 2000, doi: 10.1126/science.290.5489.147.
- [19] R. A. Fisher, "Retrospect of the criticisms of the theory of natural selection," 1930.

## References from Chapter 1

---

- [20] J. B. S. Haldane, *New Paths in Genetics*. 1941.
- [21] W. D. Hamilton, "The moulding of senescence by natural selection," *J Theor Biol*, vol. 12, no. 1, pp. 12–45, 1966, doi: 10.1016/0022-5193(66)90184-6.
- [22] D. H. Nussey, H. Froy, J.-F. Lemaitre, J.-M. Gaillard, and S. N. Austad, "Senescence in natural populations of animals: Widespread evidence and its implications for bio-gerontology," *Ageing Res Rev*, vol. 12, no. 1, pp. 214–225, 2013, doi: 10.1016/j.arr.2012.07.004.
- [23] R. E. Ricklefs, "Evolutionary Theories of Aging: Confirmation of a Fundamental Prediction, with Implications for the Genetic Basis and Evolution of Life Span," *Am Nat*, vol. 152, no. 1, pp. 24–44, 1998, doi: 10.1086/286147.
- [24] G. Libertini, "An adaptive theory of the increasing mortality with increasing chronological age in populations in the wild," *J Theor Biol*, vol. 132, no. 2, pp. 145–162, 1988, doi: 10.1016/s0022-5193(88)80153-x.
- [25] G. Libertini, "Evidence for aging theories from the study of a hunter—gatherer people (Ache of Paraguay)," *Biochem Mosc*, vol. 78, no. 9, pp. 1023–1032, 2013, doi: 10.1134/s0006297913090083.
- [26] R. D. Lee, "Rethinking the evolutionary theory of aging: Transfers, not births, shape senescence in social species," *Proc National Acad Sci*, vol. 100, no. 16, pp. 9637–9642, 2003, doi: 10.1073/pnas.1530303100.
- [27] T. Y. Pang, "On age-specific selection and extensive lifespan beyond menopause," *Roy Soc Open Sci*, vol. 7, no. 5, p. 191972, 2020, doi: 10.1098/rsos.191972.
- [28] S. N. Chapman, J. E. Pettay, V. Lummaa, and M. Lahdenperä, "Limits to Fitness Benefits of Prolonged Post-reproductive Lifespan in Women," *Curr Biol*, vol. 29, no. 4, pp. 645–650.e3, 2019, doi: 10.1016/j.cub.2018.12.052.
- [29] P. S. Kim, J. S. McQueen, and K. Hawkes, "Why does women's fertility end in mid-life? Grandmothering and age at last birth," *J Theor Biol*, vol. 461, pp. 84–91, 2019, doi: 10.1016/j.jtbi.2018.10.035.
- [30] K. Hawkes, J. F. O'Connell, and N. G. B. Jones, "Hadza Women's Time Allocation, Offspring Provisioning, and the Evolution of Long Postmenopausal Life Spans," *Curr Anthropol*, vol. 38, no. 4, pp. 551–577, 1997, doi: 10.1086/204646.
- [31] T. B. L. Kirkwood, "Evolution of ageing," *Mech Ageing Dev*, vol. 123, no. 7, pp. 737–745, 2002, doi: 10.1016/s0047-6374(01)00419-5.
- [32] A. M. Leroi et al., "What evidence is there for the existence of individual genes with antagonistic pleiotropic effects?", *Mech Ageing Dev*, vol. 126, no. 3, pp. 421–429, 2005, doi: 10.1016/j.mad.2004.07.012.
- [33] N. L. Jenkins, G. McColl, and G. J. Lithgow, "Fitness cost of extended lifespan in *Caenorhabditis elegans*," *Proc Royal Soc Lond Ser B Biological Sci*, vol. 271, no. 1556, pp. 2523–2526, 2004, doi: 10.1098/rspb.2004.2897.
- [34] J. R. Delaney, C. J. Murakami, B. Olsen, B. K. Kennedy, and M. Kaeberlein, "Quantitative evidence for early life fitness defects from 32 longevity-associated alleles in yeast," *Cell Cycle*, vol. 10, no. 1, pp. 156–165, 2011, doi: 10.4161/cc.10.1.14457.
- [35] D. M. Garcia et al., "A prion accelerates proliferation at the expense of lifespan," *Elife*, vol. 10, p. e60917, 2021, doi: 10.7554/elife.60917.
- [36] D. W. Walker, G. McColl, N. L. Jenkins, J. Harris, and G. J. Lithgow, "Evolution of lifespan in *C. elegans*," *Nature*, vol. 405, no. 6784, pp. 296–297, 2000, doi: 10.1038/35012693.
- [37] S. N. Austad and J. M. Hoffman, "Is Antagonistic Pleiotropy Ubiquitous in Aging Biology?," *Evol Medicine Public Heal*, vol. 2018, no. 1, pp. eoy033-, 2018, doi: 10.1093/emph/eoy033.
- [38] T. Flatt and D. E. L. Promislow, "Still Pondering an Age-Old Question," *Science*, vol. 318, no. 5854, pp. 1255–1256, 2007, doi: 10.1126/science.1147491.
- [39] B. Charlesworth, *Evolution in Age-structured Populations*. 1994.

## References from Chapter 1

---

- [40] S. N. Austad, "Retarded senescence in an insular population of Virginia opossums (*Didelphis virginiana*)," *J Zool*, vol. 229, no. 4, pp. 695–708, 1993, doi: 10.1111/j.1469-7998.1993.tb02665.x.
- [41] S. C. Stearns, M. Ackermann, M. Doebeli, and M. Kaiser, "Experimental evolution of aging, growth, and reproduction in fruitflies," *Proc National Acad Sci*, vol. 97, no. 7, pp. 3309–3313, 2000, doi: 10.1073/pnas.97.7.3309.
- [42] M. Rose and B. Charlesworth, "A test of evolutionary theories of senescence," *Nature*, vol. 287, no. 5778, pp. 141–142, 1980, doi: 10.1038/287141a0.
- [43] A. M. Leroi, A. K. Chippindale, and M. R. Rose, "Long-term laboratory evolution of a genetic life-history trade-off in *drosophila melanogaster*," *Evolution*, vol. 48, no. 4, pp. 1244–1257, 1994, doi: 10.1111/j.1558-5646.1994.tb05309.x.
- [44] M. R. Rose, "Laboratory Evolution of Postponed Senescence in *Drosophila melanogaster*," *Evolution*, vol. 38, no. 5, p. 1004, 1984, doi: 10.2307/2408434.
- [45] L. S. Luckinbill, R. Arking, M. J. Clare, W. C. Cirocco, and S. A. Buck, "Selection for delayed senescence in *drosophila melanogaster*," *Evolution*, vol. 38, no. 5, pp. 996–1003, 1984, doi: 10.1111/j.1558-5646.1984.tb00369.x.
- [46] J. Nielsen et al., "Eye lens radiocarbon reveals centuries of longevity in the Greenland shark (*Somniosus microcephalus*)," *Science*, vol. 353, no. 6300, pp. 702–704, 2016, doi: 10.1126/science.aaf1703.
- [47] T. B. L. Kirkwood, "Evolution of ageing," *Nature*, vol. 270, no. 5635, pp. 301–304, 1977, doi: 10.1038/270301a0.
- [48] R. B. Cervantes, J. R. Stringer, C. Shao, J. A. Tischfield, and P. J. Stambrook, "Embryonic stem cells and somatic cells differ in mutation frequency and type," *Proc National Acad Sci*, vol. 99, no. 6, pp. 3586–3590, 2002, doi: 10.1073/pnas.062527199.
- [49] G. Saretzki, L. Armstrong, A. Leake, M. Lako, and T. von Zglinicki, "Stress Defense in Murine Embryonic *Stem Cells* Is Superior to That of Various Differentiated Murine Cells," *Stem Cells*, vol. 22, no. 6, pp. 962–971, 2004, doi: 10.1634/stemcells.22-6-962.
- [50] T. B. L. Kirkwood and S. N. Austad, "Why do we age?," *Nature*, vol. 408, no. 6809, pp. 233–238, 2000, doi: 10.1038/35041682.
- [51] T. B. L. Kirkwood, "Asymmetry and the origins of ageing," *Mech Ageing Dev*, vol. 126, no. 5, pp. 533–534, 2005, doi: 10.1016/j.mad.2005.02.001.
- [52] M. Ackermann, L. Chao, C. T. Bergstrom, and M. Doebeli, "On the evolutionary origin of aging," *Aging Cell*, vol. 6, no. 2, pp. 235–244, 2007, doi: 10.1111/j.1474-9726.2007.00281.x.
- [53] L. E. Orgel, "The maintenance of the accuracy of protein synthesis and its relevance to ageing," *Proc National Acad Sci*, vol. 49, no. 4, pp. 517–521, 1963, doi: 10.1073/pnas.49.4.517.
- [54] D. Harman, "Aging: A Theory Based on Free Radical and Radiation Chemistry," *J Gerontology*, vol. 11, no. 3, pp. 298–300, 1956, doi: 10.1093/geronj/11.3.298.
- [55] D. Harman, "Free radical theory of aging," *Mutat Res Dnaging*, vol. 275, no. 3–6, pp. 257–266, 1992, doi: 10.1016/0921-8734(92)90030-s.
- [56] A. B. Lindner and A. Demarez, "Protein aggregation as a paradigm of aging," *Biochimica Et Biophysica Acta Bba - Gen Subj*, vol. 1790, no. 10, pp. 980–996, 2009, doi: 10.1016/j.bbagen.2009.06.005.
- [57] G. Failla, "The Aging Process And Cancerogenesis," *Ann Ny Acad Sci*, vol. 71, no. 6 Genetic Conce, pp. 1124–1140, 1958, doi: 10.1111/j.1749-6632.1958.tb46828.x.
- [58] L. Szilard, "On the Nature of the Aging Process," *Proc National Acad Sci*, vol. 45, no. 1, pp. 30–45, 1959, doi: 10.1073/pnas.45.1.30.
- [59] H. L. Gensler and H. Bernstein, "DNA Damage as the Primary Cause of Aging," *Q Rev Biology*, vol. 56, no. 3, pp. 279–303, 1981, doi: 10.1086/412317.
- [60] G. A. Garinis, G. T. J. van der Horst, J. Vijg, and J. H. J. Hoeijmakers, "DNA damage and ageing: new-age ideas for an age-old problem," *Nat Cell Biol*, vol. 10, no. 11, pp.

## References from Chapter 1

---

- 1241–1247, 2008, doi: 10.1038/ncb1108-1241.
- [61] P. Alexander, "The role of DNA lesions in the processes leading to aging in mice.,," *Sym Soc Exp Biol*, vol. 21, pp. 29–50, 1967.
- [62] B. Sarg, E. Koutzamani, W. Helliger, I. Rundquist, and H. H. Lindner, "Postsynthetic Trimethylation of Histone H4 at Lysine 20 in Mammalian Tissues Is Associated with Aging\*," *J Biol Chem*, vol. 277, no. 42, pp. 39195–39201, 2002, doi: 10.1074/jbc.m205166200.
- [63] M. F. Fraga and M. Esteller, "Epigenetics and aging: the targets and the marks," *Trends Genet*, vol. 23, no. 8, pp. 413–418, 2007, doi: 10.1016/j.tig.2007.05.008.
- [64] I. Cheung et al., "Developmental regulation and individual differences of neuronal H3K4me3 epigenomes in the prefrontal cortex," *Proc National Acad Sci*, vol. 107, no. 19, pp. 8824–8829, 2010, doi: 10.1073/pnas.1001702107.
- [65] S. Han and A. Brunet, "Histone methylation makes its mark on longevity," *Trends Cell Biol*, vol. 22, no. 1, pp. 42–49, 2012, doi: 10.1016/j.tcb.2011.11.001.
- [66] D. Harman, "The Biologic Clock: The Mitochondria?," *J Am Geriatr Soc*, vol. 20, no. 4, pp. 145–147, 1972, doi: 10.1111/j.1532-5415.1972.tb00787.x.
- [67] B. N. Ames, M. K. Shigenaga, and T. M. Hagen, "Oxidants, antioxidants, and the degenerative diseases of aging," *Proc National Acad Sci*, vol. 90, no. 17, pp. 7915–7922, 1993, doi: 10.1073/pnas.90.17.7915.
- [68] E. S. Unlun and A. Koc, "Effects of Deleting Mitochondrial Antioxidant Genes on Life Span," *Ann Ny Acad Sci*, vol. 1100, no. 1, pp. 505–509, 2007, doi: 10.1196/annals.1395.055.
- [69] J. Wawryn, A. Krzeplko, A. Myszka, and T. Biliński, "Deficiency in superoxide dismutases shortens life span of yeast cells.," *Acta Biochim Pol*, vol. 46, no. 2, pp. 249–53, 1999.
- [70] V. D. Longo, L.-L. Liou, J. S. Valentine, and E. B. Gralla, "Mitochondrial Superoxide Decreases Yeast Survival in Stationary Phase," *Arch Biochem Biophys*, vol. 365, no. 1, pp. 131–142, 1999, doi: 10.1006/abbi.1999.1158.
- [71] V. D. Longo, G. S. Shadel, M. Kaeberlein, and B. Kennedy, "Replicative and Chronological Aging in *Saccharomyces cerevisiae*," *Cell Metab*, vol. 16, no. 1, pp. 18–31, 2012, doi: 10.1016/j.cmet.2012.06.002.
- [72] S. Melov et al., "Extension of Life-Span with Superoxide Dismutase/Catalase Mimetics," *Science*, vol. 289, no. 5484, pp. 1567–1569, 2000, doi: 10.1126/science.289.5484.1567.
- [73] J. M. V. Raamsdonk and S. Hekimi, "Deletion of the Mitochondrial Superoxide Dismutase sod-2 Extends Lifespan in *Caenorhabditis elegans*," *Plos Genet*, vol. 5, no. 2, p. e1000361, 2009, doi: 10.1371/journal.pgen.1000361.
- [74] T. L. Parkes, A. J. Elia, D. Dickinson, A. J. Hilliker, J. P. Phillips, and G. L. Boulian, "Extension of *Drosophila* lifespan by overexpression of human SOD1 in motorneurons," *Nat Genet*, vol. 19, no. 2, pp. 171–174, 1998, doi: 10.1038/534.
- [75] J. P. Phillips, S. D. Campbell, D. Michaud, M. Charbonneau, and A. J. Hilliker, "Null mutation of copper/zinc superoxide dismutase in *Drosophila* confers hypersensitivity to paraquat and reduced longevity," *Proc National Acad Sci*, vol. 86, no. 8, pp. 2761–2765, 1989, doi: 10.1073/pnas.86.8.2761.
- [76] J. D. Parker, K. M. Parker, B. H. Sohal, R. S. Sohal, and L. Keller, "Decreased expression of Cu–Zn superoxide dismutase 1 in ants with extreme lifespan," *P Natl Acad Sci Usa*, vol. 101, no. 10, pp. 3486–3489, 2004, doi: 10.1073/pnas.0400222101.
- [77] S. Elchuri et al., "CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life," *Oncogene*, vol. 24, no. 3, pp. 367–380, 2005, doi: 10.1038/sj.onc.1208207.
- [78] V. I. Pérez, H. V. Remmen, A. Bokov, C. J. Epstein, J. Vijg, and A. Richardson, "The overexpression of major antioxidant enzymes does not extend the lifespan of mice," *Aging Cell*, vol. 8, no. 1, pp. 73–75, 2009, doi: 10.1111/j.1474-9726.2008.00449.x.
- [79] J. Viña, C. Borras, K. M. Abdelaziz, R. Garcia-Valles, and M. C. Gomez-Cabrera, "The

## References from Chapter 1

---

- Free Radical Theory of Aging Revisited: The Cell Signaling Disruption Theory of Aging," *Antioxid Redox Sign*, vol. 19, no. 8, pp. 779–787, 2013, doi: 10.1089/ars.2012.5111.
- [80] Y. Goulev et al., "Nonlinear feedback drives homeostatic plasticity in H<sub>2</sub>O<sub>2</sub> stress response," *Elife*, vol. 6, p. e23971, 2017, doi: 10.7554/elife.23971.
- [81] E. R. Miller, R. Pastor-Barriuso, D. Dalal, R. A. Riemersma, L. J. Appel, and E. Guallar, "Meta-Analysis: High-Dosage Vitamin E Supplementation May Increase All-Cause Mortality," *Ann Intern Med*, vol. 142, no. 1, p. 37, 2005, doi: 10.7326/0003-4819-142-1-200501040-00110.
- [82] G. S. Omenn et al., "Effects of a Combination of Beta Carotene and Vitamin A on Lung Cancer and Cardiovascular Disease," *New Engl J Medicine*, vol. 334, no. 18, pp. 1150–1155, 1996, doi: 10.1056/nejm199605023341802.
- [83] T. A. Group, "The Effect of Vitamin E and Beta Carotene on the Incidence of Lung Cancer and Other Cancers in Male Smokers," *New Engl J Medicine*, vol. 330, no. 15, pp. 1029–1035, 1994, doi: 10.1056/nejm199404143301501.
- [84] J. H. J. Hoeijmakers, "DNA Damage, Aging, and Cancer," *New Engl J Medicine*, vol. 361, no. 15, pp. 1475–1485, 2009, doi: 10.1056/nejmra0804615.
- [85] Wikipedia, "*DNA damage (naturally occurring)*," DNA damage (naturally occurring), n.d. [https://en.wikipedia.org/wiki/DNA\\_damage\\_\(naturally\\_occuring\)#A\\_major\\_problem\\_for\\_life](https://en.wikipedia.org/wiki/DNA_damage_(naturally_occuring)#A_major_problem_for_life) (accessed Nov. 14, 2021).
- [86] A. A. Moskalev et al., "The role of DNA damage and repair in aging through the prism of Koch-like criteria," *Ageing Res Rev*, vol. 12, no. 2, pp. 661–684, 2013, doi: 10.1016/j.arr.2012.02.001.
- [87] L. Narayanan, J. A. Fritzell, S. M. Baker, R. M. Liskay, and P. M. Glazer, "Elevated levels of mutation in multiple tissues of mice deficient in the DNA mismatch repair gene Pms2," *Proc National Acad Sci*, vol. 94, no. 7, pp. 3122–3127, 1997, doi: 10.1073/pnas.94.7.3122.
- [88] A. Kaya, A. V. Lobanov, and V. N. Gladyshev, "Evidence that mutation accumulation does not cause aging in *Saccharomyces cerevisiae*," *Aging Cell*, vol. 14, no. 3, pp. 366–371, 2015, doi: 10.1111/acel.12290.
- [89] P. S. Robinson et al., "Increased somatic mutation burdens in normal human cells due to defective DNA polymerases," *Nat Genet*, pp. 1–9, 2021, doi: 10.1038/s41588-021-00930-y.
- [90] B. N. Ames and L. S. Gold, "Endogenous mutagens and the causes of aging and cancer," *Mutat Res Fundam Mol Mech Mutagen*, vol. 250, no. 1–2, pp. 3–16, 1991, doi: 10.1016/0027-5107(91)90157-j.
- [91] G. E. Holmes, C. Bernstein, and H. Bernstein, "Oxidative and other DNA damages as the basis of aging: a review," *Mutat Res Dnaging*, vol. 275, no. 3–6, pp. 305–315, 1992, doi: 10.1016/0921-8734(92)90034-m.
- [92] K. S. Rao and L. A. Loeb, "DNA damage and repair in brain: relationship to aging," *Mutat Res Dnaging*, vol. 275, no. 3–6, pp. 317–329, 1992, doi: 10.1016/0921-8734(92)90035-n.
- [93] S. R. R. Kolora et al., "Origins and evolution of extreme life span in Pacific Ocean rockfishes," *Science*, vol. 374, no. 6569, pp. 842–847, 2021, doi: 10.1126/science.abg5332.
- [94] S. Pal and J. K. Tyler, "Epigenetics and aging," *Sci Adv*, vol. 2, no. 7, p. e1600584, 2016, doi: 10.1126/sciadv.1600584.
- [95] S. Horvath and K. Raj, "DNA methylation-based biomarkers and the epigenetic clock theory of ageing," *Nat Rev Genet*, vol. 19, no. 6, pp. 371–384, 2018, doi: 10.1038/s41576-018-0004-3.
- [96] J.-H. Yang et al., "Loss of Epigenetic Information as a Cause of Mammalian Aging," *Ssrn Electron J*, 2021, doi: 10.2139/ssrn.3951490.
- [97] M. J. Jones, S. J. Goodman, and M. S. Kobor, "DNA methylation and healthy human aging," *Aging Cell*, vol. 14, no. 6, pp. 924–932, 2015, doi: 10.1111/acel.12349.

## References from Chapter 1

---

- [98] R. E. Marioni et al., "DNA methylation age of blood predicts all-cause mortality in later life," *Genome Biol.*, vol. 16, no. 1, p. 25, 2015, doi: 10.1186/s13059-015-0584-6.
- [99] J. Feser et al., "Elevated Histone Expression Promotes Life Span Extension," *Mol Cell*, vol. 39, no. 5, pp. 724–735, 2010, doi: 10.1016/j.molcel.2010.08.015.
- [100] E. L. Greer et al., "Members of the Histone H3 Lysine 4 Trimethylation Complex Regulate Lifespan in a Germline-dependent Manner in *C. elegans*," *Nature*, vol. 466, no. 7304, pp. 383–387, 2010, doi: 10.1038/nature09195.
- [101] A. P. Siebold, R. Banerjee, F. Tie, D. L. Kiss, J. Moskowitz, and P. J. Harte, "Polycomb Repressive Complex 2 and Trithorax modulate *Drosophila* longevity and stress resistance," *Proc National Acad Sci*, vol. 107, no. 1, pp. 169–174, 2010, doi: 10.1073/pnas.0907739107.
- [102] C. Jin et al., "Histone Demethylase UTX-1 Regulates *C. elegans* Life Span by Targeting the Insulin/IGF-1 Signaling Pathway," *Cell Metab*, vol. 14, no. 2, pp. 161–172, 2011, doi: 10.1016/j.cmet.2011.07.001.
- [103] C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, "The Hallmarks of Aging," *Cell*, vol. 153, no. 6, pp. 1194–1217, 2013, doi: 10.1016/j.cell.2013.05.039.
- [104] H. Rubin, "The disparity between human cell senescence in vitro and lifelong replication in vivo," *Nat Biotechnol*, vol. 20, no. 7, pp. 675–681, 2002, doi: 10.1038/nbt0702-675.
- [105] M. H. Goyns and W. L. Lavery, "Telomerase and mammalian ageing: a critical appraisal," *Mech Ageing Dev*, vol. 114, no. 2, pp. 69–77, 2000, doi: 10.1016/s0047-6374(00)00095-6.
- [106] J. Campisi, "The biology of replicative senescence," *Eur J Cancer*, vol. 33, no. 5, pp. 703–709, 1997, doi: 10.1016/s0959-8049(96)00058-5.
- [107] M. A. Muñoz-Lorente, A. C. Cano-Martin, and M. A. Blasco, "Mice with hyper-long telomeres show less metabolic aging and longer lifespans," *Nat Commun*, vol. 10, no. 1, p. 4723, 2019, doi: 10.1038/s41467-019-12664-x.
- [108] K. L. Rudolph et al., "Longevity, Stress Response, and Cancer in Aging Telomerase-Deficient Mice," *Cell*, vol. 96, no. 5, pp. 701–712, 1999, doi: 10.1016/s0092-8674(00)80580-2.
- [109] P. J. Hornsby, "Telomerase and the aging process," *Exp Gerontol*, vol. 42, no. 7, pp. 575–581, 2007, doi: 10.1016/j.exger.2007.03.007.
- [110] H. Koga, S. Kaushik, and A. M. Cuervo, "Protein homeostasis and aging: The importance of exquisite quality control," *Ageing Res Rev*, vol. 10, no. 2, pp. 205–215, 2011, doi: 10.1016/j.arr.2010.02.001.
- [111] I. Saez and D. Vilchez, "The Mechanistic Links Between Proteasome Activity, Aging and Age-related Diseases," *Curr Genomics*, vol. 15, no. 1, pp. 38–51, 2014, doi: 10.2174/138920291501140306113344.
- [112] G. Morrow, M. Samson, S. Michaud, and R. M. Tanguay, "Overexpression of the small mitochondrial Hsp22 extends *Drosophila* life span and increases resistance to oxidative stress," *Faseb J*, vol. 18, no. 3, pp. 598–599, 2004, doi: 10.1096/fj.03-0860fje.
- [113] G. A. Walker and G. J. Lithgow, "Lifespan extension in *C. elegans* by a molecular chaperone dependent upon insulin-like signals," *Aging Cell*, vol. 2, no. 2, pp. 131–139, 2003, doi: 10.1046/j.1474-9728.2003.00045.x.
- [114] W.-C. Chiang, T.-T. Ching, H. C. Lee, C. Mousigian, and A.-L. Hsu, "HSF-1 Regulators DDL-1/2 Link Insulin-like Signaling to Heat-Shock Responses and Modulation of Longevity," *Cell*, vol. 148, no. 1–2, pp. 322–334, 2012, doi: 10.1016/j.cell.2011.12.019.
- [115] A.-L. Hsu, C. T. Murphy, and C. Kenyon, "Regulation of Aging and Age-Related Disease by DAF-16 and Heat-Shock Factor," *Science*, vol. 300, no. 5622, pp. 1142–1145, 2003, doi: 10.1126/science.1083701.
- [116] J.-O. Pyo et al., "Overexpression of Atg5 in mice activates autophagy and extends lifespan," *Nat Commun*, vol. 4, no. 1, p. 2300, 2013, doi: 10.1038/ncomms3300.

## References from Chapter 1

---

- [117] B. Friguet, A. BULTEAU, N. CHONDROGIANNI, M. CONCONI, and I. Petropoulos, "Protein Degradation by the Proteasome and Its Implications in Aging," *Ann Ny Acad Sci*, vol. 908, no. 1, pp. 143–154, 2000, doi: 10.1111/j.1749-6632.2000.tb06643.x.
- [118] A. J. L. Macario and E. C. de Macario, "Sick Chaperones, Cellular Stress, and Disease," *New Engl J Medicine*, vol. 353, no. 14, pp. 1489–1501, 2005, doi: 10.1056/nejmra050111.
- [119] D. C. Rubinsztein, G. Mariño, and G. Kroemer, "Autophagy and Aging," *Cell*, vol. 146, no. 5, pp. 682–695, 2011, doi: 10.1016/j.cell.2011.07.030.
- [120] V. Gorbunova, A. Seluanov, Z. Mao, and C. Hine, "Changes in DNA repair during aging," *Nucleic Acids Res*, vol. 35, no. 22, pp. 7466–7474, 2007, doi: 10.1093/nar/gkm756.
- [121] U. Herbig, M. Ferreira, L. Condel, D. Carey, and J. M. Sedivy, "Cellular Senescence in Aging Primates," *Science*, vol. 311, no. 5765, pp. 1257–1257, 2006, doi: 10.1126/science.1122446.
- [122] M. L. Idda et al., "Survey of senescent cell markers with age in human tissues," *Aging*, vol. 12, no. 5, pp. 4052–4066, 2020, doi: 10.18632/aging.102903.
- [123] J.-P. Coppé, P.-Y. Desprez, A. Krtolica, and J. Campisi, "The Senescence-Associated Secretory Phenotype: The Dark Side of Tumor Suppression," *Pathology Mech Dis*, vol. 5, no. 1, pp. 99–118, 2010, doi: 10.1146/annurev-pathol-121808-102144.
- [124] D. McHugh and J. Gil, "Senescence and aging: Causes, consequences, and therapeutic avenues," *J Cell Biol*, vol. 217, no. 1, pp. 65–77, 2018, doi: 10.1083/jcb.201708092.
- [125] M. Lemoine, "The Evolution of the Hallmarks of Aging," *Frontiers Genetics*, vol. 12, p. 693071, 2021, doi: 10.3389/fgene.2021.693071.
- [126] A. Dillin, D. E. Gottschling, and T. Nyström, "The good and the bad of being connected: the integrons of aging," *Curr Opin Cell Biol*, vol. 26, pp. 107–112, 2014, doi: 10.1016/j.ceb.2013.12.003.
- [127] A. Kowald and T. B. L. Kirkwood, "A network theory of ageing: the interactions of defective mitochondria, aberrant proteins, free radicals and scavengers in the ageing process," *Mutat Res Aging*, vol. 316, no. 5–6, pp. 209–236, 1996, doi: 10.1016/s0921-8734(96)90005-3.
- [128] T. B. L. Kirkwood and A. Kowald, "Network theory of aging," *Exp Gerontol*, vol. 32, no. 4–5, pp. 395–399, 1997, doi: 10.1016/s0531-5565(96)00171-4.
- [129] T. B. L. Kirkwood, "Systems biology of ageing and longevity," *Philosophical Transactions Royal Soc B Biological Sci*, vol. 366, no. 1561, pp. 64–70, 2011, doi: 10.1098/rstb.2010.0275.
- [130] R. Song, E. A. Sarnoski, and M. Acar, "The Systems Biology of Single-Cell Aging," *Science*, vol. 7, pp. 154–169, 2018, doi: 10.1101/10.1016/j.isci.2018.08.023.
- [131] D. E. Martinez, "Mortality Patterns Suggest Lack of Senescence in Hydra," *Exp Gerontol*, vol. 33, no. 3, pp. 217–225, 1998, doi: 10.1016/s0531-5565(97)00113-7.
- [132] C. E. Finch, *Longevity, Senescence, and the Genome*. 1994.
- [133] J. W. Vaupel, A. Baudisch, M. Dölling, D. A. Roach, and J. Gampe, "The case for negative senescence," *Theor Popul Biol*, vol. 65, no. 4, pp. 339–351, 2004, doi: 10.1016/j.tpb.2003.12.003.
- [134] J. G. Ruby, M. Smith, and R. Buffenstein, "Naked Mole-Rat mortality rates defy gompertzian laws by not increasing with age," *Elife*, vol. 7, p. e31157, 2018, doi: 10.7554/elife.31157.
- [135] J. Kim, B.-K. Koo, and J. A. Knoblich, "Human organoids: model systems for human biology and medicine," *Nat Rev Mol Cell Bio*, vol. 21, no. 10, pp. 571–584, 2020, doi: 10.1038/s41580-020-0259-3.
- [136] L. Mesch et al., "Aged human iPSC-RPE organoid cultures display hallmarks of drusen formation," *bioRxiv*, 2021, doi: 10.1101/2021.10.12.463899.
- [137] J. L. Hu, M. E. Todhunter, M. A. LaBarge, and Z. J. Gartner, "Opportunities for

## References from Chapter 1

---

- organoids as new models of aging," *J Cell Biol*, vol. 217, no. 1, pp. 39–50, 2018, doi: 10.1083/jcb.201709054.
- [138] M. Tatar, A. Kopelman, D. Epstein, M.-P. Tu, C.-M. Yin, and R. S. Garofalo, "A Mutant Drosophila Insulin Receptor Homolog That Extends Life-Span and Impairs Neuroendocrine Function," *Science*, vol. 292, no. 5514, pp. 107–110, 2001, doi: 10.1126/science.1057987.
- [139] Y.-J. Lin, L. Seroude, and S. Benzer, "Extended Life-Span and Stress Resistance in the Drosophila Mutant *methuselah*," *Science*, vol. 282, no. 5390, pp. 943–946, 1998, doi: 10.1126/science.282.5390.943.
- [140] D. J. Clancy et al., "Extension of Life-Span by Loss of CHICO, a Drosophila Insulin Receptor Substrate Protein," *Science*, vol. 292, no. 5514, pp. 104–106, 2001, doi: 10.1126/science.1057991.
- [141] B. Rogina, S. L. Helfand, and S. Frankel, "Longevity Regulation by Drosophila Rpd3 Deacetylase and Caloric Restriction," *Science*, vol. 298, no. 5599, pp. 1745–1745, 2002, doi: 10.1126/science.1078986.
- [142] T. Maruzs, Z. Simon-Vecsei, V. Kiss, T. Csizmadia, and G. Juhász, "On the Fly: Recent Progress on Autophagy and Aging in Drosophila," *Frontiers Cell Dev Biology*, vol. 7, p. 140, 2019, doi: 10.3389/fcell.2019.00140.
- [143] M. D. W. Piper and L. Partridge, "Drosophila as a model for ageing," *Biochimica Et Biophysica Acta Bba - Mol Basis Dis*, vol. 1864, no. 9, pp. 2707–2717, 2018, doi: 10.1016/j.bbadi.2017.09.016.
- [144] A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello, "Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*," *Nature*, vol. 391, no. 6669, pp. 806–811, 1998, doi: 10.1038/35888.
- [145] B. Hamilton et al., "A systematic RNAi screen for longevity genes in *C. elegans*," *Gene Dev*, vol. 19, no. 13, pp. 1544–1555, 2005, doi: 10.1101/gad.1308205.
- [146] L. R. Lapierre and M. Hansen, "Lessons from *C. elegans*: signaling pathways for longevity," *Trends Endocrinol Metabolism*, vol. 23, no. 12, pp. 637–644, 2012, doi: 10.1016/j.tem.2012.07.007.
- [147] K. Flurkey, J. Papaconstantinou, R. A. Miller, and D. E. Harrison, "Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production," *Proc National Acad Sci*, vol. 98, no. 12, pp. 6736–6741, 2001, doi: 10.1073/pnas.111158898.
- [148] R. K. Mortimer and J. R. Johnston, "Life Span of Individual Yeast Cells," *Nature*, vol. 183, no. 4677, pp. 1751–1752, 1959, doi: 10.1038/1831751a0.
- [149] A. A. Barton, "Some Aspects of Cell Division in *Saccharomyces cerevisiae*," *Microbiology+*, vol. 4, no. 1, pp. 84–86, 1950, doi: 10.1099/00221287-4-1-84.
- [150] S. C. Johnson, P. S. Rabinovitch, and M. Kaeberlein, "mTOR is a key modulator of ageing and age-related disease," *Nature*, vol. 493, no. 7432, pp. 338–345, 2013, doi: 10.1038/nature11861.
- [151] C. M. McCay, M. F. Crowell, and L. A. Maynard, "The Effect of Retarded Growth Upon the Length of Life Span and Upon the Ultimate Body Size," *J Nutrition*, vol. 10, no. 1, pp. 63–79, 1935, doi: 10.1093/jn/10.1.63.
- [152] M. J. P. Simons, W. Koch, and S. Verhulst, "Dietary restriction of rodents decreases aging rate without affecting initial mortality rate – a meta-analysis," *Aging Cell*, vol. 12, no. 3, pp. 410–414, 2013, doi: 10.1111/acel.12061.
- [153] F. Pifferi et al., "Caloric restriction increases lifespan but affects brain integrity in grey mouse lemur primates," *Commun Biology*, vol. 1, no. 1, p. 30, 2018, doi: 10.1038/s42003-018-0024-8.
- [154] R. Weindruch, R. L. Walford, S. Fligiel, and D. Guthrie, "The Retardation of Aging in Mice by Dietary Restriction: Longevity, Cancer, Immunity and Lifetime Energy Intake," *J Nutrition*, vol. 116, no. 4, pp. 641–654, 1986, doi: 10.1093/jn/116.4.641.
- [155] H. H. Pak et al., "Fasting drives the metabolic, molecular and geroprotective effects of

## References from Chapter 1

---

- a calorie-restricted diet in mice," *Nat Metabolism*, pp. 1–15, 2021, doi: 10.1038/s42255-021-00466-9.
- [156] E. L. Greer and A. Brunet, "Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*," *Aging Cell*, vol. 8, no. 2, pp. 113–127, 2009, doi: 10.1111/j.1474-9726.2009.00459.x.
- [157] S.-J. Lin et al., "Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration," *Nature*, vol. 418, no. 6895, pp. 344–348, 2002, doi: 10.1038/nature00829.
- [158] J. C. Jiang, E. Jaruga, M. V. Repnevskaya, and S. M. Jazwinski, "An intervention resembling caloric restriction prolongs life span and retards aging in yeast," *Faseb J*, vol. 14, no. 14, pp. 2135–2137, 2000, doi: 10.1096/fj.00-0242fje.
- [159] E. C. Spivey, S. K. Jones, J. R. Rybarski, F. A. Saifuddin, and I. J. Finkelstein, "An aging-independent replicative lifespan in a symmetrically dividing eukaryote," *Elife*, vol. 6, p. e20340, 2017, doi: 10.7554/elife.20340.
- [160] J. A. Mattison et al., "Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study," *Nature*, vol. 489, no. 7415, pp. 318–321, 2012, doi: 10.1038/nature11432.
- [161] R. J. Colman et al., "Caloric Restriction Delays Disease Onset and Mortality in Rhesus Monkeys," *Science*, vol. 325, no. 5937, pp. 201–204, 2009, doi: 10.1126/science.1173635.
- [162] J. A. Mattison et al., "Caloric restriction improves health and survival of rhesus monkeys," *Nat Commun*, vol. 8, no. 1, p. 14063, 2017, doi: 10.1038/ncomms14063.
- [163] R. S. Sohal and M. J. Forster, "Caloric restriction and the aging process: a critique," *Free Radical Bio Med*, vol. 73, pp. 366–382, 2014, doi: 10.1016/j.freeradbiomed.2014.05.015.
- [164] S. Caristia et al., "Is Caloric Restriction Associated with Better Healthy Aging Outcomes? A Systematic Review and Meta-Analysis of Randomized Controlled Trials," *Nutrients*, vol. 12, no. 8, p. 2290, 2020, doi: 10.3390/nu12082290.
- [165] M. Ulgherait et al., "Circadian autophagy drives iTRF-mediated longevity," *Nature*, vol. 598, no. 7880, pp. 353–358, 2021, doi: 10.1038/s41586-021-03934-0.
- [166] B. K. Kennedy, K. K. Steffen, and M. Kaeberlein, "Ruminations on dietary restriction and aging," *Cell Mol Life Sci*, vol. 64, no. 11, pp. 1323–1328, 2007, doi: 10.1007/s00018-007-6470-y.
- [167] E. D. Smith et al., "Quantitative evidence for conserved longevity pathways between divergent eukaryotic species," *Genome Res*, vol. 18, no. 4, pp. 564–570, 2008, doi: 10.1101/gr.074724.107.
- [168] E. J. Stewart, R. Madden, G. Paul, and F. Taddei, "Aging and Death in an Organism That Reproduces by Morphologically Symmetric Division," *Plos Biol*, vol. 3, no. 2, p. e45, 2005, doi: 10.1371/journal.pbio.0030045.
- [169] C. U. Rang, A. Y. Peng, and L. Chao, "Temporal Dynamics of Bacterial Aging and Rejuvenation," *Curr Biol*, vol. 21, no. 21, pp. 1813–1816, 2011, doi: 10.1016/j.cub.2011.09.018.
- [170] P. Wang et al., "Robust Growth of *Escherichia coli*," *Curr Biol*, vol. 20, no. 12, pp. 1099–1103, 2010, doi: 10.1016/j.cub.2010.04.045.
- [171] L. G. Burman, J. Raichler, and J. T. Park, "Evidence for diffuse growth of the cylindrical portion of the *Escherichia coli* murein sacculus," *J Bacteriol*, vol. 155, no. 3, pp. 983–988, 1983, doi: 10.1128/jb.155.3.983-988.1983.
- [172] M. A. de Pedro, J. C. Quintela, J. V. Höltje, and H. Schwarz, "Murein segregation in *Escherichia coli*," *J Bacteriol*, vol. 179, no. 9, pp. 2823–2834, 1997, doi: 10.1128/jb.179.9.2823-2834.1997.
- [173] D.-J. Scheffers and M. G. Pinho, "Bacterial Cell Wall Synthesis: New Insights from Localization Studies," *Microbiol Mol Biol R*, vol. 69, no. 4, pp. 585–607, 2005, doi: 10.1128/mmbr.69.4.585-607.2005.

## References from Chapter 1

---

- [174] A. B. Lindner, R. Madden, A. Demarez, E. J. Stewart, and F. Taddei, "Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation," *Proc National Acad Sci*, vol. 105, no. 8, pp. 3076–3081, 2008, doi: 10.1073/pnas.0708931105.
- [175] J. Winkler et al., "Quantitative and spatio-temporal features of protein aggregation in *Escherichia coli* and consequences on protein quality control and cellular ageing," *Embo J*, vol. 29, no. 5, pp. 910–923, 2010, doi: 10.1038/emboj.2009.412.
- [176] M. G. Lee and P. Nurse, "Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2," *Nature*, vol. 327, no. 6117, pp. 31–35, 1987, doi: 10.1038/327031a0.
- [177] P. Fantes and P. Nurse, "Control of cell size at division in fission yeast by a growth-modulated size control over nuclear division," *Exp Cell Res*, vol. 107, no. 2, pp. 377–386, 1977, doi: 10.1016/0014-4827(77)90359-7.
- [178] P. A. Fantes and P. Nurse, "Control of the timing of cell division in fission yeast Cell size mutants reveal a second control pathway," *Exp Cell Res*, vol. 115, no. 2, pp. 317–329, 1978, doi: 10.1016/0014-4827(78)90286-0.
- [179] J. M. Mitchison and P. Nurse, "Growth in cell length in the fission yeast *Schizosaccharomyces pombe*," *J Cell Sci*, vol. 75, pp. 357–76, 1985.
- [180] P. Nurse, P. Thuriaux, and K. Nasmyth, "Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*," *Mol Gen Genetics Mgg*, vol. 146, no. 2, pp. 167–178, 1976, doi: 10.1007/bf00268085.
- [181] D. Coudreuse and P. Nurse, "Driving the cell cycle with a minimal CDK control network," *Nature*, vol. 468, no. 7327, pp. 1074–1079, 2010, doi: 10.1038/nature09543.
- [182] D. García-Ruano et al., "Fluorescence exclusion: a rapid, accurate and powerful method for measuring yeast cell volume," *Biorxiv*, p. 2021.10.07.463508, 2021, doi: 10.1101/2021.10.07.463508.
- [183] D. O. Morgan, *The Cell Cycle: Principles of Control*. 2007.
- [184] M. Coelho et al., "Fission Yeast Does Not Age under Favorable Conditions, but Does So after Stress," *Curr Biol*, vol. 23, no. 19, pp. 1844–1852, 2013, doi: 10.1016/j.cub.2013.07.084.
- [185] M. G. Barker and R. M. Walmsley, "Replicative ageing in the fission yeast *Schizosaccharomyces pombe*," *Yeast*, vol. 15, no. 14, pp. 1511–1518, 1999, doi: 10.1002/(sici)1097-0061(199910)15:14<1511::aid-yea482>3.0.co;2-y.
- [186] H. Nakaoka and Y. Wakamoto, "Aging, mortality, and the fast growth trade-off of *Schizosaccharomyces pombe*," *Plos Biol*, vol. 15, no. 6, p. e2001109, 2017, doi: 10.1371/journal.pbio.2001109.
- [187] J. M. Mitchison, "The growth of single cells I. *Schizosaccharomyces pombe*," *Exp Cell Res*, vol. 13, no. 2, pp. 244–262, 1957, doi: 10.1016/0014-4827(57)90005-8.
- [188] B. F. Johnson, "Autoradiographic analysis of regional cell wall growth of yeasts *Schizosaccharomyces pombe*," *Exp Cell Res*, vol. 39, no. 2–3, pp. 613–624, 1965, doi: 10.1016/0014-4827(65)90064-9.
- [189] J. W. May, "Sites of cell-wall extension demonstrated by the use of fluorescent antibody," *Exp Cell Res*, vol. 27, no. 1, pp. 170–172, 1962, doi: 10.1016/0014-4827(62)90060-5.
- [190] S. Baumgärtner and I. M. Tolić-Nørrelykke, "Growth Pattern of Single Fission Yeast Cells Is Bilinear and Depends on Temperature and DNA Synthesis," *Biophys J*, vol. 96, no. 10, pp. 4336–4347, 2009, doi: 10.1016/j.bpj.2009.02.051.
- [191] M. Sohrmann, S. Schmidt, I. Hagan, and V. Simanis, "Asymmetric segregation on spindle poles of the *Schizosaccharomyces pombe* septum-inducing protein kinase Cdc7p," *Gene Dev*, vol. 12, no. 1, pp. 84–94, 1998, doi: 10.1101/gad.12.1.84.
- [192] M. Ackermann, S. C. Stearns, and U. Jenal, "Senescence in a Bacterium with Asymmetric Division," *Science*, vol. 300, no. 5627, pp. 1920–1920, 2003, doi: 10.1126/science.1083532.

## References from Chapter 1

---

- [193] M. D. Murtey and P. Ramasamy, "Modern Electron Microscopy in Physical and Life Sciences," 2016, doi: 10.5772/61720.
- [194] A. D. Lippuner, T. Julou, and Y. Barral, "Budding yeast as a model organism to study the effects of age," *Fems Microbiol Rev*, vol. 38, no. 2, pp. 300–325, 2014, doi: 10.1111/1574-6976.12060.
- [195] J. Saarikangas and Y. Barral, "Protein aggregates are associated with replicative aging without compromising protein quality control," *Elife*, vol. 4, p. e06197, 2015, doi: 10.7554/elife.06197.
- [196] H. Gershon and D. Gershon, "The budding yeast, *Saccharomyces cerevisiae*, as a model for aging research: a critical review," *Mech Ageing Dev*, vol. 120, no. 1–3, pp. 1–22, 2000, doi: 10.1016/s0047-6374(00)00182-2.
- [197] C. He, C. Zhou, and B. K. Kennedy, "The yeast replicative aging model," *Biochimica Et Biophysica Acta Bba - Mol Basis Dis*, vol. 1864, no. 9, pp. 2690–2696, 2018, doi: 10.1016/j.bbadi.2018.02.023.
- [198] G. E. Janssens and L. M. Veenhoff, "Evidence for the hallmarks of human aging in replicatively aging yeast," *Microb Cell*, vol. 3, no. 7, pp. 263–274, 2016, doi: 10.15698/mic2016.07.510.
- [199] W.-K. Huh et al., "Global analysis of protein localization in budding yeast," *Nature*, vol. 425, no. 6959, pp. 686–691, 2003, doi: 10.1038/nature02026.
- [200] J. Peter et al., "Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates," *Nature*, vol. 556, no. 7701, pp. 339–344, 2018, doi: 10.1038/s41586-018-0030-5.
- [201] E. X. Kwan, E. Foss, L. Kruglyak, and A. Bedalov, "Natural Polymorphism in BUL2 Links Cellular Amino Acid Availability with Chronological Aging and Telomere Maintenance in Yeast," *Plos Genet*, vol. 7, no. 8, p. e1002250, 2011, doi: 10.1371/journal.pgen.1002250.
- [202] E. X. Kwan et al., "A Natural Polymorphism in rDNA Replication Origins Links Origin Activation with Calorie Restriction and Lifespan," *Plos Genet*, vol. 9, no. 3, p. e1003329, 2013, doi: 10.1371/journal.pgen.1003329.
- [203] A. Kaya, S. Ma, B. Wasko, M. Lee, M. Kaeberlein, and V. N. Gladyshev, "Defining molecular basis for longevity traits in natural yeast isolates," *Npj Aging Mech Dis*, vol. 1, no. 1, p. 15001, 2015, doi: 10.1038/npjamd.2015.1.
- [204] A. Kaya et al., "Evolution of natural lifespan variation and molecular strategies of extended lifespan," *Elife*, vol. 10, p. e64860, 2021, doi: 10.7554/elife.64860.
- [205] P. Laun, M. Rinnerthaler, E. Bogengruber, G. Heeren, and M. Breitenbach, "Yeast as a model for chronological and reproductive aging – A comparison," *Exp Gerontol*, vol. 41, no. 12, pp. 1208–1212, 2006, doi: 10.1016/j.exger.2006.11.001.
- [206] M. S. Singer and D. E. Gottschling, "TLC1: Template RNA Component of *Saccharomyces cerevisiae* Telomerase," *Science*, vol. 266, no. 5184, pp. 404–409, 1994, doi: 10.1126/science.7545955.
- [207] V. Lundblad and J. W. Szostak, "A mutant with a defect in telomere elongation leads to senescence in yeast," *Cell*, vol. 57, no. 4, pp. 633–643, 1989, doi: 10.1016/0092-8674(89)90132-3.
- [208] H. Martin, M. Doumic, M. T. Teixeira, and Z. Xu, "Telomere shortening causes distinct cell division regimes during replicative senescence in *Saccharomyces cerevisiae*," *Biorxiv*, p. 2021.06.16.448683, 2021, doi: 10.1101/2021.06.16.448683.
- [209] M. Kaeberlein and B. K. Kennedy, "Large-scale identification in yeast of conserved ageing genes," *Mech Ageing Dev*, vol. 126, no. 1, pp. 17–21, 2005, doi: 10.1016/j.mad.2004.09.013.
- [210] S. W. Stumpferl et al., "Natural genetic variation in yeast longevity," *Genome Res*, vol. 22, no. 10, pp. 1963–1973, 2012, doi: 10.1101/gr.136549.111.
- [211] N. K. Egilmez and S. M. Jazwinski, "Evidence for the involvement of a cytoplasmic factor in the aging of the yeast *Saccharomyces cerevisiae*," *J Bacteriol*, vol. 171, no. 1, pp. 37–42, 1989, doi: 10.1128/jb.171.1.37-42.1989.

## References from Chapter 1

---

- [212] B. K. Kennedy, N. R. Austriaco, and L. Guarente, "Daughter cells of *Saccharomyces cerevisiae* from old mothers display a reduced life span.," *J Cell Biology*, vol. 127, no. 6, pp. 1985–1993, 1994, doi: 10.1083/jcb.127.6.1985.
- [213] K. A. Henderson and D. E. Gottschling, "A mother's sacrifice: what is she keeping for herself?," *Curr Opin Cell Biol*, vol. 20, no. 6, pp. 723–728, 2008, doi: 10.1016/j.ceb.2008.09.004.
- [214] T. Nyström and B. Liu, "The mystery of aging and rejuvenation—a budding topic," *Curr Opin Microbiol*, vol. 18, pp. 61–67, 2014, doi: 10.1016/j.mib.2014.02.003.
- [215] D. Sinclair, K. Mills, and L. Guarente, "Aging in *Saccharomyces Cerevisiae*," *Annu Rev Microbiol*, vol. 52, no. 1, pp. 533–560, 1998, doi: 10.1146/annurev.micro.52.1.533.
- [216] B. M. Wasko and M. Kaeberlein, "Yeast replicative aging: a paradigm for defining conserved longevity interventions," *Fems Yeast Res*, vol. 14, no. 1, pp. 148–159, 2014, doi: 10.1111/1567-1364.12104.
- [217] C. D. Powell, D. E. Quain, and K. A. Smart, "Chitin scar breaks in aged *Saccharomyces cerevisiae*," *Microbiology+*, vol. 149, no. 11, pp. 3129–3137, 2003, doi: 10.1099/mic.0.25940-0.
- [218] J. W. Bartholomew and T. Mittwer, "Demonstration of yeast bud scars with the electron microscope.," *J Bacteriol*, vol. 65, no. 3, pp. 272–5, 1953.
- [219] J. R. Johnston, "Reproductive capacity and mode of death of yeast cells," *Antonie Van Leeuwenhoek*, vol. 32, no. 1, pp. 94–98, 1966, doi: 10.1007/bf02097448.
- [220] S. M. Jazwinski, S. Kim, C.-Y. Lai, and A. Benguria, "Epigenetic stratification: the role of individual change in the biological aging process," *Exp Gerontol*, vol. 33, no. 6, pp. 571–580, 1998, doi: 10.1016/s0531-5565(98)00029-1.
- [221] S. S. Lee, I. A. Vizcarra, D. H. E. W. Huberts, L. P. Lee, and M. Heinemann, "Whole lifespan microscopic observation of budding yeast aging through a microfluidic dissection platform," *Proc National Acad Sci*, vol. 109, no. 13, pp. 4916–4920, 2012, doi: 10.1073/pnas.1113505109.
- [222] Y. Zhang et al., "Single Cell Analysis of Yeast Replicative Aging Using a New Generation of Microfluidic Device," *Plos One*, vol. 7, no. 11, p. e48275, 2012, doi: 10.1371/journal.pone.0048275.
- [223] M. Jin et al., "Divergent Aging of Isogenic Yeast Cells Revealed through Single-Cell Phenotypic Dynamics," *Cell Syst*, vol. 8, no. 3, pp. 242–253.e3, 2019, doi: 10.1016/j.cels.2019.02.002.
- [224] N. K. Egilmez, J. B. Chen, and S. M. Jazwinski, "Preparation and Partial Characterization of Old Yeast Cells," *J Gerontology*, vol. 45, no. 1, pp. B9–B17, 1990, doi: 10.1093/geronj/45.1.b9.
- [225] J. Yang et al., "Cell size and growth rate are major determinants of replicative lifespan," *Cell Cycle*, vol. 10, no. 1, pp. 144–155, 2011, doi: 10.4161/cc.10.1.14455.
- [226] S. Morlot, J. Song, I. Léger-Silvestre, A. Matifas, O. Gadal, and G. Charvin, "Excessive rDNA Transcription Drives the Disruption in Nuclear Homeostasis during Entry into Senescence in Budding Yeast," *Cell Reports*, vol. 28, no. 2, pp. 408–422.e4, 2019, doi: 10.1016/j.celrep.2019.06.032.
- [227] R. Zadrag, G. Bartosz, and T. Bilinski, "Replicative aging of the yeast does not require DNA replication," *Biochem Biophys Res Co*, vol. 333, no. 1, pp. 138–141, 2005, doi: 10.1016/j.bbrc.2005.05.081.
- [228] R. Zadrag, M. Kwolek-Mirek, G. Bartosz, and T. Bilinski, "Relationship between the replicative age and cell volume in *Saccharomyces cerevisiae*," *Acta Biochim Pol*, vol. 53, no. 4, pp. 747–51, 2006.
- [229] G. E. Neurohr et al., "Excessive Cell Growth Causes Cytoplasm Dilution And Contributes to Senescence," *Cell*, vol. 176, no. 5, pp. 1083–1097.e18, 2019, doi: 10.1016/j.cell.2019.01.018.
- [230] J. Wright, H. Dungrawala, R. K. Bright, and B. L. Schneider, "A growing role for

## References from Chapter 1

---

- hypertrophy in senescence," *Fems Yeast Res*, vol. 13, no. 1, pp. 2–6, 2013, doi: 10.1111/1567-1364.12015.
- [231] M. C. Jo, W. Liu, L. Gu, W. Dang, and L. Qin, "High-throughput analysis of yeast replicative aging using a microfluidic system," *Proc National Acad Sci*, vol. 112, no. 30, pp. 9364–9369, 2015, doi: 10.1073/pnas.1510328112.
- [232] T. Biliński, R. Zadraż-Tęcza, and G. Bartosz, "Hypertrophy hypothesis as an alternative explanation of the phenomenon of replicative aging of yeast," *Fems Yeast Res*, vol. 12, no. 1, pp. 97–101, 2012, doi: 10.1111/j.1567-1364.2011.00759.x.
- [233] A. R. D. Ganley, M. Breitenbach, B. K. Kennedy, and T. Kobayashi, "Yeast hypertrophy: cause or consequence of aging? Reply to Bilinski et al," *Fems Yeast Res*, vol. 12, no. 3, pp. 267–268, 2012, doi: 10.1111/j.1567-1364.2012.00796.x.
- [234] G. E. Janssens and L. M. Veenhoff, "The Natural Variation in Lifespans of Single Yeast Cells Is Related to Variation in Cell Size, Ribosomal Protein, and Division Time," *Plos One*, vol. 11, no. 12, p. e0167394, 2016, doi: 10.1371/journal.pone.0167394.
- [235] J. T. Smith, J. W. White, H. Dungrawala, H. Hua, and B. L. Schneider, "Yeast lifespan variation correlates with cell growth and SIR2 expression," *Plos One*, vol. 13, no. 7, p. e0200275, 2018, doi: 10.1371/journal.pone.0200275.
- [236] M. A. McCormick et al., "A Comprehensive Analysis of Replicative Lifespan in 4,698 Single-Gene Deletion Strains Uncovers Conserved Mechanisms of Aging," *Cell Metab*, vol. 22, no. 5, pp. 895–906, 2015, doi: 10.1016/j.cmet.2015.09.008.
- [237] J. R. Delaney et al., "Sir2 deletion prevents lifespan extension in 32 long-lived mutants," *Aging Cell*, vol. 10, no. 6, pp. 1089–1091, 2011, doi: 10.1111/j.1474-9726.2011.00742.x.
- [238] S. N. Mouton et al., "A physicochemical perspective of aging from single-cell analysis of pH, macromolecular and organellar crowding in yeast," *Elife*, vol. 9, p. e54707, 2020, doi: 10.7554/elife.54707.
- [239] M. Kaeberlein, "Hypertrophy and senescence factors in yeast aging. A reply to Bilinski et al.," *Fems Yeast Res*, vol. 12, no. 3, pp. 269–270, 2012, doi: 10.1111/j.1567-1364.2012.00798.x.
- [240] J. E. Pérez-Ortíz, A. Mena, M. Barba-Aliaga, A. Singh, S. Chávez, and J. García-Martínez, "Cell volume homeostatically controls the rDNA repeat copy number and rRNA synthesis rate in yeast," *Plos Genet*, vol. 17, no. 4, p. e1009520, 2021, doi: 10.1371/journal.pgen.1009520.
- [241] X.-M. Sun et al., "Size-Dependent Increase in RNA Polymerase II Initiation Rates Mediates Gene Expression Scaling with Cell Size," *Curr Biol*, vol. 30, no. 7, pp. 1217–1230.e7, 2020, doi: 10.1016/j.cub.2020.01.053.
- [242] M. C. Lanz et al., "Increasing cell size remodels the proteome and promotes senescence," *Biorxiv*, p. 2021.07.29.454227, 2021, doi: 10.1101/2021.07.29.454227.
- [243] S. Anzi et al., "Postnatal Exocrine Pancreas Growth by Cellular Hypertrophy Correlates with a Shorter Lifespan in Mammals," *Dev Cell*, vol. 45, no. 6, pp. 726–737.e3, 2018, doi: 10.1016/j.devcel.2018.05.024.
- [244] L. Cheng, J. Chen, Y. Kong, C. Tan, R. Kafri, and M. Björklund, "Size-scaling promotes senescence-like changes in proteome and organelle content," *Biorxiv*, p. 2021.08.05.455193, 2021, doi: 10.1101/2021.08.05.455193.
- [244b] J. Lengefeld et al., "Cell size is a determinant of stem cell potential during aging," *Sci Adv*, vol. 7, no. 46, p. eabk0271, 2021, doi: 10.1126/sciadv.abk0271.
- [245] Z. Xie et al., "Molecular phenotyping of aging in single yeast cells using a novel microfluidic device," *Aging Cell*, vol. 11, no. 4, pp. 599–606, 2012, doi: 10.1111/j.1474-9726.2012.00821.x.
- [246] S. Fehrmann, C. Paoletti, Y. Goulev, A. Ungureanu, H. Aguilaniu, and G. Charvin, "Aging Yeast Cells Undergo a Sharp Entry into Senescence Unrelated to the Loss of Mitochondrial Membrane Potential," *Cell Reports*, vol. 5, no. 6, pp. 1589–1599, 2013, doi:

## References from Chapter 1

---

- 10.1016/j.celrep.2013.11.013.
- [247] E. A. Sarnoski, R. Song, E. Ertekin, N. Koonce, and M. Acar, "Fundamental characteristics of single-cell aging in diploid yeast," *Iscience*, vol. 7, pp. 96–109, 2018, doi: 10.1016/j.isci.2018.08.011.
- [248] G. E. Neurohr, R. L. Terry, A. Sandikci, K. Zou, H. Li, and A. Amon, "Deregulation of the G1/S-phase transition is the proximal cause of mortality in old yeast mother cells," *Gene Dev*, vol. 32, no. 15–16, pp. 1075–1084, 2018, doi: 10.1101/gad.312140.118.
- [249] M. M. Crane et al., "Rb analog Whi5 regulates G1 to S transition and cell size but not replicative lifespan in budding yeast," *Transl Medicine Aging*, vol. 3, pp. 104–108, 2019, doi: 10.1016/j.tma.2019.10.002.
- [250] G. Schlissel, M. K. Krzyzanowski, F. Caudron, Y. Barral, and J. Rine, "Aggregation of the Whi3 protein, not loss of heterochromatin, causes sterility in old yeast cells," *Science*, vol. 355, no. 6330, pp. 1184–1187, 2017, doi: 10.1126/science.aaj2103.
- [251] T. Smeal, J. Claus, B. Kennedy, F. Cole, and L. Guarente, "Loss of Transcriptional Silencing Causes Sterility in Old Mother Cells of *S. cerevisiae*," *Cell*, vol. 84, no. 4, pp. 633–642, 1996, doi: 10.1016/s0092-8674(00)81038-7.
- [252] I. Müller, "Parental age and the life-span of zygotes of *Saccharomyces cerevisiae*," *Antonie Van Leeuwenhoek*, vol. 51, no. 1, pp. 1–10, 1985, doi: 10.1007/bf00444223.
- [253] M. Boselli, J. Rock, E. Ünal, S. S. Levine, and A. Amon, "Effects of Age on Meiosis in Budding Yeast," *Dev Cell*, vol. 16, no. 6, pp. 844–855, 2009, doi: 10.1016/j.devcel.2009.05.013.
- [254] T. Z. Young, P. Liu, G. Urbonaitė, and M. Acar, "Quantitative Insights into Age-Associated DNA-Repair Inefficiency in Single Cells," *Cell Reports*, vol. 28, no. 8, pp. 2220–2230.e7, 2019, doi: 10.1016/j.celrep.2019.07.082.
- [255] M. A. McMurray and D. E. Gottschling, "An Age-Induced Switch to a Hyper-Recombinational State," *Science*, vol. 301, no. 5641, pp. 1908–1911, 2003, doi: 10.1126/science.1087706.
- [256] L. L. Carr and D. E. Gottschling, "Does age influence loss of heterozygosity?," *Exp Gerontol*, vol. 43, no. 3, pp. 123–129, 2008, doi: 10.1016/j.exger.2007.10.010.
- [257] D. L. Lindstrom, C. K. Leverich, K. A. Henderson, and D. E. Gottschling, "Reproductive Age Induces Mitotic Recombination in the Ribosomal RNA Gene Cluster of *Saccharomyces cerevisiae*," *Plos Genet*, vol. 7, no. 3, p. e1002015, 2011, doi: 10.1371/journal.pgen.1002015.
- [258] M. M. Crane et al., "DNA damage checkpoint activation impairs chromatin homeostasis and promotes mitotic catastrophe during aging," *Elife*, vol. 8, p. e50778, 2019, doi: 10.7554/elife.50778.
- [259] N. P. D'Mello and S. M. Jazwinski, "Telomere length constancy during aging of *Saccharomyces cerevisiae*," *J Bacteriol*, vol. 173, no. 21, pp. 6709–6713, 1991, doi: 10.1128/jb.173.21.6709-6713.1991.
- [260] N. R. Austriaco and L. P. Guarente, "Changes of telomere length cause reciprocal changes in the lifespan of mother cells in *Saccharomyces cerevisiae*," *Proc National Acad Sci*, vol. 94, no. 18, pp. 9768–9772, 1997, doi: 10.1073/pnas.94.18.9768.
- [261] S. Kim, B. Villeponteau, and S. M. Jazwinski, "Effect of Replicative Age on Transcriptional Silencing Near Telomeres in *Saccharomyces cerevisiae*," *Biochem Bioph Res Co*, vol. 219, no. 2, pp. 370–376, 1996, doi: 10.1006/bbrc.1996.0240.
- [262] J. R. Veatch, M. A. McMurray, Z. W. Nelson, and D. E. Gottschling, "Mitochondrial Dysfunction Leads to Nuclear Genome Instability via an Iron-Sulfur Cluster Defect," *Cell*, vol. 137, no. 7, pp. 1247–1258, 2009, doi: 10.1016/j.cell.2009.04.014.
- [263] C. Q. Scheckhuber, N. Erjavec, A. Tinazli, A. Hamann, T. Nyström, and H. D. Osiewacz, "Reducing mitochondrial fission results in increased life span and fitness of two fungal ageing models," *Nat Cell Biol*, vol. 9, no. 1, pp. 99–105, 2007, doi: 10.1038/ncb1524.

## References from Chapter 1

---

- [264] A. L. Hughes and D. E. Gottschling, "An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast," *Nature*, vol. 492, no. 7428, pp. 261–265, 2012, doi: 10.1038/nature11654.
- [265] R. S. Balaban, S. Nemoto, and T. Finkel, "Mitochondria, Oxidants, and Aging," *Cell*, vol. 120, no. 4, pp. 483–495, 2005, doi: 10.1016/j.cell.2005.02.001.
- [266] A. Bratic and N.-G. Larsson, "The role of mitochondria in aging," *J Clin Invest*, vol. 123, no. 3, pp. 951–957, 2013, doi: 10.1172/jci64125.
- [267] N. Sun, R. J. Youle, and T. Finkel, "The Mitochondrial Basis of Aging," *Mol Cell*, vol. 61, no. 5, pp. 654–666, 2016, doi: 10.1016/j.molcel.2016.01.028.
- [268] J. Nunnari and A. Suomalainen, "Mitochondria: In Sickness and in Health," *Cell*, vol. 148, no. 6, pp. 1145–1159, 2012, doi: 10.1016/j.cell.2012.02.035.
- [269] D. C. Wallace, "A Mitochondrial Paradigm of Metabolic and Degenerative Diseases, Aging, and Cancer: a Dawn for Evolutionary Medicine," *Genetics*, vol. 39, no. 1, pp. 359–407, 2005, doi: 10.1146/annurev.genet.39.110304.095751.
- [270] L. N. Dimitrov, R. B. Brem, L. Kruglyak, and D. E. Gottschling, "Polymorphisms in Multiple Genes Contribute to the Spontaneous Mitochondrial Genome Instability of *Saccharomyces cerevisiae* S288C Strains," *Genetics*, vol. 183, no. 1, pp. 365–383, 2009, doi: 10.1534/genetics.109.104497.
- [271] M. D. Chiara et al., "Domestication reprogrammed the budding yeast life cycle," *Biorxiv*, p. 2020.02.08.939314, 2020, doi: 10.1101/2020.02.08.939314.
- [272] C.-Y. Lai, E. Jaruga, C. Borghouts, and S. M. Jazwinski, "A Mutation in the ATP2 Gene Abrogates the Age Asymmetry Between Mother and Daughter Cells of the Yeast *Saccharomyces cerevisiae*," *Genetics*, vol. 162, no. 1, pp. 73–87, 2002, doi: 10.1093/genetics/162.1.73.
- [273] S. M. Jazwinski, "Yeast replicative life span – the mitochondrial connection," *Fems Yeast Res*, vol. 5, no. 2, pp. 119–125, 2004, doi: 10.1016/j.femsyr.2004.04.005.
- [274] H. Klinger et al., "Quantitation of (a)symmetric inheritance of functional and of oxidatively damaged mitochondrial aconitase in the cell division of old yeast mother cells," *Exp Gerontol*, vol. 45, no. 7–8, pp. 533–542, 2010, doi: 10.1016/j.exger.2010.03.016.
- [275] J. R. McFaline-Figueroa et al., "Mitochondrial quality control during inheritance is associated with lifespan and mother–daughter age asymmetry in budding yeast," *Aging Cell*, vol. 10, no. 5, pp. 885–895, 2011, doi: 10.1111/j.1474-9726.2011.00731.x.
- [276] R. Higuchi et al., "Actin Dynamics Affect Mitochondrial Quality Control and Aging in Budding Yeast," *Curr Biol*, vol. 23, no. 23, pp. 2417–2422, 2013, doi: 10.1016/j.cub.2013.10.022.
- [277] P. W. Piper, G. W. Jones, D. Bringloe, N. Harris, M. MacLean, and M. Mollapour, "The shortened replicative life span of prohibitin mutants of yeast appears to be due to defective mitochondrial segregation in old mother cells," *Aging Cell*, vol. 1, no. 2, pp. 149–157, 2002, doi: 10.1046/j.1474-9728.2002.00018.x.
- [278] D. Laporte, L. Gouleme, L. Jimenez, I. Khemiri, and I. Sagot, "Mitochondria reorganization upon proliferation arrest predicts individual yeast cell fate," *Elife*, vol. 7, p. e35685, 2018, doi: 10.7554/elife.35685.
- [279] S. Leupold et al., "Saccharomyces cerevisiae goes through distinct metabolic phases during its replicative lifespan," *Elife*, vol. 8, p. e41046, 2019, doi: 10.7554/elife.41046.
- [280] K. L. Chen et al., "Loss of vacuolar acidity results in iron-sulfur cluster defects and divergent homeostatic responses during aging in *Saccharomyces cerevisiae*," *Geroscience*, vol. 42, no. 2, pp. 749–764, 2020, doi: 10.1007/s11357-020-00159-3.
- [281] C. E. Hughes, T. K. Coody, M.-Y. Jeong, J. A. Berg, D. R. Winge, and A. L. Hughes, "Cysteine Toxicity Drives Age-Related Mitochondrial Decline by Altering Iron Homeostasis," *Cell*, vol. 180, no. 2, pp. 296–310.e18, 2020, doi: 10.1016/j.cell.2019.12.035.
- [282] H. Atamna, D. W. Killilea, A. N. Killilea, and B. N. Ames, "Heme deficiency may be a factor in the mitochondrial and neuronal decay of aging," *Proc National Acad Sci*, vol. 99,

## References from Chapter 1

---

- no. 23, pp. 14807–14812, 2002, doi: 10.1073/pnas.192585799.
- [283] Y. Li et al., “A programmable fate decision landscape underlies single-cell aging in yeast.,” *Sci New York N Y*, vol. 369, no. 6501, pp. 325–329, 2020, doi: 10.1126/science.aax9552.
- [284] A. Y. Seo, A.-M. Joseph, D. Dutta, J. C. Y. Hwang, J. P. Aris, and C. Leeuwenburgh, “New insights into the role of mitochondria in aging: mitochondrial dynamics and more,” *J Cell Sci*, vol. 123, no. 15, pp. 2533–2542, 2010, doi: 10.1242/jcs.070490.
- [285] P. Laun et al., “Aged mother cells of *Saccharomyces cerevisiae* show markers of oxidative stress and apoptosis,” *Mol Microbiol*, vol. 39, no. 5, pp. 1166–1173, 2001, doi: 10.1111/j.1365-2958.2001.02317.x.
- [286] Y. Ohya, N. Umemoto, I. Tanida, A. Ohta, H. Iida, and Y. Anraku, “Calcium-sensitive *cls* mutants of *Saccharomyces cerevisiae* showing a Pet- phenotype are ascribable to defects of vacuolar membrane H(+)-ATPase activity,” *J Biol Chem*, vol. 266, no. 21, pp. 13971–13977, 1991, doi: 10.1016/s0021-9258(18)92798-5.
- [287] A. L. Hughes, C. E. Hughes, K. A. Henderson, N. Yazvenko, and D. E. Gottschling, “Selective sorting and destruction of mitochondrial membrane proteins in aged yeast,” *Elife*, vol. 5, p. e13943, 2016, doi: 10.7554/elife.13943.
- [288] M. Thumm, “Structure and function of the yeast vacuole and its role in autophagy,” *Microsc Res Techniq*, vol. 51, no. 6, pp. 563–572, 2000, doi: 10.1002/1097-0029(20001215)51:6<563::aid-jemt6>3.0.co;2-8.
- [289] S. C. Li and P. M. Kane, “The yeast lysosome-like vacuole: Endpoint and crossroads,” *Biochimica Et Biophysica Acta Bba - Mol Cell Res*, vol. 1793, no. 4, pp. 650–663, 2009, doi: 10.1016/j.bbamcr.2008.08.003.
- [290] F. Tang et al., “A lifespan-extending form of autophagy employs the vacuole-vacuole fusion machinery,” *Autophagy*, vol. 4, no. 7, pp. 874–886, 2008, doi: 10.4161/auto.6556.
- [291] S. Gebre et al., “Osh6 overexpression extends the lifespan of yeast by increasing vacuole fusion,” *Cell Cycle*, vol. 11, no. 11, pp. 2176–2188, 2014, doi: 10.4161/cc.20691.
- [292] J. Choi, S. Wang, Y. Li, N. Hao, and B. M. Zid, “Age-induced P-bodies become detrimental and shorten the lifespan of yeast,” *bioRxiv*, 2021, doi: 10.1101/2021.11.05.467477.
- [293] K. A. Henderson, A. L. Hughes, and D. E. Gottschling, “Mother-daughter asymmetry of pH underlies aging and rejuvenation in yeast,” *Elife*, vol. 3, p. e03504, 2014, doi: 10.7554/elife.03504.
- [294] G. E. Janssens et al., “Protein biogenesis machinery is a driver of replicative aging in yeast,” *Elife*, vol. 4, p. e08527, 2015, doi: 10.7554/elife.08527.
- [295] R. A. Knieß and M. P. Mayer, “The oxidation state of the cytoplasmic glutathione redox system does not correlate with replicative lifespan in yeast,” *Npj Aging Mech Dis*, vol. 2, no. 1, p. 16028, 2016, doi: 10.1038/npjamd.2016.28.
- [296] M. C. Munder et al., “A pH-driven transition of the cytoplasm from a fluid- to a solid-like state promotes entry into dormancy,” *Elife*, vol. 5, p. e09347, 2016, doi: 10.7554/elife.09347.
- [297] B. Jacquel, T. Aspert, D. Laporte, I. Sagot, and G. Charvin, “Monitoring single-cell dynamics of entry into quiescence during an unperturbed lifecycle,” *Biorxiv*, p. 2020.11.25.395608, 2021, doi: 10.1101/2020.11.25.395608.
- [298] R. Orij, S. Brul, and G. J. Smits, “Intracellular pH is a tightly controlled signal in yeast,” *Biochimica Et Biophysica Acta Bba - Gen Subj*, vol. 1810, no. 10, pp. 933–944, 2011, doi: 10.1016/j.bbagen.2011.03.011.
- [299] M. H. Barros, B. Bandy, E. B. Tahara, and A. J. Kowaltowski, “Higher Respiratory Activity Decreases Mitochondrial Reactive Oxygen Release and Increases Life Span in *Saccharomyces cerevisiae* \*,” *J Biol Chem*, vol. 279, no. 48, pp. 49883–49888, 2004, doi: 10.1074/jbc.m408918200.
- [300] P. K. Sharma, V. Agrawal, and N. Roy, “Mitochondria-mediated hormetic response in

## References from Chapter 1

---

- life span extension of calorie-restricted *Saccharomyces cerevisiae*," *Age*, vol. 33, no. 2, pp. 143–154, 2011, doi: 10.1007/s11357-010-9169-1.
- [301] M. Ristow and S. Schmeisser, "Extending life span by increasing oxidative stress," *Free Radical Bio Med*, vol. 51, no. 2, pp. 327–336, 2011, doi: 10.1016/j.freeradbiomed.2011.05.010.
- [302] A. J. Kowaltowski, N. C. de Souza-Pinto, R. F. Castilho, and A. E. Vercesi, "Mitochondria and reactive oxygen species," *Free Radical Bio Med*, vol. 47, no. 4, pp. 333–343, 2009, doi: 10.1016/j.freeradbiomed.2009.05.004.
- [303] A. Koc, A. P. Gasch, J. C. Rutherford, H.-Y. Kim, and V. N. Gladyshev, "Methionine sulfoxide reductase regulation of yeast lifespan reveals reactive oxygen species-dependent and -independent components of aging," *P Natl Acad Sci Usa*, vol. 101, no. 21, pp. 7999–8004, 2004, doi: 10.1073/pnas.0307929101.
- [304] Y. J. Suzuki, M. Carini, and D. A. Butterfield, "Protein Carbonylation," *Antioxid Redox Sign*, vol. 12, no. 3, pp. 323–325, 2010, doi: 10.1089/ars.2009.2887.
- [305] E. R. Stadtman, "Protein oxidation and aging," *Free Radical Res*, vol. 40, no. 12, pp. 1250–1258, 2009, doi: 10.1080/10715760600918142.
- [306] D. A. Parsell, A. S. Kowal, M. A. Singer, and S. Lindquist, "Protein disaggregation mediated by heat-shock protein Hsp104," *Nature*, vol. 372, no. 6505, pp. 475–478, 1994, doi: 10.1038/372475a0.
- [307] E. Ünal, B. Kinde, and A. Amon, "Gametogenesis Eliminates Age-Induced Cellular Damage and Resets Life Span in Yeast," *Science*, vol. 332, no. 6037, pp. 1554–1557, 2011, doi: 10.1126/science.1204349.
- [308] H. Aguilaniu, L. Gustafsson, M. Rigoulet, and T. Nyström, "Asymmetric Inheritance of Oxidatively Damaged Proteins During Cytokinesis," *Science*, vol. 299, no. 5613, pp. 1751–1753, 2003, doi: 10.1126/science.1080418.
- [309] N. Erjavec, L. Larsson, J. Grantham, and T. Nyström, "Accelerated aging and failure to segregate damaged proteins in Sir2 mutants can be suppressed by overproducing the protein aggregation-remodeling factor Hsp104p," *Gene Dev*, vol. 21, no. 19, pp. 2410–2421, 2007, doi: 10.1101/gad.439307.
- [310] C. Zhou, B. D. Slaughter, J. R. Unruh, A. Eldakak, B. Rubinstein, and R. Li, "Motility and Segregation of Hsp104-Associated Protein Aggregates in Budding Yeast," *Cell*, vol. 147, no. 5, pp. 1186–1196, 2011, doi: 10.1016/j.cell.2011.11.002.
- [311] B. Liu et al., "The Polarisome Is Required for Segregation and Retrograde Transport of Protein Aggregates," *Cell*, vol. 140, no. 2, pp. 257–267, 2010, doi: 10.1016/j.cell.2009.12.031.
- [312] T. Nyström, J. Yang, and M. Molin, "Peroxiredoxins, gerontogenes linking aging to genome instability and cancer," *Gene Dev*, vol. 26, no. 18, pp. 2001–2008, 2012, doi: 10.1101/gad.200006.112.
- [313] D. A. Knorre et al., "Sir2-dependent daughter-to-mother transport of the damaged proteins in yeast is required to prevent high stress sensitivity of the daughters," *Cell Cycle*, vol. 9, no. 22, pp. 4501–4505, 2010, doi: 10.4161/cc.9.22.13683.
- [314] P. Tessarz, M. Schwarz, A. Mogk, and B. Bukau, "The Yeast AAA+ Chaperone Hsp104 Is Part of a Network That Links the Actin Cytoskeleton with the Inheritance of Damaged Proteins v," *Mol Cell Biol*, vol. 29, no. 13, pp. 3738–3745, 2009, doi: 10.1128/mcb.00201-09.
- [315] C. Paoletti, S. Quintin, A. Matifas, and G. Charvin, "Kinetics of Formation and Asymmetrical Distribution of Hsp104-Bound Protein Aggregates in Yeast," *Biophys J*, vol. 110, no. 7, pp. 1605–1614, 2016, doi: 10.1016/j.bpj.2016.02.034.
- [316] D. F. Moreno, K. Jenkins, S. Morlot, G. Charvin, A. Csikasz-Nagy, and M. Aldea, "Proteostasis collapse, a hallmark of aging, hinders the chaperone-Start network and arrests cells in G1," *Elife*, vol. 8, p. e48240, 2019, doi: 10.7554/elife.48240.
- [317] U. Kruegel et al., "Elevated Proteasome Capacity Extends Replicative Lifespan in

## References from Chapter 1

---

- Saccharomyces cerevisiae," Plos Genet, vol. 7, no. 9, p. e1002253, 2011, doi: 10.1371/journal.pgen.1002253.
- [318] D. G. Hendrickson et al., "A new experimental platform facilitates assessment of the transcriptional and chromatin landscapes of aging yeast," Elife, vol. 7, p. e39911, 2018, doi: 10.7554/elife.39911.
- [319] K. K. Steffen et al., "Yeast Life Span Extension by Depletion of 60S Ribosomal Subunits Is Mediated by Gcn4," Cell, vol. 133, no. 2, pp. 292–302, 2008, doi: 10.1016/j.cell.2008.02.037.
- [320] F. Capuano, M. Mülleter, R. Kok, H. J. Blom, and M. Ralser, "Cytosine DNA Methylation Is Found in *Drosophila melanogaster* but Absent in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and Other Yeast Species," *Anal Chem*, vol. 86, no. 8, pp. 3697–3702, 2014, doi: 10.1021/ac500447w.
- [321] W. Dang et al., "Histone H4 lysine 16 acetylation regulates cellular lifespan," Nature, vol. 459, no. 7248, pp. 802–807, 2009, doi: 10.1038/nature08085.
- [322] M. Kaeberlein, M. McVey, and L. Guarente, "The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms," Gene Dev, vol. 13, no. 19, pp. 2570–2580, 1999, doi: 10.1101/gad.13.19.2570.
- [323] M. A. McCormick et al., "The SAGA Histone Deubiquitinase Module Controls Yeast Replicative Lifespan via Sir2 Interaction," Cell Reports, vol. 8, no. 2, pp. 477–486, 2014, doi: 10.1016/j.celrep.2014.06.037.
- [324] S. Giavara et al., "Yeast Nhp6A/B and Mammalian Hmgb1 Facilitate the Maintenance of Genome Stability," Curr Biol, vol. 15, no. 1, pp. 68–72, 2005, doi: 10.1016/j.cub.2004.12.065.
- [325] R. Yu, L. Sun, Y. Sun, X. Han, L. Qin, and W. Dang, "Cellular response to moderate chromatin architectural defects promotes longevity," Sci Adv, vol. 5, no. 7, p. eaav1165, 2019, doi: 10.1126/sciadv.aav1165.
- [326] Z. Hu et al., "Nucleosome loss leads to global transcriptional up-regulation and genomic instability during yeast aging," Gene Dev, vol. 28, no. 4, pp. 396–408, 2014, doi: 10.1101/gad.233221.113.
- [327] W. Dang et al., "Inactivation of Yeast Isw2 Chromatin Remodeling Enzyme Mimics Longevity Effect of Calorie Restriction via Induction of Genotoxic Stress Response," Cell Metab, vol. 19, no. 6, pp. 952–966, 2014, doi: 10.1016/j.cmet.2014.04.004.
- [328] Y. Li et al., "Multigenerational silencing dynamics control cell aging," Proc National Acad Sci, vol. 114, no. 42, pp. 11253–11258, 2017, doi: 10.1073/pnas.1703379114.
- [329] K. Saka, S. Ide, A. R. D. Ganley, and T. Kobayashi, "Cellular Senescence in Yeast Is Regulated by rDNA Noncoding Transcription," Curr Biol, vol. 23, no. 18, pp. 1794–1798, 2013, doi: 10.1016/j.cub.2013.07.048.
- [330] S. Pal, S. D. Postnikoff, M. Chavez, and J. K. Tyler, "Impaired cohesion and homologous recombination during replicative aging in budding yeast," Sci Adv, vol. 4, no. 2, p. eaaoq0236, 2018, doi: 10.1126/sciadv.aaoq0236.
- [331] S. S. Lin, J. K. Manchester, and J. I. Gordon, "Enhanced Gluconeogenesis and Increased Energy Storage as Hallmarks of Aging in *Saccharomyces cerevisiae*\* 210," J Biol Chem, vol. 276, no. 38, pp. 36000–36007, 2001, doi: 10.1074/jbc.m103509200.
- [332] G. Yiu et al., "Pathways Change in Expression During Replicative Aging in *Saccharomyces cerevisiae*," *Journals Gerontology Ser*, vol. 63, no. 1, pp. 21–34, 2008, doi: 10.1093/gerona/63.1.21.
- [333] Y. Kamei, Y. Tamada, Y. Nakayama, E. Fukusaki, and Y. Mukai, "Changes in Transcription and Metabolism During the Early Stage of Replicative Cellular Senescence in Budding Yeast\*," J Biol Chem, vol. 289, no. 46, pp. 32081–32093, 2014, doi: 10.1074/jbc.m114.600528.
- [334] S.-J. Lin, P.-A. Defossez, and L. Guarente, "Requirement of NAD and SIR2 for Life-Span Extension by Calorie Restriction in *Saccharomyces cerevisiae*," Science, vol. 289, no.

## References from Chapter 1

---

- 5487, pp. 2126–2128, 2000, doi: 10.1126/science.289.5487.2126.
- [335] D. H. E. W. Huberts et al., “Calorie restriction does not elicit a robust extension of replicative lifespan in *Saccharomyces cerevisiae*,” *Proc National Acad Sci*, vol. 111, no. 32, pp. 11727–11731, 2014, doi: 10.1073/pnas.1410024111.
- [336] C. V. Jack, C. Cruz, R. M. Hull, M. A. Keller, M. Ralser, and J. Houseley, “Regulation of ribosomal DNA amplification by the TOR pathway,” *Proc National Acad Sci*, vol. 112, no. 31, pp. 9674–9679, 2015, doi: 10.1073/pnas.1505015112.
- [337] F. R. Neumann and P. Nurse, “Nuclear size control in fission yeast,” *J Cell Biology*, vol. 179, no. 4, pp. 593–600, 2007, doi: 10.1083/jcb.200708054.
- [338] P. Jorgensen, N. P. Edgington, B. L. Schneider, I. Rupeš, M. Tyers, and B. Futcher, “The Size of the Nucleus Increases as Yeast Cells Grow,” *Mol Biol Cell*, vol. 18, no. 9, pp. 3523–3532, 2007, doi: 10.1091/mbc.e06-10-0973.
- [339] D. L. Levy and R. Heald, “Nuclear Size Is Regulated by Importin  $\alpha$  and Ntf2 in *Xenopus*,” *Cell*, vol. 143, no. 2, pp. 288–298, 2010, doi: 10.1016/j.cell.2010.09.012.
- [340] K. Kume, H. Cantwell, F. R. Neumann, A. W. Jones, A. P. Snijders, and P. Nurse, “A systematic genomic screen implicates nucleocytoplasmic transport and membrane growth in nuclear size control,” *Plos Genet*, vol. 13, no. 5, p. e1006767, 2017, doi: 10.1371/journal.pgen.1006767.
- [341] I. L. Rempel et al., “Age-dependent deterioration of nuclear pore assembly in mitotic cells decreases transport dynamics,” *Elife*, vol. 8, p. e48186, 2019, doi: 10.7554/elife.48186.
- [342] D. H. Lin and A. Hoelz, “Infographic: The Nuclear Pore Complex,” 2016. <https://www.the-scientist.com/infographics/infographic-the-nuclear-pore-complex-32456> (accessed 2021).
- [343] C. W. Akey et al., “Comprehensive Structure and Functional Adaptations of the Yeast Nuclear Pore Complex,” *Biorxiv*, p. 2021.10.29.466335, 2021, doi: 10.1101/2021.10.29.466335.
- [344] A. Taddei et al., “Nuclear pore association confers optimal expression levels for an inducible yeast gene,” *Nature*, vol. 441, no. 7094, pp. 774–778, 2006, doi: 10.1038/nature04845.
- [345] R. Reed, “Coupling transcription, splicing and mRNA export,” *Curr Opin Cell Biol*, vol. 15, no. 3, pp. 326–331, 2003, doi: 10.1016/s0955-0674(03)00048-6.
- [346] K. Sträßer et al., “TREX is a conserved complex coupling transcription with messenger RNA export,” *Nature*, vol. 417, no. 6886, pp. 304–308, 2002, doi: 10.1038/nature746.
- [347] V. Galy, O. Gadal, M. Fromont-Racine, A. Romano, A. Jacquier, and U. Nehrbass, “Nuclear Retention of Unspliced mRNAs in Yeast Is Mediated by Perinuclear Mlp1,” *Cell*, vol. 116, no. 1, pp. 63–73, 2004, doi: 10.1016/s0092-8674(03)01026-2.
- [348] B. Khadaroo et al., “The DNA damage response at eroded telomeres and tethering to the nuclear pore complex,” *Nat Cell Biol*, vol. 11, no. 8, pp. 980–987, 2009, doi: 10.1038/ncb1910.
- [349] C. Lemaître et al., “The nucleoporin 153, a novel factor in double-strand break repair and DNA damage response,” *Oncogene*, vol. 31, no. 45, pp. 4803–4809, 2012, doi: 10.1038/onc.2011.638.
- [350] P. Heun, T. Laroche, K. Shimada, P. Furrer, and S. M. Gasser, “Chromosome Dynamics in the Yeast Interphase Nucleus,” *Science*, vol. 294, no. 5549, pp. 2181–2186, 2001, doi: 10.1126/science.1065366.
- [351] C. Horigome, E. Unoza, T. Ooki, and T. Kobayashi, “Ribosomal RNA gene repeats associate with the nuclear pore complex for maintenance after DNA damage,” *Plos Genet*, vol. 15, no. 4, p. e1008103, 2019, doi: 10.1371/journal.pgen.1008103.
- [352] M. Gomar-Alba and M. Mendoza, “Modulation of Cell Identity by Modification of Nuclear Pore Complexes,” *Frontiers Genetics*, vol. 10, p. 1301, 2020, doi: 10.3389/fgene.2019.01301.
- [353] A. Kumar et al., “Daughter-cell-specific modulation of nuclear pore complexes

## References from Chapter 1

---

- controls cell cycle entry during asymmetric division," *Nat Cell Biol*, vol. 20, no. 4, pp. 432–442, 2018, doi: 10.1038/s41556-018-0056-9.
- [354] Z. Shcheprova, S. Baldi, S. B. Frei, G. Gonnet, and Y. Barral, "A mechanism for asymmetric segregation of age during yeast budding," *Nature*, vol. 454, no. 7205, pp. 728–734, 2008, doi: 10.1038/nature07212.
- [355] B. M. Webster, P. Colombi, J. Jäger, and C. P. Lusk, "Surveillance of Nuclear Pore Complex Assembly by ESCRT-III/Vps4," *Cell*, vol. 159, no. 2, pp. 388–401, 2014, doi: 10.1016/j.cell.2014.09.012.
- [356] A. Denoth-Lippuner, M. K. Krzyzanowski, C. Stober, and Y. Barral, "Role of SAGA in the asymmetric segregation of DNA circles during yeast ageing," *Elife*, vol. 3, p. e03790, 2014, doi: 10.7554/elife.03790.
- [357] I. L. Rempel, A. Steen, and L. M. Veenhoff, "Poor old pores—The challenge of making and maintaining nuclear pore complexes in aging," *Febs J*, vol. 287, no. 6, pp. 1058–1075, 2020, doi: 10.1111/febs.15205.
- [358] A. C. Meinema, T. Aspert, S. S. Lee, G. Charvin, and Y. Barral, "Specialization of chromatin-bound nuclear pore complexes promotes yeast ageing," *Biorxiv*, p. 2021.06.28.450139, 2021, doi: 10.1101/2021.06.28.450139.
- [359] A. Khmelinskii et al., "Tandem fluorescent protein timers for in vivo analysis of protein dynamics," *Nat Biotechnol*, vol. 30, no. 7, pp. 708–714, 2012, doi: 10.1038/nbt.2281.
- [360] C. L. Lord, B. L. Timney, M. P. Rout, and S. R. Wente, "Altering nuclear pore complex function impacts longevity and mitochondrial function in *S. cerevisiae* NPCs and nuclear transport regulate longevity," *J Cell Biology*, vol. 208, no. 6, pp. 729–744, 2015, doi: 10.1083/jcb.201412024.
- [361] C. L. Lord, O. Ospovat, and S. R. Wente, "Nup100 regulates *Saccharomyces cerevisiae* replicative life span by mediating the nuclear export of specific tRNAs," *Rna*, vol. 23, no. 3, pp. 365–377, 2017, doi: 10.1261/rna.057612.116.
- [362] M. A. D'Angelo, M. Raices, S. H. Panowski, and M. W. Hetzer, "Age-Dependent Deterioration of Nuclear Pore Complexes Causes a Loss of Nuclear Integrity in Postmitotic Cells," *Cell*, vol. 136, no. 2, pp. 284–295, 2009, doi: 10.1016/j.cell.2008.11.037.
- [363] A. Ghavami, L. M. Veenhoff, E. van der Giessen, and P. R. Onck, "Probing the Disordered Domain of the Nuclear Pore Complex through Coarse-Grained Molecular Dynamics Simulations," *Biophys J*, vol. 107, no. 6, pp. 1393–1402, 2014, doi: 10.1016/j.bpj.2014.07.060.
- [364] J. Venema and D. Tollervey, "Ribosome synthesis in *Saccharomyces cerevisiae*," *Annu Rev Genet*, vol. 33, no. 1, pp. 261–311, 1999, doi: 10.1146/annurev.genet.33.1.261.
- [365] J. L. Woolford and S. J. Baserga, "Ribosome Biogenesis in the Yeast *Saccharomyces cerevisiae*," *Genetics*, vol. 195, no. 3, pp. 643–681, 2013, doi: 10.1534/genetics.113.153197.
- [366] T. Kobayashi, "How does genome instability affect lifespan?," *Genes Cells*, vol. 16, no. 6, pp. 617–624, 2011, doi: 10.1111/j.1365-2443.2011.01519.x.
- [367] E. Matos-Perdomo and F. Machín, "Nucleolar and Ribosomal DNA Structure under Stress: Yeast Lessons for Aging and Cancer," *Cells*, vol. 8, no. 8, p. 779, 2019, doi: 10.3390/cells8080779.
- [368] D. A. Sinclair, K. Mills, and L. Guarente, "Accelerated Aging and Nucleolar Fragmentation in Yeast sgs1 Mutants," *Science*, vol. 277, no. 5330, pp. 1313–1316, 1997, doi: 10.1126/science.277.5330.1313.
- [369] V. Tiku et al., "Small nucleoli are a cellular hallmark of longevity," *Nat Commun*, vol. 8, no. 1, p. 16083, 2017, doi: 10.1038/ncomms16083.
- [370] V. Tiku and A. Antebi, "Nucleolar Function in Lifespan Regulation," *Trends Cell Biol*, vol. 28, no. 8, pp. 662–672, 2018, doi: 10.1016/j.tcb.2018.03.007.
- [371] S. Hamperl, M. Wittner, V. Babl, J. Perez-Fernandez, H. Tschochner, and J. Griesenbeck, "Chromatin states at ribosomal DNA loci," *Biochimica Et Biophysica Acta Bba*

## References from Chapter 1

---

- *Gene Regul Mech*, vol. 1829, no. 3–4, pp. 405–417, 2013, doi: 10.1016/j.bbagr.2012.12.007.
- [372] J. O. Nelson, G. J. Watase, N. Warsinger-Pepe, and Y. M. Yamashita, "Mechanisms of rDNA Copy Number Maintenance," *Trends Genet*, vol. 35, no. 10, pp. 734–742, 2019, doi: 10.1016/j.tig.2019.07.006.
- [373] T. Kobayashi, "Regulation of ribosomal RNA gene copy number and its role in modulating genome integrity and evolutionary adaptability in yeast," *Cell Mol Life Sci*, vol. 68, no. 8, pp. 1395–1403, 2011, doi: 10.1007/s00018-010-0613-2.
- [374] T. Kobayashi and M. Sasaki, "Ribosomal DNA stability is supported by many 'buffer genes'—introduction to the Yeast rDNA Stability Database," *Fems Yeast Res*, vol. 17, no. 1, p. fox001, 2017, doi: 10.1093/femsyr/fox001.
- [375] J. R. Warner, "The economics of ribosome biosynthesis in yeast," *Trends Biochem Sci*, vol. 24, no. 11, pp. 437–440, 1999, doi: 10.1016/s0968-0004(99)01460-7.
- [376] J. E. Pérez-Ortín, V. Tordera, and S. Chávez, "Homeostasis in the Central Dogma of molecular biology: the importance of mRNA instability," *Rna Biol*, vol. 16, no. 12, pp. 1–8, 2019, doi: 10.1080/15476286.2019.1655352.
- [377] I. Grummt and C. S. Pikaard, "Epigenetic silencing of RNA polymerase I transcription," *Nat Rev Mol Cell Bio*, vol. 4, no. 8, pp. 641–649, 2003, doi: 10.1038/nrm1171.
- [378] A. Mansisidor et al., "Genomic Copy-Number Loss Is Rescued by Self-Limiting Production of DNA Circles," *Mol Cell*, vol. 72, no. 3, pp. 583–593.e4, 2018, doi: 10.1016/j.molcel.2018.08.036.
- [379] R. Dammann, R. Lucchini, T. Koller, and J. M. Sogo, "Chromatin structures and transcription of rDNA in yeast *Saccharomyces cerevisiae*," *Nucleic Acids Res*, vol. 21, no. 10, pp. 2331–2338, 1993, doi: 10.1093/nar/21.10.2331.
- [380] M. Toussaint, G. Levasseur, M. Tremblay, M. Paquette, and A. Conconi, "Psoralen photocrosslinking, a tool to study the chromatin structure of RNA polymerase I - transcribed ribosomal genes," *Biochem Cell Biol*, vol. 83, no. 4, pp. 449–459, 2005, doi: 10.1139/o05-141.
- [381] S. L. French, Y. N. Osheim, F. Cioci, M. Nomura, and A. L. Beyer, "In Exponentially Growing *Saccharomyces cerevisiae* Cells, rRNA Synthesis Is Determined by the Summed RNA Polymerase I Loading Rate Rather than by the Number of Active Genes," *Mol Cell Biol*, vol. 23, no. 5, pp. 1558–1568, 2003, doi: 10.1128/mcb.23.5.1558-1568.2003.
- [382] D. R. Kief and J. R. Warner, "Hierarchy of elements regulating synthesis of ribosomal proteins in *Saccharomyces cerevisiae*," *Mol Cell Biol*, vol. 1, no. 11, pp. 1016–1023, 1981, doi: 10.1128/mcb.1.11.1016.
- [383] D. R. Kief and J. R. Warner, "Coordinate control of syntheses of ribosomal ribonucleic acid and ribosomal proteins during nutritional shift-up in *Saccharomyces cerevisiae*," *Mol Cell Biol*, vol. 1, no. 11, pp. 1007–1015, 1981, doi: 10.1128/mcb.1.11.1007.
- [384] M. Oakes, I. Siddiqi, L. Vu, J. Aris, and M. Nomura, "Transcription Factor UAF, Expansion and Contraction of Ribosomal DNA (rDNA) Repeats, and RNA Polymerase Switch in Transcription of Yeast rDNA," *Mol Cell Biol*, vol. 19, no. 12, pp. 8559–8569, 1999, doi: 10.1128/mcb.19.12.8559.
- [385] H. Li, C. K. Tsang, M. Watkins, P. G. Bertram, and X. F. S. Zheng, "Nutrient regulates Tor1 nuclear localization and association with rDNA promoter," *Nature*, vol. 442, no. 7106, pp. 1058–1061, 2006, doi: 10.1038/nature05020.
- [386] C. W. Ha and W.-K. Huh, "Rapamycin increases rDNA stability by enhancing association of Sir2 with rDNA in *Saccharomyces cerevisiae*," *Nucleic Acids Res*, vol. 39, no. 4, pp. 1336–1350, 2011, doi: 10.1093/nar/gkq895.
- [387] Y. Wei, C. K. Tsang, and X. F. S. Zheng, "Mechanisms of regulation of RNA polymerase III-dependent transcription by TORC1," *Embo J*, vol. 28, no. 15, pp. 2220–2230, 2009, doi: 10.1038/emboj.2009.179.
- [388] T. Kobayashi and T. Horiuchi, "A yeast gene product, Fob1 protein, required for both

## References from Chapter 1

---

- replication fork blocking and recombinational hotspot activities," *Genes Cells*, vol. 1, no. 5, pp. 465–474, 1996, doi: 10.1046/j.1365-2443.1996.d01-256.x.
- [389] J. Huang and D. Moazed, "Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing," *Gene Dev*, vol. 17, no. 17, pp. 2162–2176, 2003, doi: 10.1101/gad.1108403.
- [390] T. Kobayashi, "The Replication Fork Barrier Site Forms a Unique Structure with Fob1p and Inhibits the Replication Fork," *Mol Cell Biol*, vol. 23, no. 24, pp. 9178–9188, 2003, doi: 10.1128/mcb.23.24.9178-9188.2003.
- [391] A. R. D. Ganley, K. Hayashi, T. Horiuchi, and T. Kobayashi, "Identifying gene-independent noncoding functional elements in the yeast ribosomal DNA by phylogenetic footprinting," *P Natl Acad Sci Usa*, vol. 102, no. 33, pp. 11787–11792, 2005, doi: 10.1073/pnas.0504905102.
- [392] C. Li, J. E. Mueller, and M. Bryk, "Sir2 Represses Endogenous Polymerase II Transcription Units in the Ribosomal DNA Nontranscribed Spacer," *Mol Biol Cell*, vol. 17, no. 9, pp. 3848–3859, 2006, doi: 10.1091/mbc.e06-03-0205.
- [393] L. Vasiljeva, M. Kim, N. Terzi, L. M. Soares, and S. Buratowski, "Transcription Termination and RNA Degradation Contribute to Silencing of RNA Polymerase II Transcription within Heterochromatin," *Mol Cell*, vol. 29, no. 3, pp. 313–323, 2008, doi: 10.1016/j.molcel.2008.01.011.
- [394] E. Cesarini, F. R. Mariotti, F. Cioci, and G. Camilloni, "RNA Polymerase I Transcription Silences Noncoding RNAs at the Ribosomal DNA Locus in *Saccharomyces cerevisiae*," *Eukaryot Cell*, vol. 9, no. 2, pp. 325–335, 2010, doi: 10.1128/ec.00280-09.
- [395] J. S. Smith and J. D. Boeke, "An unusual form of transcriptional silencing in yeast ribosomal DNA," *Gene Dev*, vol. 11, no. 2, pp. 241–254, 1997, doi: 10.1101/gad.11.2.241.
- [396] C. M. Armstrong, M. Kaeberlein, S. I. Imai, and L. Guarente, "Mutations in *Saccharomyces cerevisiae* GeneSIR2 Can Have Differential Effects on In Vivo Silencing Phenotypes and In Vitro Histone Deacetylation Activity," *Mol Biol Cell*, vol. 13, no. 4, pp. 1427–1438, 2002, doi: 10.1091/mbc.01-10-0482.
- [397] J. J. Sandmeier et al., "RPD3 is required for the inactivation of yeast ribosomal DNA genes in stationary phase," *Embo J*, vol. 21, no. 18, pp. 4959–4968, 2002, doi: 10.1093/emboj/cdf498.
- [398] S. W. Buck, J. J. Sandmeier, and J. S. Smith, "RNA Polymerase I Propagates Unidirectional Spreading of rDNA Silent Chromatin," *Cell*, vol. 111, no. 7, pp. 1003–1014, 2002, doi: 10.1016/s0092-8674(02)01193-5.
- [399] F. Cioci, L. Vu, K. Eliason, M. Oakes, I. N. Siddiqi, and M. Nomura, "Silencing in Yeast rDNA Chromatin Reciprocal Relationship in Gene Expression between RNA Polymerase I and II," *Mol Cell*, vol. 12, no. 1, pp. 135–145, 2003, doi: 10.1016/s1097-2765(03)00262-4.
- [400] M. R. Gartenberg and J. S. Smith, "The Nuts and Bolts of Transcriptionally Silent Chromatin in *Saccharomyces cerevisiae*," *Genetics*, vol. 203, no. 4, pp. 1563–1599, 2016, doi: 10.1534/genetics.112.145243.
- [401] H. Chen, M. Fan, L. M. Pfeffer, and R. N. Laribee, "The histone H3 lysine 56 acetylation pathway is regulated by target of rapamycin (TOR) signaling and functions directly in ribosomal RNA biogenesis," *Nucleic Acids Res*, vol. 40, no. 14, pp. 6534–6546, 2012, doi: 10.1093/nar/gks345.
- [402] P. Tessarz et al., "Glutamine methylation in Histone H2A is an RNA Polymerase I dedicated modification," *Nature*, vol. 505, no. 7484, pp. 564–568, 2014, doi: 10.1038/nature12819.
- [403] R. Srivastava, R. Srivastava, and S. H. Ahn, "The Epigenetic Pathways to Ribosomal DNA Silencing," *Microbiol Mol Biol R*, vol. 80, no. 3, pp. 545–563, 2016, doi: 10.1128/mmbr.00005-16.
- [404] R. T. Yamamoto, Y. Nogi, J. A. Dodd, and M. Nomura, "RRN3 gene of *Saccharomyces*

## References from Chapter 1

---

- cerevisiae encodes an essential RNA polymerase I transcription factor which interacts with the polymerase independently of DNA template.,” *Embo J*, vol. 15, no. 15, pp. 3964–73, 1996.
- [405] G. Peyroche et al., “The recruitment of RNA polymerase I on rDNA is mediated by the interaction of the A43 subunit with Rrn3,” *Embo J*, vol. 19, no. 20, pp. 5473–5482, 2000, doi: 10.1093/emboj/19.20.5473.
- [406] C. Blattner et al., “Molecular basis of Rrn3-regulated RNA polymerase I initiation and cell growth,” *Gene Dev*, vol. 25, no. 19, pp. 2093–2105, 2011, doi: 10.1101/gad.17363311.
- [407] K. Hannig et al., “The C-terminal region of Net1 is an activator of RNA polymerase I transcription with conserved features from yeast to human,” *Plos Genet*, vol. 15, no. 2, p. e1008006, 2019, doi: 10.1371/journal.pgen.1008006.
- [408] T. Kobayashi, “Strategies to maintain the stability of the ribosomal RNA gene repeats,” *Genes Genet Syst*, vol. 81, no. 3, pp. 155–161, 2006, doi: 10.1266/ggs.81.155.
- [409] Y. Takeuchi, T. Horiuchi, and T. Kobayashi, “Transcription-dependent recombination and the role of fork collision in yeast rDNA,” *Gene Dev*, vol. 17, no. 12, pp. 1497–1506, 2003, doi: 10.1101/gad.1085403.
- [410] T. Kobayashi, D. J. Heck, M. Nomura, and T. Horiuchi, “Expansion and contraction of ribosomal DNA repeats in *Saccharomyces cerevisiae*: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I,” *Gene Dev*, vol. 12, no. 24, pp. 3821–3830, 1998, doi: 10.1101/gad.12.24.3821.
- [411] J. W. Szostak and R. Wu, “Unequal crossing over in the ribosomal DNA of *Saccharomyces cerevisiae*,” *Nature*, vol. 284, no. 5755, pp. 426–430, 1980, doi: 10.1038/284426a0.
- [412] S. Gangloff, H. Zou, and R. Rothstein, “Gene conversion plays the major role in controlling the stability of large tandem repeats in yeast,” *Embo J*, vol. 15, no. 7, pp. 1715–25, 1996.
- [413] S. Ide, K. Saka, and T. Kobayashi, “Rtt109 Prevents Hyper-Amplification of Ribosomal RNA Genes through Histone Modification in Budding Yeast,” *Plos Genet*, vol. 9, no. 4, p. e1003410, 2013, doi: 10.1371/journal.pgen.1003410.
- [414] T. Iida and T. Kobayashi, “How do cells count multi-copy genes?: ‘Musical Chair’ model for preserving the number of rDNA copies,” *Curr Genet*, vol. 65, no. 4, pp. 883–885, 2019, doi: 10.1007/s00294-019-00956-0.
- [415] T. Iida and T. Kobayashi, “RNA Polymerase I Activators Count and Adjust Ribosomal RNA Gene Copy Number,” *Mol Cell*, vol. 73, no. 4, pp. 645–654.e13, 2019, doi: 10.1016/j.molcel.2018.11.029.
- [416] K. Saka, A. Takahashi, M. Sasaki, and T. Kobayashi, “More than 10% of yeast genes are related to genome stability and influence cellular senescence via rDNA maintenance,” *Nucleic Acids Res*, vol. 44, no. 9, pp. 4211–4221, 2016, doi: 10.1093/nar/gkw110.
- [417] A. R. D. Ganley, S. Ide, K. Saka, and T. Kobayashi, “The Effect of Replication Initiation on Gene Amplification in the rDNA and Its Relationship to Aging,” *Mol Cell*, vol. 35, no. 5, pp. 683–693, 2009, doi: 10.1016/j.molcel.2009.07.012.
- [418] T. Kobayashi, T. Horiuchi, P. Tongaonkar, L. Vu, and M. Nomura, “SIR2 Regulates Recombination between Different rDNA Repeats, but Not Recombination within Individual rRNA Genes in Yeast,” *Cell*, vol. 117, no. 4, pp. 441–453, 2004, doi: 10.1016/s0092-8674(04)00414-3.
- [419] S. Gottlieb and R. E. Esposito, “A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA,” *Cell*, vol. 56, no. 5, pp. 771–776, 1989, doi: 10.1016/0092-8674(89)90681-8.
- [420] T. Kobayashi and A. R. D. Ganley, “Recombination Regulation by Transcription-Induced Cohesin Dissociation in rDNA Repeats,” *Science*, vol. 309, no. 5740, pp. 1581–1584, 2005, doi: 10.1126/science.1116102.
- [421] T. Kobayashi, “A new role of the rDNA and nucleolus in the nucleus—rDNA instability

## References from Chapter 1

---

- maintains genome integrity," *Bioessays*, vol. 30, no. 3, pp. 267–272, 2008, doi: 10.1002/bies.20723.
- [422] A. R. D. Ganley and T. Kobayashi, "Ribosomal DNA and cellular senescence: new evidence supporting the connection between rDNA and aging," *Fems Yeast Res*, vol. 14, no. 1, pp. 49–59, 2014, doi: 10.1111/1567-1364.12133.
- [423] E. Cesarini, A. D'Alfonso, and G. Camilloni, "H4K16 acetylation affects recombination and ncRNA transcription at rDNA in *Saccharomyces cerevisiae*," *Mol Biol Cell*, vol. 23, no. 14, pp. 2770–2781, 2012, doi: 10.1091/mbc.e12-02-0095.
- [424] D. A. Sinclair and L. Guarente, "Extrachromosomal rDNA Circles— A Cause of Aging in Yeast," *Cell*, vol. 91, no. 7, pp. 1033–1042, 1997, doi: 10.1016/s0092-8674(00)80493-6.
- [425] P.-A. Defossez et al., "Elimination of Replication Block Protein Fob1 Extends the Life Span of Yeast Mother Cells," *Mol Cell*, vol. 3, no. 4, pp. 447–455, 1999, doi: 10.1016/s1097-2765(00)80472-4.
- [426] C. S. Gillespie, C. J. Proctor, R. J. Boys, D. P. Shanley, D. J. Wilkinson, and T. B. L. Kirkwood, "A mathematical model of ageing in yeast," *J Theor Biol*, vol. 229, no. 2, pp. 189–196, 2004, doi: 10.1016/j.jtbi.2004.03.015.
- [427] L. R. Gehlen, S. Nagai, K. Shimada, P. Meister, A. Taddei, and S. M. Gasser, "Nuclear Geometry and Rapid Mitosis Ensure Asymmetric Episome Segregation in Yeast," *Curr Biol*, vol. 21, no. 1, pp. 25–33, 2011, doi: 10.1016/j.cub.2010.12.016.
- [428] B. Boettcher, T. T. Marquez-Lago, M. Bayer, E. L. Weiss, and Y. Barral, "Nuclear envelope morphology constrains diffusion and promotes asymmetric protein segregation in closed mitosis," *J Cell Biol*, vol. 197, no. 7, pp. 921–937, 2012, doi: 10.1083/jcb.201112117.
- [429] A. Khmelinskii, M. Meurer, M. Knop, and E. Schiebel, "Artificial tethering to nuclear pores promotes partitioning of extrachromosomal DNA during yeast asymmetric cell division," *Curr Biol*, vol. 21, no. 1, pp. R17–R18, 2011, doi: 10.1016/j.cub.2010.11.034.
- [430] A. W. Murray and J. W. Szostak, "Pedigree analysis of plasmid segregation in yeast," *Cell*, vol. 34, no. 3, pp. 961–970, 1983, doi: 10.1016/0092-8674(83)90553-6.
- [431] A. B. Futcher and B. S. Cox, "Copy number and the stability of 2-micron circle-based artificial plasmids of *Saccharomyces cerevisiae*," *J Bacteriol*, vol. 157, no. 1, pp. 283–90, 1984.
- [432] M. C. Espinosa, M. A. Rehman, P. Chisamore-Robert, D. Jeffery, and K. Yankulov, "GCN5 Is a Positive Regulator of Origins of DNA Replication in *Saccharomyces cerevisiae*," *Plos One*, vol. 5, no. 1, p. e8964, 2010, doi: 10.1371/journal.pone.0008964.
- [433] A. A. Falcón and J. P. Aris, "Plasmid Accumulation Reduces Life Span in *Saccharomyces cerevisiae*," *J Biol Chem*, vol. 278, no. 43, pp. 41607–41617, 2003, doi: 10.1074/jbc.m307025200.
- [434] R. Sweeney and V. A. Zakian, "Extrachromosomal elements cause a reduced division potential in nib 1 strains of *Saccharomyces cerevisiae*," *Genetics*, vol. 122, no. 4, pp. 749–757, 1989, doi: 10.1093/genetics/122.4.749.
- [435] H. Wang, L. B. Carey, Y. Cai, H. Wijnen, and B. Futcher, "Recruitment of Cln3 Cyclin to Promoters Controls Cell Cycle Entry via Histone Deacetylase and Other Targets," *Plos Biol*, vol. 7, no. 9, p. e1000189, 2009, doi: 10.1371/journal.pbio.1000189.
- [436] P. Bensidoun, T. Reiter, B. Montpetit, D. Zenklusen, and M. Oeffinger, "Nuclear mRNA metabolism drives selective basket assembly on a subset of nuclear pores in budding yeast," *Biorxiv*, p. 2021.11.07.467636, 2021, doi: 10.1101/2021.11.07.467636.
- [437] C. L. WoldeRingh, K. Fluiter, and P. G. Huls, "Production of senescent cells of *Saccharomyces cerevisiae* by centrifugal elutriation," *Yeast*, vol. 11, no. 4, pp. 361–369, 1995, doi: 10.1002/yea.320110409.
- [438] I. Lesur and J. L. Campbell, "The Transcriptome of Prematurely Aging Yeast Cells Is Similar to That of Telomerase-deficient Cells," *Mol Biol Cell*, vol. 15, no. 3, pp. 1297–1312, 2004, doi: 10.1091/mbc.e03-10-0742.

## References from Chapter 1

---

- [439] D. L. Lindstrom and D. E. Gottschling, "The Mother Enrichment Program: A Genetic System for Facile Replicative Life Span Analysis in *Saccharomyces cerevisiae*," *Genetics*, vol. 183, no. 2, pp. 413–422, 2009, doi: 10.1534/genetics.109.106229.
- [440] B. C.E, "The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression," *Cold Spring Harbor Laboratory Press*, 1982.
- [441] E. A. Sarnoski, P. Liu, and M. Acar, "A High-Throughput Screen for Yeast Replicative Lifespan Identifies Lifespan-Extending Compounds," *Cell Reports*, vol. 21, no. 9, pp. 2639–2646, 2017, doi: 10.1016/j.celrep.2017.11.002.
- [442] T. Chen et al., "A drug-compatible and temperature-controlled microfluidic device for live-cell imaging," *Open Biol*, vol. 6, no. 8, p. 160156, 2016, doi: 10.1098/rsob.160156.
- [443] R. B. Kopito and E. Levine, "Durable spatiotemporal surveillance of *Caenorhabditis elegans* response to environmental cues," *Lab Chip*, vol. 14, no. 4, pp. 764–770, 2013, doi: 10.1039/c3lc51061a.
- [444] R. Gómez-Sjöberg, A. A. Leyrat, D. M. Pirone, C. S. Chen, and S. R. Quake, "Versatile, Fully Automated, Microfluidic Cell Culture System," *Anal Chem*, vol. 79, no. 22, pp. 8557–8563, 2007, doi: 10.1021/ac071311w.
- [445] A. M. Taylor, D. C. Dieterich, H. T. Ito, S. A. Kim, and E. M. Schuman, "Microfluidic Local Perfusion Chambers for the Visualization and Manipulation of Synapses," *Neuron*, vol. 66, no. 1, pp. 57–68, 2010, doi: 10.1016/j.neuron.2010.03.022.
- [446] D. Qin, Y. Xia, and G. M. Whitesides, "Soft lithography for micro- and nanoscale patterning," *Nat Protoc*, vol. 5, no. 3, pp. 491–502, 2010, doi: 10.1038/nprot.2009.234.
- [447] R. O'Laughlin et al., "Advances in quantitative biology methods for studying replicative aging in *Saccharomyces cerevisiae*," *Transl Medicine Aging*, 2019, doi: 10.1016/j.tma.2019.09.002.
- [448] M. M. Crane, I. B. N. Clark, E. Bakker, S. Smith, and P. S. Swain, "A Microfluidic System for Studying Ageing and Dynamic Single-Cell Responses in Budding Yeast," *Plos One*, vol. 9, no. 6, p. e100042, 2014, doi: 10.1371/journal.pone.0100042.
- [449] P. Liu, T. Z. Young, and M. Acar, "Yeast Replicator: A High-Throughput Multiplexed Microfluidics Platform for Automated Measurements of Single-Cell Aging," *Cell Reports*, vol. 13, no. 3, pp. 634–644, 2015, doi: 10.1016/j.celrep.2015.09.012.
- [450] J. Ryley and O. M. Pereira-Smith, "Microfluidics device for single cell gene expression analysis in *Saccharomyces cerevisiae*," *Yeast*, vol. 23, no. 14-15, pp. 1065–1073, 2006, doi: 10.1002/yea.1412.
- [451] S. Beucher, "The Watershed Transformation Applied to Image Segmentation," *Scanning Microscopy*, vol. 6, 1992.
- [452] E. Bakker, P. S. Swain, and M. M. Crane, "Morphologically constrained and data informed cell segmentation of budding yeast," *Bioinformatics*, vol. 34, no. 1, pp. 88–96, 2017, doi: 10.1093/bioinformatics/btx550.
- [453] N. E. Wood and A. Doncic, "A fully-automated, robust, and versatile algorithm for long-term budding yeast segmentation and tracking," *Plos One*, vol. 14, no. 3, p. e0206395, 2019, doi: 10.1371/journal.pone.0206395.
- [453b] K. W. Eliceiri et al., "Biological imaging software tools," *Nat Methods*, vol. 9, no. 7, pp. 697–710, 2012, doi: 10.1038/nmeth.2084.
- [454] I. Walsh et al., "DOME: recommendations for supervised machine learning validation in biology," *Nat Methods*, vol. 18, no. 10, pp. 1122–1127, 2021, doi: 10.1038/s41592-021-01205-4.
- [455] M. Weigert et al., "Content-aware image restoration: pushing the limits of fluorescence microscopy," *Nat Methods*, vol. 15, no. 12, pp. 1090–1097, 2018, doi: 10.1038/s41592-018-0216-7.
- [456] M. Priesnner, et al., and R. Laine, "Content-aware frame interpolation (CAFI): Deep Learning-based temporal super-resolution for fast bioimaging," *biorxiv*, 2021, doi: 10.1101/2021.11.02.466664.

## References from Chapter 1

---

- [457] J. Jumper et al., "Highly accurate protein structure prediction with AlphaFold," *Nature*, vol. 596, no. 7873, pp. 583–589, 2021, doi: 10.1038/s41586-021-03819-2.
- [458] S. Berg et al., "ilastik: interactive machine learning for (bio)image analysis," *Nat Methods*, vol. 16, no. 12, pp. 1226–1232, 2019, doi: 10.1038/s41592-019-0582-9.
- [459] Y. LeCun and Y. Bengio, "Convolutional networks for images, speech, and time series," *The handbook of brain theory and neural networks*, 1995.
- [460] O. Ronneberger, P. Fischer, and T. Brox, "U-Net: Convolutional Networks for Biomedical Image Segmentation," *Arxiv*, 2015.
- [461] C. Stringer, T. Wang, M. Michaelos, and M. Pachitariu, "Cellpose: a generalist algorithm for cellular segmentation," *Nat Methods*, vol. 18, no. 1, pp. 100–106, 2021, doi: 10.1038/s41592-020-01018-x.
- [462] U. Schmidt, M. Weigert, C. Broaddus, and G. Myers, "Cell Detection with Star-convex Polygons," *Arxiv*, 2018, doi: 10.1007/978-3-030-00934-2\_30.
- [463] N. Dietler et al., "A convolutional neural network segments yeast microscopy images with high accuracy," *Nat Commun*, vol. 11, no. 1, p. 5723, 2020, doi: 10.1038/s41467-020-19557-4.
- [464] D. Salem, Y. Li, P. Xi, H. Phenix, M. Cuperlovic-Culf, and M. Kærn, "YeastNet: Deep-Learning-Enabled Accurate Segmentation of Budding Yeast Cells in Bright-Field Microscopy," *Appl Sci*, vol. 11, no. 6, p. 2692, 2021, doi: 10.3390/app11062692.
- [465] A. X. Lu, T. Zarin, I. S. Hsu, and A. M. Moses, "YeastSpotter: Accurate and parameter-free web segmentation for microscopy images of yeast cells," *Bioinformatics*, vol. 35, no. 21, pp. 4525–4527, 2019, doi: 10.1093/bioinformatics/btz402.
- [466] D. Bunk, J. Moriasy, F. Thoma, C. Jakubke, C. Osman, and D. Hörl, "YeastMate: Neural network-assisted segmentation of mating and budding events in *S. cerevisiae*," 2021, doi: 10.1101/2021.10.13.464238.
- [467] T. Prangemeier, C. Reich, and H. Koepll, "Attention-Based Transformers for Instance Segmentation of Cells in Microstructures," *Arxiv*, 2020, doi: 10.1109/bibm49941.2020.9313305.
- [468] T. Prangemeier, C. Wildner, A. O. Françani, C. Reich, and H. Koepll, "Multiclass Yeast Segmentation in Microstructured Environments with Deep Learning," *Arxiv*, 2020, doi: 10.1109/cibcb48159.2020.9277693.
- [469] J.-Y. Tinevez et al., "TrackMate: An open and extensible platform for single-particle tracking," *Methods*, vol. 115, pp. 80–90, 2017, doi: 10.1016/j.jymeth.2016.09.016.
- [470] J.-B. Lugagne, H. Lin, and M. J. Dunlop, "DeLTA: Automated cell segmentation, tracking, and lineage reconstruction using deep learning," *Plos Comput Biol*, vol. 16, no. 4, p. e1007673, 2020, doi: 10.1371/journal.pcbi.1007673.
- [471] S. Hochreiter and J. Schmidhuber, "Long Short-Term Memory," *Neural Comput*, vol. 9, no. 8, pp. 1735–1780, 1997, doi: 10.1162/neco.1997.9.8.1735.
- [472] P. B. Dennis, S. Fumagalli, and G. Thomas, "Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation," *Curr Opin Genet Dev*, vol. 9, no. 1, pp. 49–54, 1999, doi: 10.1016/s0959-437x(99)80007-0.
- [473] S.-B. Qian, X. Zhang, J. Sun, J. R. Bennink, J. W. Yewdell, and C. Patterson, "mTORC1 Links Protein Quality and Quantity Control by Sensing Chaperone Availability\*", *J Biol Chem*, vol. 285, no. 35, pp. 27385–27395, 2010, doi: 10.1074/jbc.m110.120295.
- [474] M. Perić et al., "TORC1-mediated sensing of chaperone activity alters glucose metabolism and extends lifespan," *Aging Cell*, vol. 16, no. 5, pp. 994–1005, 2017, doi: 10.1111/ace.12623.
- [475] R. Hatakeyama et al., "Spatially Distinct Pools of TORC1 Balance Protein Homeostasis," *Mol Cell*, vol. 73, no. 2, pp. 325-338.e8, 2019, doi: 10.1016/j.molcel.2018.10.040.
- [476] M. N. Devare, Y. H. Kim, J. Jung, W. K. Kang, K. Kwon, and J. Kim, "TORC1 signaling regulates cytoplasmic pH through Sir2 in yeast," *Aging Cell*, vol. 19, no. 6, p. e13151, 2020,

## References from Chapter 1

---

- doi: 10.1111/acel.13151.
- [477] A. Caballero et al., "Absence of Mitochondrial Translation Control Proteins Extends Life Span by Activating Sirtuin-Dependent Silencing," *Mol Cell*, vol. 42, no. 3, pp. 390–400, 2011, doi: 10.1016/j.molcel.2011.03.021.
- [478] I. Orlandi, M. Bettiga, L. Alberghina, T. Nyström, and M. Vai, "Sir2-dependent asymmetric segregation of damaged proteins in ubp10 null mutants is independent of genomic silencing," *Biochimica Et Biophysica Acta Bba - Mol Cell Res*, vol. 1803, no. 5, pp. 630–638, 2010, doi: 10.1016/j.bbamcr.2010.02.009.
- [479] B. Sampaio-Marques et al., "SNCA ( $\alpha$ -synuclein)-induced toxicity in yeast cells is dependent on Sir2-mediated mitophagy," *Autophagy*, vol. 8, no. 10, pp. 79–78, 2012, doi: 10.4161/auto.21275.
- [480] J. Song et al., "Essential Genetic Interactors of SIR2 Required for Spatial Sequestration and Asymmetrical Inheritance of Protein Aggregates," *Plos Genet*, vol. 10, no. 7, p. e1004539, 2014, doi: 10.1371/journal.pgen.1004539.
- [481] M. Kaeberlein, K. T. Kirkland, S. Fields, and B. K. Kennedy, "Sir2-Independent Life Span Extension by Calorie Restriction in Yeast," *Plos Biol*, vol. 2, no. 9, p. e296, 2004, doi: 10.1371/journal.pbio.0020296.
- [482] D. Koshland, J. C. Kent, and L. H. Hartwell, "Genetic analysis of the mitotic transmission of minichromosomes," *Cell*, vol. 40, no. 2, pp. 393–403, 1985, doi: 10.1016/0092-8674(85)90153-9.
- [483] I. Prada-Luengo et al., "Relicative aging is associated with loss of genetic heterogeneity from extrachromosomal circular DNA in *Saccharomyces cerevisiae*," *Nucleic Acids Res*, vol. 48, no. 14, pp. gkaa545-, 2020, doi: 10.1093/nar/gkaa545.
- [484] S. Baldi, A. Bolognesi, A. C. Meinema, and Y. Barral, "Heat stress promotes longevity in budding yeast by relaxing the confinement of age-promoting factors in the mother cell," *Elife*, vol. 6, p. e28329, 2017, doi: 10.7554/elife.28329.
- [485] A. Mena et al., "Asymmetric cell division requires specific mechanisms for adjusting global transcription," *Nucleic Acids Res*, vol. 45, no. 21, pp. 12401–12412, 2017, doi: 10.1093/nar/gkx974.
- [486] M. Niepel et al., "The nuclear basket proteins Mlp1p and Mlp2p are part of a dynamic interactome including Esc1p and the proteasome," *Mol Biol Cell*, vol. 24, no. 24, pp. 3920–3938, 2013, doi: 10.1091/mbc.e13-07-0412.
- [487] J. Miné-Hattab and A. Taddei, "Physical principles and functional consequences of nuclear compartmentalization in budding yeast," *Curr Opin Cell Biol*, vol. 58, pp. 105–113, 2019, doi: 10.1016/j.ceb.2019.02.005.
- [488] L. E. Orgel and F. H. C. Crick, "Selfish DNA: the ultimate parasite," *Nature*, vol. 284, no. 5757, pp. 604–607, 1980, doi: 10.1038/284604a0.
- [489] T. Cavalier-Smith, "Skeletal DNA and the Evolution of Genome Size," *Annu Rev Biophys Bio*, vol. 11, no. 1, pp. 273–302, 1982, doi: 10.1146/annurev.bb.11.060182.001421.
- [490] T. R. Gregory, "The Evolution of the Genome," *Part C-value Enigma*, no. Adv Enzymol161955, pp. 3–87, 2005, doi: 10.1016/b978-012301463-4/50003-6.
- [491] M. P. Swaffer et al., "Transcriptional and chromatin-based partitioning mechanisms uncouple protein scaling from cell size," *Mol Cell*, 2021, doi: 10.1016/j.molcel.2021.10.007.
- [492] Z. Liao, W. Jiang, L. Ye, T. Li, X. Yu, and L. Liu, "Classification of extrachromosomal circular DNA with a focus on the role of extrachromosomal DNA (ecDNA) in tumor heterogeneity and progression," *Biochimica Et Biophysica Acta Bba - Rev Cancer*, vol. 1874, no. 1, p. 188392, 2020, doi: 10.1016/j.bbcan.2020.188392.
- [493] H. D. Møller et al., "Circular DNA elements of chromosomal origin are common in healthy human somatic tissue," *Nat Commun*, vol. 9, no. 1, p. 1069, 2018, doi: 10.1038/s41467-018-03369-8.
- [494] R. P. Koche et al., "Extrachromosomal circular DNA drives oncogenic genome

## References from Chapter 1

---

- remodeling in neuroblastoma," *Nat Genet*, vol. 52, no. 1, pp. 29–34, 2020, doi: 10.1038/s41588-019-0547-z.
- [495] R. G. W. Verhaak, V. Bafna, and P. S. Mischel, "Extrachromosomal oncogene amplification in tumour pathogenesis and evolution," *Nat Rev Cancer*, vol. 19, no. 5, pp. 283–288, 2019, doi: 10.1038/s41568-019-0128-6.
- [496] R. M. Hull, M. King, G. Pizza, F. Krueger, X. Vergara, and J. Houseley, "Transcription-induced formation of extrachromosomal DNA during yeast ageing," *Plos Biol*, vol. 17, no. 12, p. e3000471, 2019, doi: 10.1371/journal.pbio.3000471.
- [497] R. M. Hull and J. Houseley, "The adaptive potential of circular DNA accumulation in ageing cells," *Curr Genet*, vol. 66, no. 5, pp. 889–894, 2020, doi: 10.1007/s00294-020-01069-9.
- [498] Q. Ain, C. Schmeer, D. Wengerdt, O. W. Witte, and A. Kretz, "Extrachromosomal Circular DNA: Current Knowledge and Implications for CNS Aging and Neurodegeneration," *Int J Mol Sci*, vol. 21, no. 7, p. 2477, 2020, doi: 10.3390/ijms21072477.
- [499] B. Xu et al., "Ribosomal DNA copy number loss and sequence variation in cancer," *Plos Genet*, vol. 13, no. 6, p. e1006771, 2017, doi: 10.1371/journal.pgen.1006771.
- [500] R. Ren et al., "Visualization of aging-associated chromatin alterations with an engineered TALE system," *Cell Res*, vol. 27, no. 4, pp. 483–504, 2017, doi: 10.1038/cr.2017.18.
- [501] M. Wang and B. Lemos, "Ribosomal DNA harbors an evolutionarily conserved clock of biological aging," *Genome Res*, vol. 29, no. 3, pp. 325–333, 2019, doi: 10.1101/gr.241745.118.
- [502] K. Nishimura et al., "Perturbation of Ribosome Biogenesis Drives Cells into Senescence through 5S RNP-Mediated p53 Activation," *Cell Reports*, vol. 10, no. 8, pp. 1310–1323, 2015, doi: 10.1016/j.celrep.2015.01.055.
- [503] F. Lessard et al., "Senescence-associated ribosome biogenesis defects contributes to cell cycle arrest through the Rb pathway," *Nat Cell Biol*, vol. 20, no. 7, pp. 789–799, 2018, doi: 10.1038/s41556-018-0127-y.
- [504] E. Zlotorynski, "Live longer with small nucleoli," *Nat Rev Mol Cell Bio*, vol. 18, no. 11, pp. 651–651, 2017, doi: 10.1038/nrm.2017.100.
- [505] C. K. Tsang, P. G. Bertram, W. Ai, R. Drenan, and X. F. S. Zheng, "Chromatin-mediated regulation of nucleolar structure and RNA Pol I localization by TOR," *Embo J*, vol. 22, no. 22, pp. 6045–6056, 2003, doi: 10.1093/emboj/cdg578.
- [506] M. M. Crane and M. Kaeberlein, "The paths of mortality: How understanding the biology of aging can help explain systems behavior of single cells," *Curr Opin Syst Biology*, vol. 8, pp. 25–31, 2018, doi: 10.1016/j.coisb.2017.11.010.
- [507] M. M. Crane, K. L. Chen, B. W. Blue, and M. Kaeberlein, "Trajectories of Aging: How Systems Biology in Yeast Can Illuminate Mechanisms of Personalized Aging," *Proteomics*, vol. 20, no. 5–6, p. 1800420, 2020, doi: 10.1002/pmic.201800420.
- [508] M. Morawska and H. D. Ulrich, "An expanded tool kit for the auxin-inducible degron system in budding yeast," *Yeast Chichester Engl*, vol. 30, no. 9, pp. 341–351, 2013, doi: 10.1002/yea.296

# Chapter 2: Monitoring single-cell dynamics of entry into quiescence during an unperturbed life-cycle

This work is the result of a side-project in close collaboration with Basile Jacquel (another PhD student from the lab). It was recently published in Elife. I will shortly introduce the background and the result part will consist in the published paper.

The chapter ends with a short discussion.

## Introduction

### 1. General concepts of quiescence

Microorganisms are tightly controlling their proliferation in response to nutrient availability from their immediate environment. In fact, in their natural habitat they spend most of the time under nutrient depletion [1,2]. Therefore, prokaryotic and eukaryotic microbes have evolved complex programs to maintain viability in the absence of metabolizable carbon, nitrogen, or amino acids, in order to be able to proliferate again when these nutrients are available again. For example, starved diploid budding yeast can perform meiosis and form spores that can survive for extended periods [3]. Similarly, cells can enter **quiescence, defined as a reversible nonproliferating state** [1]. In this state, microbial cells can survive for many years without nutrients and tolerate stresses several orders of magnitude higher than what would kill them in a proliferative state.

Quiescence also occurs in mammal cells, especially in stem cells, when no proliferation is needed [4,5]. It is also a property of certain cancer cells which allows them to survive chemotherapies [6].

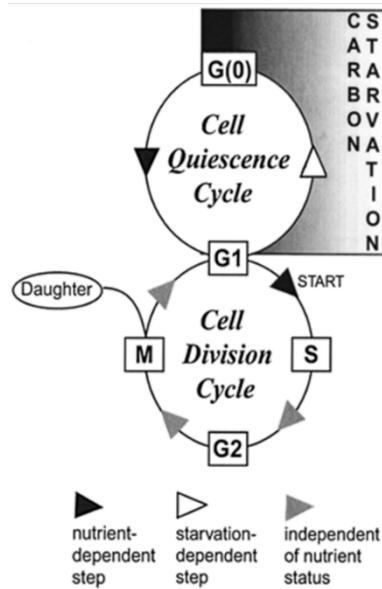
Of note, the molecular pathways involved in the entry into quiescence are partially conserved across eukaryotes (notably driven by the TOR and PKA metabolic pathways) [7].

Overall, and similarly to replicative aging, budding yeast is a relevant organism to study quiescence.

### 2. The molecular pathways of entry into quiescence

Originally, cells were thought to be able to enter quiescence only by exiting the cell cycle from the G1 phase, which is why quiescence is sometimes termed G0 phase (Figure 75) [8].

In line with that, many genes blocking the G1/S transition are repressed during the entry into quiescence [9,10]. However, later studies established that *S. cerevisiae*, *Cryptococcus neoformans*, and *Tetrahymena pyriformis* could enter quiescence in G2 [2,11]. Therefore, cells could prefer to stay in G1 before entering quiescence when the nutrients start waning, but being in G1 is not necessary to enter it.



*Figure 75: Relation between the cell cycle and quiescence, as historically stated (From [1]).*

The molecular pathways driving the transition to quiescence are partially known and rely on TORC1 and PKA (major nutrient sensing and metabolic pathways, see p.44). Their repression leads to the transcription and activation of several hub repressor genes such as Rim15, Xbp1, and Stb3. Rim15 induces the general stress response, indirectly inhibits Pol II transcription, and promotes glycogen and trehalose storage (important for longevity in quiescence, as discussed later) [12–14]. Similarly, Xbp1 represses 800 genes, and Stb3 represses ribosomal protein transcripts.

Overall, quiescence is regulated molecularly with specifically dedicated programs. However, these molecular pathways are not the only layer of control.

### 3. Structural rearrangements in quiescence

Indeed, the entry into quiescence is also characterized by a wide reorganization of cellular structures, presumed to be involved in the long-term viability in this state [2] (Figure 76).

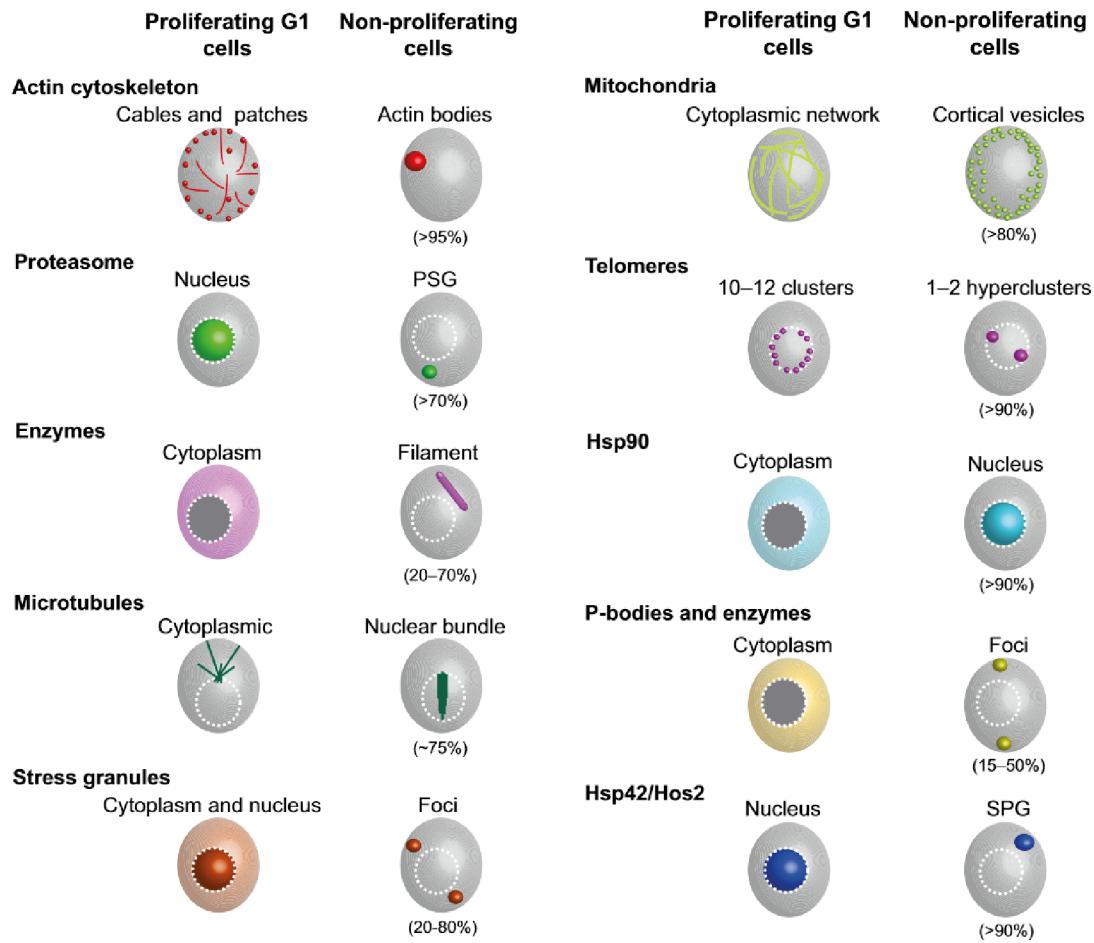


Figure 76: Cellular and organellar reorganizations upon proliferation cessation in budding yeast. The percentages indicate the number of cells displaying these changes in the population among all other possible forms (From [2]).

The following subsections will briefly present some of these changes.

### 3.1. Cytoskeleton

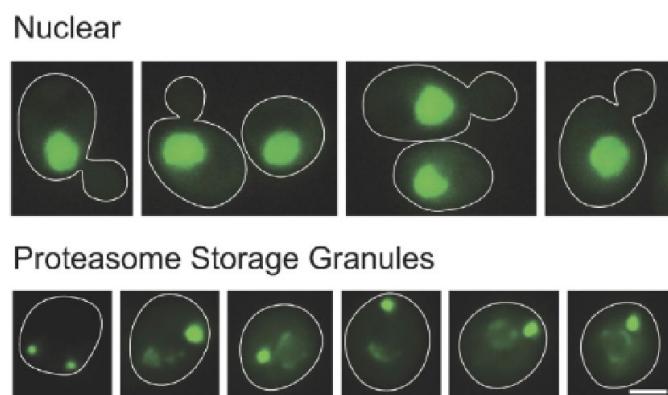
The cytoskeleton is the central controller of the dynamical mechanical properties of cells and is involved in intracellular transports, polarized growth, and material segregation during division [15]. Upon nutrient depletion and proliferation arrest, it is remodeled with the actin network transitioning into single bodies (called actin bodies) [16], and microtubules forming a single tubular structure called nuclear bundle [17] (Figure 76). Importantly, these quiescence-specific structures are reversible and return to their functional state when nutrients are available again, suggesting that they act as a storage compartment to efficiently re-enter proliferation.

### 3.2. Mitochondrial network and respiration

As seen previously (Chapter I, p.36), mitochondria are an important metabolic hub and are necessary for respiration. In response to abrupt starvation, cells need to have a flux in the respiration metabolism in order to survive [18]. Besides, the mitochondrial network (which directly depends on the mitochondrial activity) is also remodeled during quiescence, going from a tubular network during proliferation to small vesicles (Figure 76). Importantly, cells that do not display this remodeling do not survive longer than a few days (discussed later) [19]. Overall, this suggests that respiration and mitochondrial reorganization play a critical role in the establishment of a "healthy" quiescent state.

### 3.3. Proteasome

As discussed in Chapter I (p.42), the proteasome is a group of enzymatic complexes which control the proteostasis and the degradation of damaged proteins [20]. During the entry into quiescence, the proteasome is relocalized from the nucleus to the cytoplasm and forms Proteasome Storage Granules (PSG) [21] (Figure 77). This sequestering protects the proteasome from autophagic degradation occurring upon carbon starvation [22], and could explain why the cells have evolved to form these structures during the entry into quiescence. Like precedently, this structure is reversible since the PSG can rapidly be transformed into a functional proteasome upon nutrient availability [21].

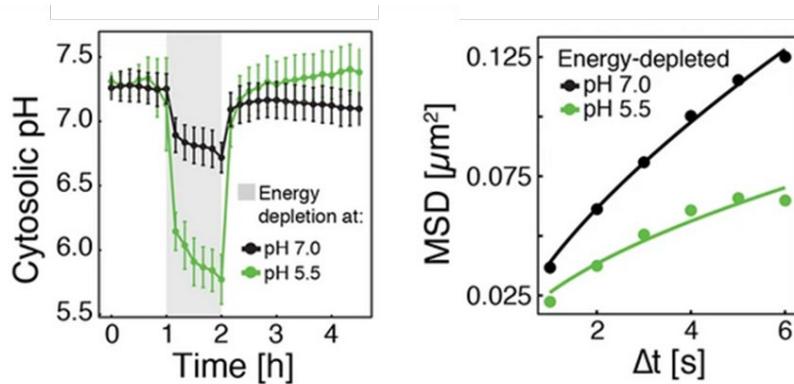


*Figure 77: Images from the different types of organizations the proteasome can adopt. Most of the cells display a « nuclear » proteasome during proliferation, and a PSG during proliferation arrest (From [21]).*

### 3.4. Phase transition of the cytoplasm

Beyond cellular structures, the whole cytoplasm organization could be modified in quiescence. In fact, it was shown that the cytoplasm of the cell was transitioning from a liquid-like state to a gel-like state upon starvation, not only in budding yeast but also in fission yeast and bacteria [23–26]. This state is caused by a decrease in metabolic activity and is associated

with reduced mobility of cytoplasmic particles above a certain size, as well as an increase in molecular crowding (Figure 78). Two biophysical phenomena can explain this phenomenon: the decrease in cell volume during nutrient stress [23] and the acidification of the cytosol [24]. Indeed, the cytosolic pH is mainly controlled by ATP-dependent proton pumps (such as Pma1) [27] and since ATP levels drop upon starvation [18], the cytosol becomes acid [24]. In turn, a low pH can induce the aggregation of several proteins such as metabolic enzymes [24,28], proteasomal proteins [29], stress granules, and P-bodies [30–32].



*Figure 78: Link between energy, pH, and cytosolic physical state. (Left) : Cytosolic pH vs. Time. The gray area indicates an induced energy depletion, in a media of pH 7.0 (black) or 5.5 (green). When cells are depleted from energy, intracellular pH becomes close to extracellular pH. (Right) : Mean Square Displacement of a given fluorescent particle in energy-depleted cells in different pH. The mobility is decreased in low-pH cytosols (From [24]).*

If the transition of the cytosol into a gel-like state is the result of a global aggregation of proteins or just an independent effect of a drop of pH and volume decrease, is still debated. Nonetheless, the aggregation of the proteins described previously participates in the cellular fitness in starvation and quiescence [22,24,30,31].

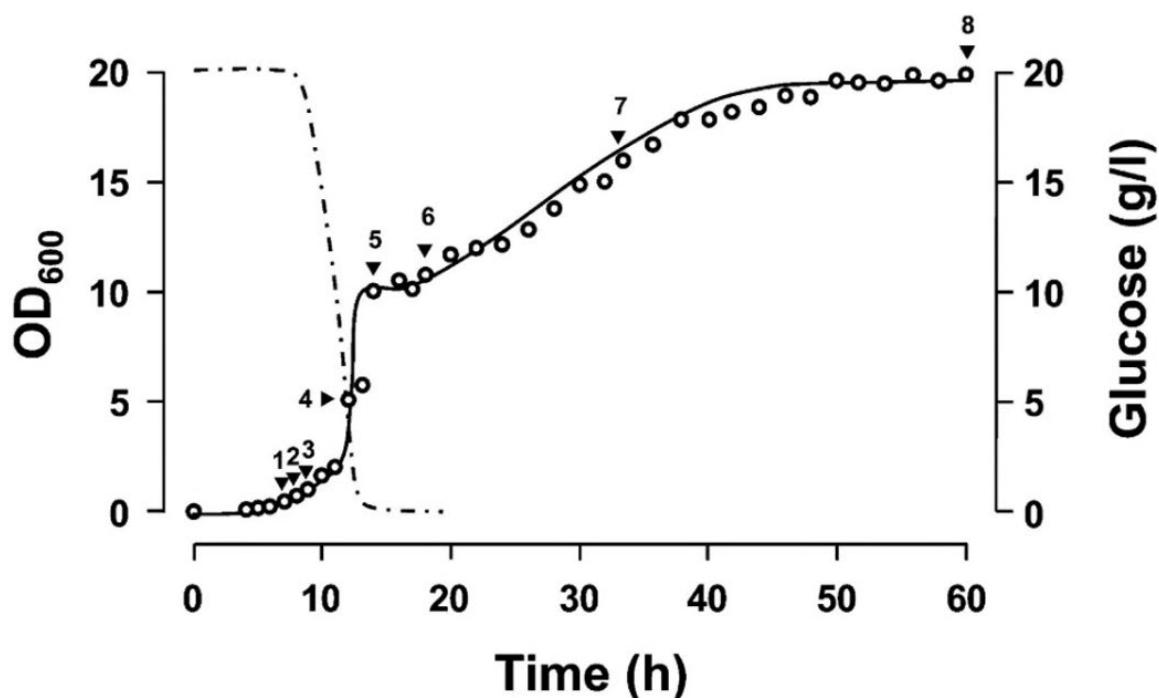
Furthermore, the phase transition of proteins is also known to depend on their concentration [30,54–56], and a reduction in volume during starvation could help achieve a gel-like state. More generally, this state is also thought to confer stress resistance properties and protect the proteins and structures by reducing their interactions [30].

Overall, in the absence of nutrients, protein aggregation and the phase transition of the cytosol are driven by a drop of pH, and are an efficient and energy-free mechanism for the cell to keep its integrity.

## 4. Quiescence and life-cycle of budding yeast

### 4.1. The life-cycle of budding yeast

In most ecological niches, yeast experience different metabolic phases where they catabolize several carbon sources. For example, in the wine-making process, they start by fermenting the sugars from the media until their exhaustion. Then, after a diauxic shift period, they can proliferate again by respiring the ethanol produced during the fermentative phase. Finally, when all the carbon sources are exhausted, the population reaches the stationary phase and cells enter quiescence (Figure 79). Hence, quiescence in the wild rarely happens after a sharp nutrient depletion, but rather after a dynamic change of environments.



*Figure 79: Optical density (i.e., indirect measurement of the cell concentration)(solid line and dots) and glucose concentration (dashed line) vs. Time of a population of cells put in a glucose-based media. The points 1-5 represent the **fermentative phase**, 5-6 the **diauxic shift**, 6-7.5 the **respirative phase**, and from 7.5, the **stationary phase** (From [33]).*

### 4.2. Quiescence: one unique cellular state?

Do cells from an isogenic population reach the same quiescent state after this dynamic change of environment? In other words, is there a single genetically encoded quiescent state and if not, what factors determine the population's heterogeneity?

## Chapter II : Introduction

---

If the different hallmarks presented previously are happening in most cells, there is always a subpopulation not displaying them, or displaying another form of hallmark (see % in Figure 76). In line with that, two subpopulations were identified based on more general markers such as cell density [34]. The population with the lowest density was called 'non-quiescent' because its viability was null after 21 days of culture, while the other was called quiescent. Futher study identified the 'non-quiescent' cells as cells that could not respire during the respiration phase [19,35] and show a globularization of their mitochondrial network in respirative phase (instead of fragmentation). They have been termed senescent cells and represent the same population as the 'non-quiescent' cells.

In agreement with this, cells with a null respiratory capacity ( $\rho O$  cells) rapidly die upon stationary phase. Interestingly, the longevity of these cells can be rescued when adding trehalose to the media [36], a sugar that is normally produced and stored in the cells during respiration.

Hence, to respire or not during the respirative phase of the culture can lead to a different final state once in stationary phase.

Besides, the appearance of these two populations during the respirative phase has been recently proposed as a bet-edging strategy for the cells to adapt to environmental instability [37]. In this study, they performed an abrupt switch between a fermentative and a respiratory media, and found that the cells that could not respire after the switch were growing slightly faster than the cells that could do the metabolic switch. However, how one cell decides to be of one or the other type is unclear.

More generally, it is known that how yeast and other microorganisms respond to their environment is dependent on their history ('termed history-dependent behavior') [38]. Along the same line, cells can anticipate and predict future environmental changes in the context of the standard life-cycle (fermentative phase ->respirative phase->stationary phase). It was indeed shown that exposition to ethanol during the fermentative phase was protective against ROS produced during the respirative phase [39].

Thus, the quiescence state is most likely shaped by the previous experiences of the cell (for example, respiration).

As an illustration, cells can reach a different quiescent state depending on the type of nutrient depletion they experience (glucose, nitrogen, or phosphate), although several properties such as stress tolerance and increase in cell wall thickness were common [40].

Overall, this suggests that quiescence is not a unique and well-defined state, and is strongly influenced by what the cell experienced in its life. In the context of the standard life-cycle, to respire or not is identified as a source of heterogeneity later on in quiescence. Yet, what determines if a cell will respire during the respiratory phase is unclear, nor if the subpopulations pre-exist before respiration.

### 4.3. Assessing quiescence

One way of assessing the state of quiescence in which the cells are is by putting them in a glucose-based media, let them grow until the stationary phase, and measure how long they remain viable (Chronological Lifespan) (often done using Colony Formation Units (CFU) as a readout (see p.25) (Figure 80, 'Q cells')). Similarly, extracting a fraction of the cells at given timepoints to observe specific markers (see introduction) can also be informative. The caveat of this approach is the absence of information at the single-cell level. In particular, the history of the cell that recreate a colony, or that is being observed, is entirely unknown.

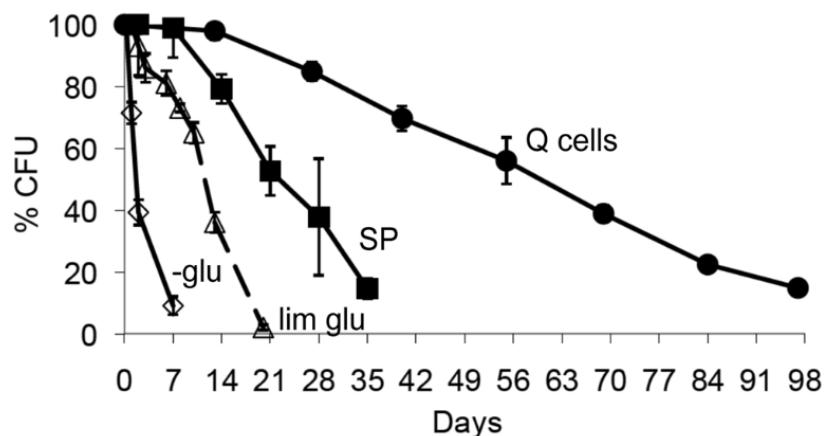


Figure 80: CFU of cells experiencing different environmental conditions? (Q): a normal life-cycle without any perturbation. (SP): cells left until the stationary phase before being put into water. (lim glu): Cells grown in a media with 0.8% glucose before being transferred to a media with 0% glucose (starvation). (-glu): Cells grown in a media with 2% glucose before being transferred to a media with 0% glucose. (From [41])

Another approach consists of observing single-cells longitudinally in microfluidics, by doing synthetic nutrient starvation such as switching from a rich media to a glucose-depleted one. Yet, such treatment ignores the complex dynamical processes that the cell put in place during evolution (see previous section). In support of that, cells experiencing an abrupt nutrient depletion can only survive a few days, while cells that underwent an entire life-cycle and were left in their media, can survive more than 100 days. This suggests that the conditions of growth can impact the quiescent state of the cells (as intuited in the previous section) [41] (Figure 80).

## Summary and motivations

Quiescence defines a reversible proliferative arrest accompanied by several molecular processes and a massive reorganization of many cellular structures. However, it does not represent a unique and well-defined state. Even an isogenic population cultured in the same environment will reach heterogeneous states. Besides, the state of quiescence is highly dependent on the cell's past experiences.

In particular, in the context of the yeast life-cycle, respiration seems a source of heterogeneity later on in stationary phase, leading to a quiescent and a 'non-quiescent' (also called senescent) population.

Relative to the methodology, populational assays lead to averaging biases of these heterogeneous populations and fail to capture the history of single-cells (similarly to traditional methods used in replicative aging). When they manage to do it, they ignore the dynamic environments for which the cells have evolved specific ordered responses.

To our knowledge, there is no available method to track single-cells longitudinally while experiencing their natural life-cycle.

Therefore, the objectives of this project were to:

- a) Develop a methodology to track single-cells experiencing all the phases from the yeast life-cycle, from the fermentation to the entry into quiescence and the survival once in quiescence.
- b) Understand the source of heterogeneities and better characterize the 'non-quiescent' population:

    What is the source of this non-quiescent population?

    Can we predict which cells will become 'non-quiescent' before stationary phase by looking at their history?

    Do these cells enter some kind of quiescence before losing viability, or do they die directly?

## Results

*The result part is composed of a published paper, but a small paragraph will introduce the details of one key component from the methodological pipeline (that is not detailed in the paper).*

As described later in the paper (Figure 1), we plugged a culture of cells to an observation microfluidic device to follow single cells throughout the life-cycle. Consequently, the cells present in the observation chambers can experience the same media as the cells in the batch culture. Yet, the concentration of cells is so high in the late stages of the life-cycle ( $5 \times 10^8$  cells/mL) that a filtration device is needed between the culture and the observation chamber to avoid unwanted cells colonizing it.

Beyond being used to trap cells, microfluidic devices can also be serve to focus particles in a thin streamline. For that, several solutions exist. If of adequate dimensions, a rectangular channel will focus particles above a certain size, along its outer walls [42] (Figure 81, top middle). That is due to an inertial force, called lift force, which is a result of a shear force induced by the flow (due to the parabolic nature of the velocity, since microfluidic flows are mostly Poiseuille flows), and a lift force exerted by the walls (said simply).

From that, adding curvatures to the channels adds other inertial forces such as the Dean force (a drag force with a vortices profil), which focuses the particles onto a unique wall (Figure 81, right)<sup>3</sup>.

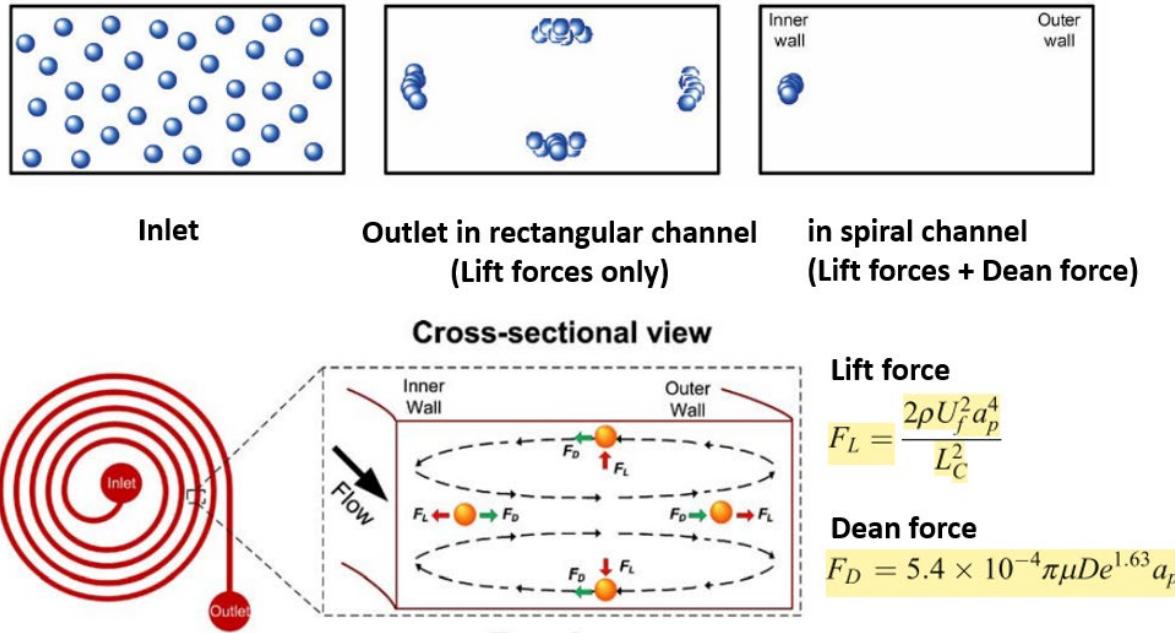
Among these channel types, we can cite "bumpy" channels [43], asymmetrically curved channels [44], or spirals [45].

Therefore, by tweaking the dimensions of these channels, it is possible to split a mixed group of objects of different sizes<sup>4</sup> into ordered focused streams [46].

---

<sup>3</sup> The physics of inertial microfluidics will not be further detailed here for the sake of conciseness (more details: [51]).

<sup>4</sup> Of note, it is also possible to sort particles by shape [48] using the same principle.



*Figure 81: Schematics of the principle of inertial separation. (Top) : Cross-section of a channel, with blue dots representing particles, at the channel's inlet (left), at the outlet of a rectangular channel (middle), or at the outlet of a spiral channel. (Bottom) : A spiral device (left) and a close up on its cross-section indicating the different non-negligible forces at stake (middle). The expression of the forces is indicated on the right, with  $a_p$  the size of the particle,  $\rho$  the density of the fluid,  $U_f$  the average fluid velocity,  $L_C$  the narrowest dimension of the channel,  $\mu$  the viscosity of the fluid, and  $D e$  the Dean number, which depends on the channel dimensions and the Reynolds number (Adapted from [47]).*

This type of particle separation is used, for example, to separate the different types of blood cells from a sample [49,50]. Yet, if the threshold side is well adapted, one can separate particles (like cells) from the media. Hence, among the different device types, we found that the spiral-like was the best compromise between continuous filtration of particles above  $3\mu\text{m}$  and hydrodynamic resistivity, and can achieve a 99% filtration efficiency.

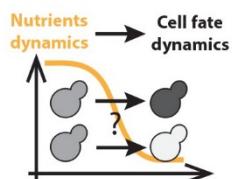
## Chapter II : Results

---

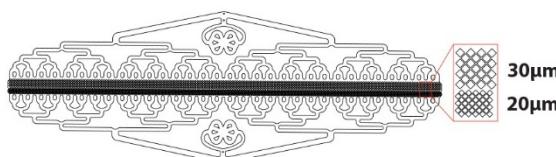
## Chapter II : Results

**Figure 1 – Supplement 1**

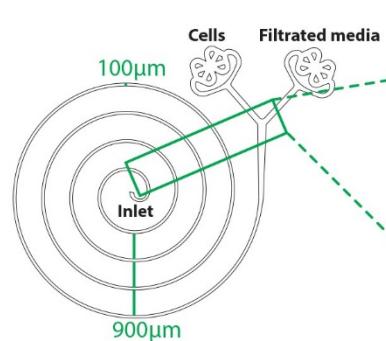
A



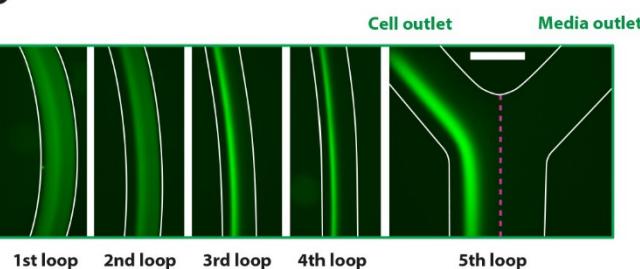
B



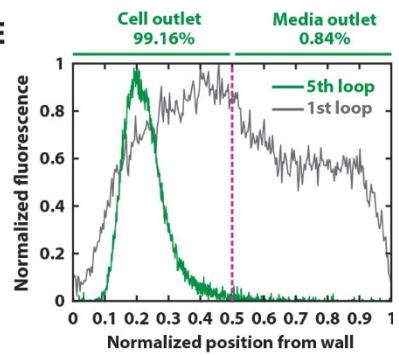
C



D



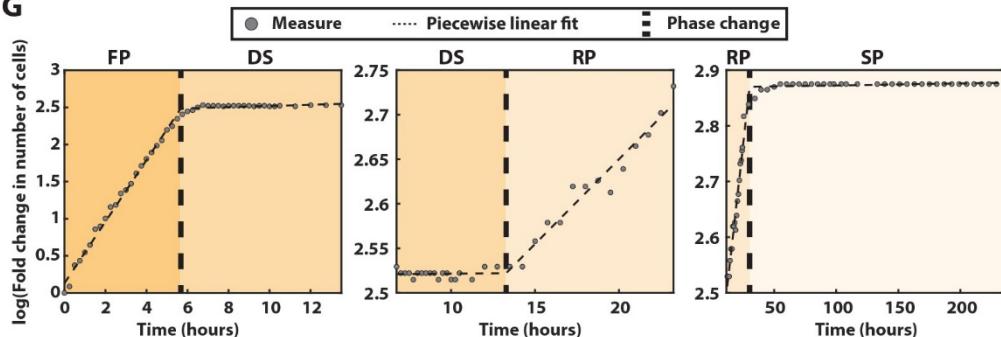
E



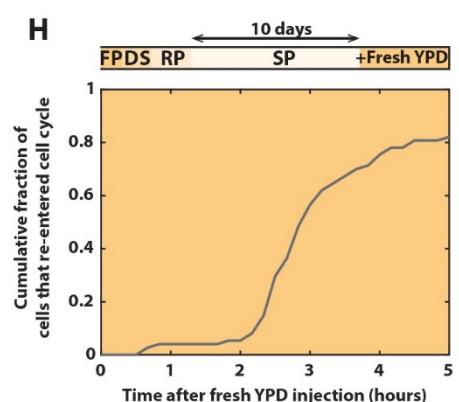
F



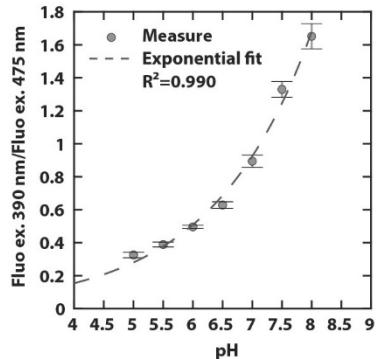
G



H



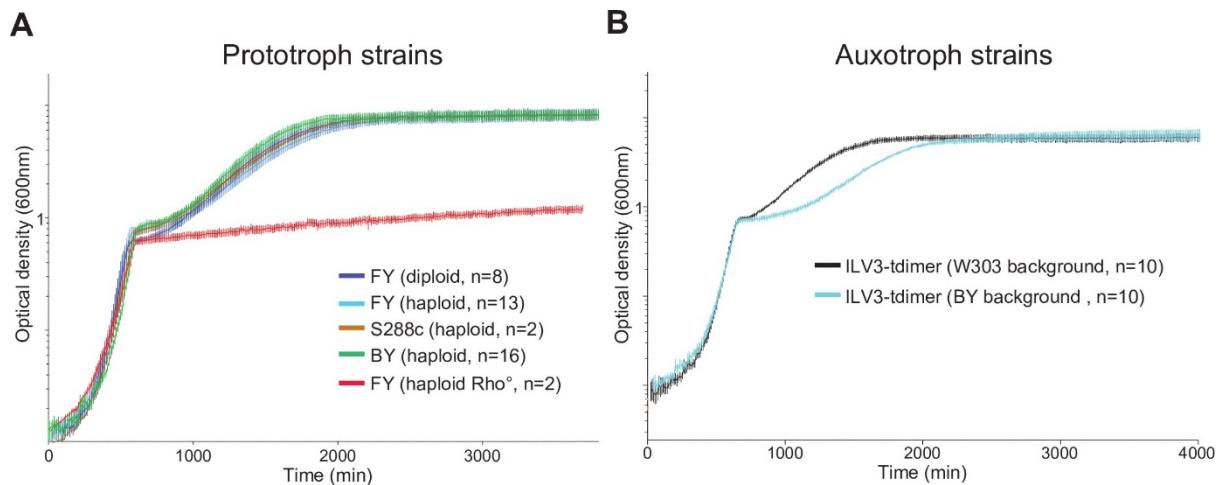
I



**Figure 1 – Supplement 1: Design and calibration of the experimental setup.**

(A) Schematics representing the main question addressed in this study, namely how the natural dynamics of environmental changes shapes nonuniform cellular responses and induces distinct cellular fates in a clonal population. (B) Schematics of the dust filter device, including a closeup of the debris retention arrays of 30 and 20  $\mu\text{m}$  size. (C) Schematics of the cell-filtering device, made of a spiraling channel with five loops of 100  $\mu\text{m}$  width, separated by 900  $\mu\text{m}$ . (D) Sample fluorescence images of TDH3-GFP cells at different positions in the filtering device. Each image represents the indicated loop. The magenta dotted line represents the middle of the channel. Scale bar = 150  $\mu\text{m}$ . (E) Fluorescence profile of the first loop (gray) and fifth loop (green) along the channel's cross-section. The value is normalized to the maximum of each condition. In the upper part is displayed the cumulative signal on each side of the channel of the fifth loop. (F) Schematics of the whole closed-loop fluidic platform. The flask is connected to a peristaltic pump, which drives media flows to the dust filter. The dust-free media then flows into the inlet of the spiral. The cell outlet of the spiral is redirected into the flask while the cell-free outlet irrigates the observation device. To close the loop, the outlet of the observation device is connected to the flask. (G) Determination of the culture metabolic phases during the yeast life cycle based on the evolution of cell number over time. A piecewise linear fit on the number of cells defines the limit between fermentation phase (FP) and diauxic shift (DS; left), DS and respiration phase (RP; middle), or RP and stationary phase (SP; right). Gray dots represent the measured number of cells in one microcolony, thin dashed lines represent the line fits and vertical dashed lines the transition time. Each shaded area represents a distinct proliferation phase, as indicated in the legend. (H) Cumulative fraction of cells reentering the cell cycle after 10 days in SP over time, upon refeeding with YPD medium ( $N = 60$ ). (I) Calibration curve of the pHluorin probe. Gray dots represent the ratio of fluorescence collected at 390 nm over the fluorescence collected at 475 nm excitations, error bars represent the standard deviation ( $N = 20$ ). The dashed line corresponds to an exponential fit  $f(x) = a \times \exp(b \times x)$  of parameters (with 95 % confidence bounds):  $a = 0.01419$  (0.005774, 0.0226) and  $b = 0.5967$  (0.5174, 0.6761).

## Figure 1 – Supplement 2

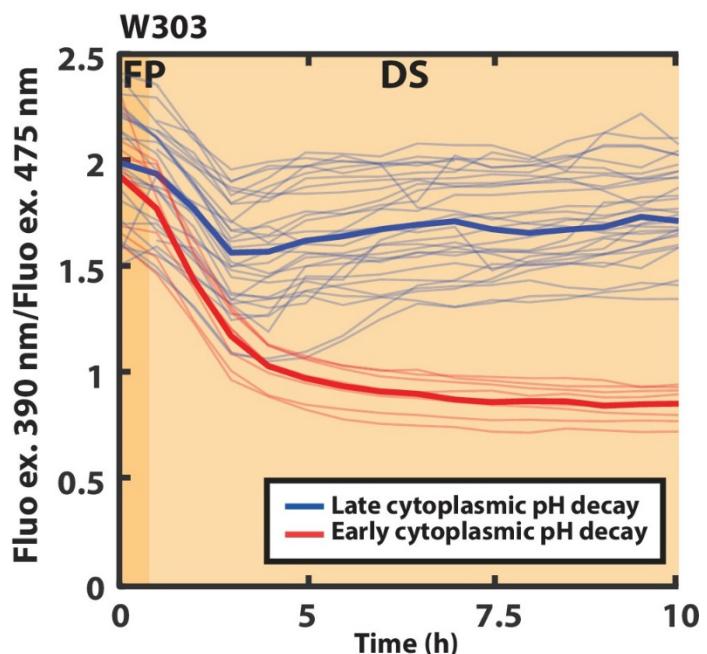
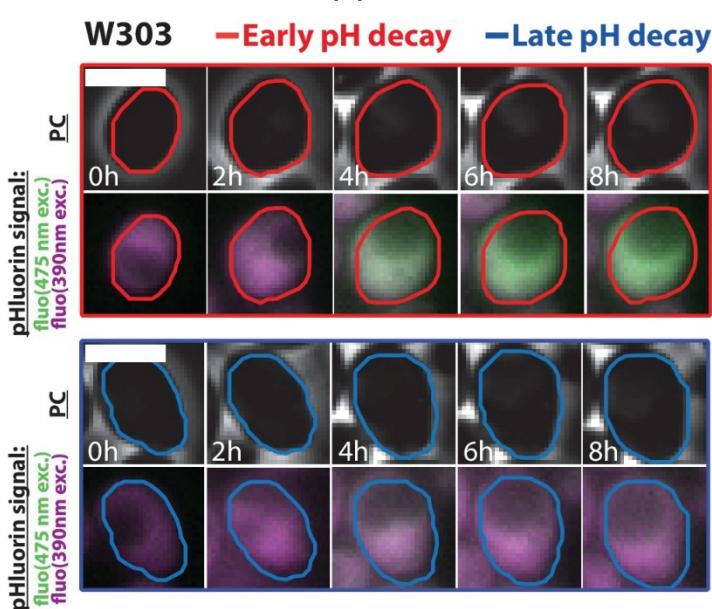


**Figure 1 – Supplement 2: Population measurements of cell growth during an entire life cycle.**

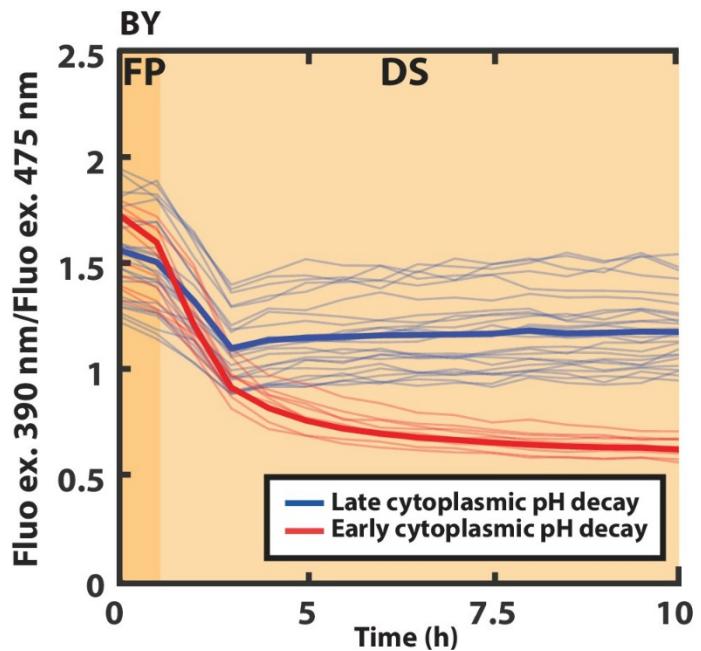
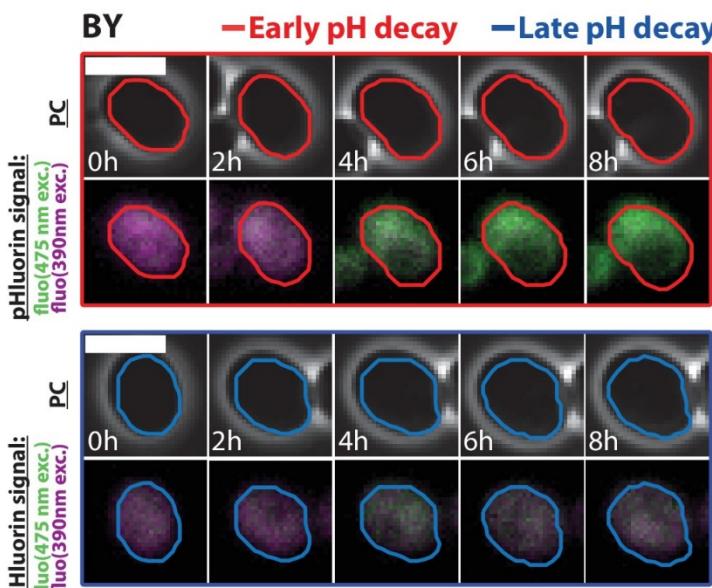
Growth curves comparison between prototrophs FY, S288c, and BY (A) and auxotrophs W303 and BY Ilv3-tdimer expressing strains (B). Haploid FY Rho (red curve) is unable to respire and stop proliferating after carbon fermentation. Automated turbidity measurement was done using the Bioscreen C device (Labsystems). Strains were maintained 24 hr in exponential phase in rich medium supplemented with Adenine (YPDA) at 30°C, then inoculated into the microplates at OD600m = 0.01, 150 µl YPDA, 30 °C. Yeasts were grown at continuous agitation, with absorbances reading every 10 min during 3 days.

Figure 1 – Supplement 3

A



B



Single-cell dynamics of entry into stationary phase in BY versus W303 strains.

(A) Left: typical sequence of phase-contrast and fluorescence images for W303 cells that display (respectively, do not display) an early cytosolic pH decay upon the diauxic shift (DS; red and blue cell contours, respectively). The fluorescence image displays an overlay of fluorescence images obtained using indicated excitation wavelengths. Right: quantification of the raw (i.e., uncalibrated) ratio of the fluorescence level obtained using indicated excitation wavelength. The colored shading indicates the fermentation phase (FP) and the DS.  $N > 30$  cells. (B) Same as A, but using the BY strain as a control.  $N > 30$  cells.

## Chapter II : Results

---

### Figure 1 – Video 1: (please see

<https://elifesciences.org/articles/73186/figures#fig1video1>)

Top: phase-contrast video of a microcolony growing in the observation device during the life cycle of the culture.

Bottom: fold increase in the number of cells during colony proliferation. The colored progress bar indicates the metabolic phase of the culture as defined in Figure 1. Scale bar: 6.3  $\mu$ m.

### Figure 1 – Video 2: (please see

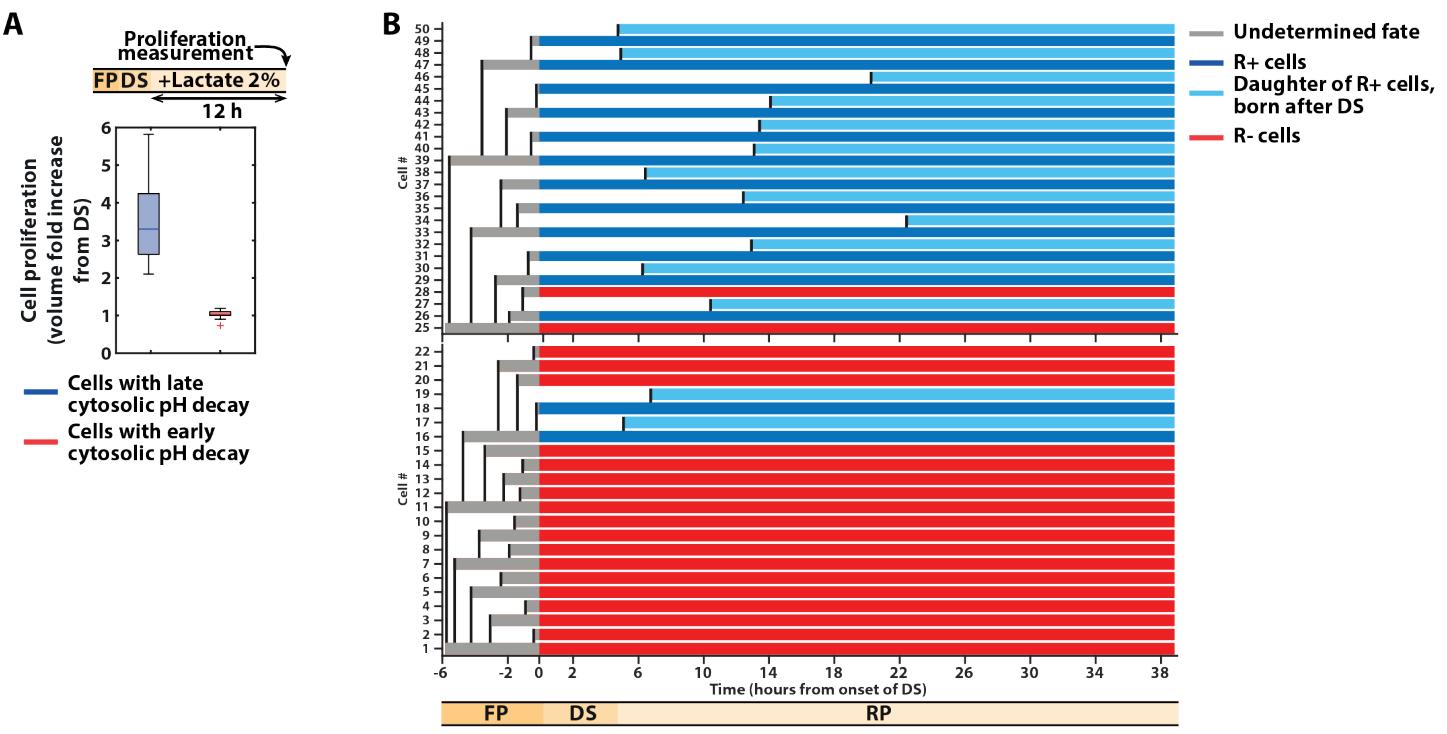
<https://elifesciences.org/articles/73186/figures#fig1video2>)

Top: phase-contrast (left) and pHluorin (right, channels 1 and 2 merged as in Figure 1) video of a microcolony growing in the observation device during the life cycle of the culture.

The colored progress bar indicates the metabolic phase of the culture as defined in Figure 1.

The red and blue contours indicate R– and R+ cells, respectively. Scale bar: 10.8  $\mu$ m. Bottom: quantification of cytosolic pH over time, as described in Figure 1.

**Figure 2 – Supplement 1:**



**Complementary analyses of the divergent cell fate at the diauxic shift.**

(A) Proliferation capacity in a respiratory media. Box plots indicate the fold increase in cell area from the diauxic shift (DS) in each subpopulation (blue and red lines for cells with a late or early cytoplasmic pH decay, respectively), after a 12 hr exposure to a 2 % lactate respiratory media after the DS ( $N = 30$  for each box plot). (B) Inheritance of the R+ and R- phenotypes. Sample pedigrees representing two single cells (#1 and #25) and their progeny; each horizontal bar represents a cell. Cells reveal their respiratory status at the DS, hence are displayed in gray before the DS, and red (for R-) or blue (R+) after the DS. Daughters born after DS are represented in light blue. Only the first daughter of mothers born before the DS are represented for clarity.

**Figure 2 – Video 1: (please see**

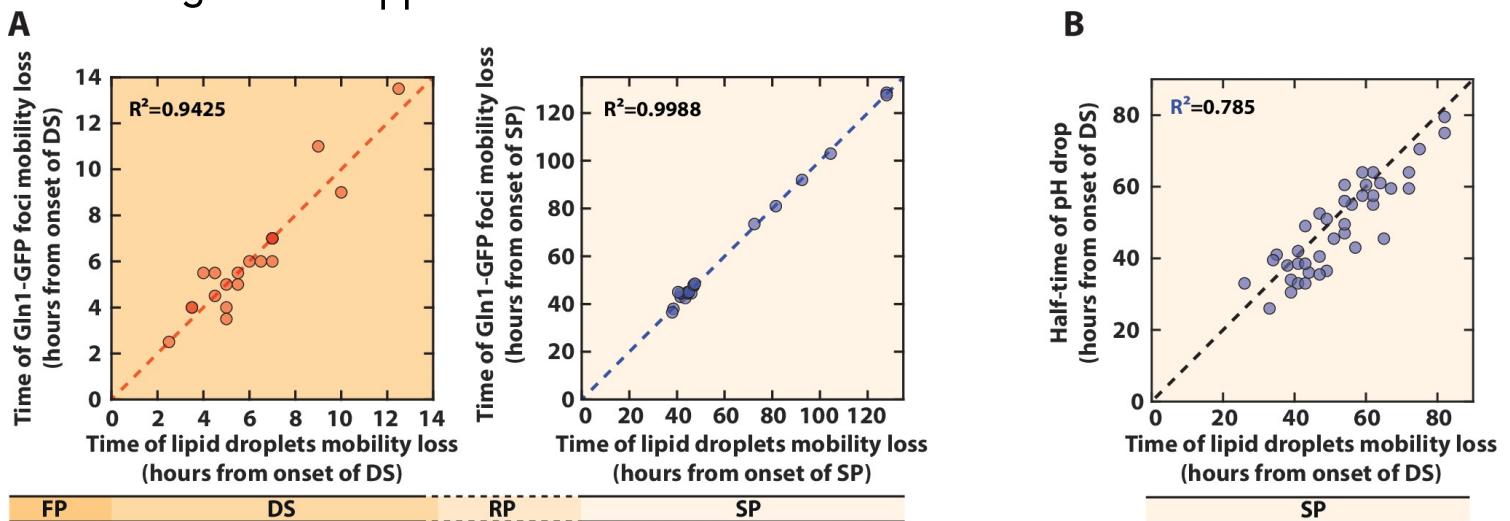
<https://elifesciences.org/articles/73186/figures#fig2video1>

Phase-contrast (left) and *Ilv3-mCherry* (right) video of a microcolony growing in the observation device during the life cycle of the culture.

The colored progress bar indicates the metabolic phase of the culture as defined in [Figure 1](#).

The thick red and blue contours indicate original R- and R+ cells, respectively, while the thin red and blue contours indicate the daughters of the original cells. Scale bar: 6.3  $\mu$ m.

**Figure 2 – Supplement 1:**



#### Coincidence of lipid droplets and Gln1-GFP foci mobility.

Correlation of the time of mobility loss of Gln1-GFP foci with that of the lipid droplet (LD) in R- (left plot) or R+ (right plot) cells. The origin of time is set as the proliferation arrest for each type of cells, that is the onset of diauxic shift (DS) and the onset of stationary phase (SP), respectively. The dashed lines represent the diagonal. (B) Correlation of the half-time of the pH drop with the time of LD mobility loss in R+ cells. The time origin the onset of SP. The dashed lines represent the diagonal.

**Figure 3 – Video 1:** (please see

<https://elifesciences.org/articles/73186/figures#fig3video1>

Phase-contrast (left) and Gln1-GFP (right) video of a microcolony growing in the observation device from fermentation phase to respiration phase.

The colored progress bar indicates the metabolic phase of the culture as defined in [Figure 1](#).

The red and blue contours indicate R- and R+ cells, respectively. Scale bar: 6.3  $\mu$ m

## Discussion

In addition to the paper's discussion:

Our methodology allowed us to follow single-cell throughout the successive phase and capture their entry into quiescence. With that, we were able to answer the following question:

What is the source of this senescent population? The inability to activate the respiratory metabolism **at the diauxic shift**, that is why we call them R- cells.

Do these cells enter some kind of quiescence before losing viability? They have been described as senescent or non-quiescent because of their short viability and because they lose quiescence markers after their death. Our data show that these cells display all the markers seen in *bona fide* quiescent cells, and that the R- cells can re-enter the cell cycle during a few days after proliferation arrest. Therefore, if these cells are unable to sustain quiescence, they can enter it. Hence, in the strict sense of the term, they are quiescent cells. The fact that they die rapidly is (at least partly) linked to their inability to form trehalose and glycogen storages [52] [36], two well-known byproducts of respiration. The R- cells are probably close to cells abruptly switched from a glucose media to glucose depleted media (See "SP" on Figure 79, p.186).

Yet, if trehalose supplementation during fermentation can restore longevity in non-respirative cells [36], it cannot do so after an abrupt starvation [53]. This suggests that other processes linked to the dynamics of the media (like a prolonged exposure to ethanol during fermentation) are necessary to achieve a prolonged quiescence.

Can we predict which cells will become R- before quiescence by looking at their history? A part is predictable and due to the loss of mtDNA ( $\varrho 0$  cells), but unexpectedly, it does not represent the majority of the R- cells. In line with that, we performed the experiment in another background known to have very few  $\varrho 0$  cells, and also observed R- cells (Figure 1 – Suppl 3). Characterizing these cells in detail and understanding why they experience this metabolic crash is made possible with this new methodology and is an interesting perspective.

## Chapter II : Discussion

---

Do we observe gelification of the cytosol like in abrupt energy depletion experiments? Yes, to our knowledge, this is the first evidence that the cytosol of cells undergoes a gel-like transition during quiescence in physiological conditions. Even if this was expected since it is the case after abrupt transitions [24,25], these experiments require drugs to completely abolish the glycolysis, and the phase transition is not observed with a simple glucose depletion.

Yet, if our data about the phase transition are weak (based on the decreased mobility of particles), they are strongly supported by in-vitro experiments that can reproduce protein phase transition by decreasing the pH of the solution [30,54–56]. Besides, *S. pombe* cells submitted to abrupt starvation with drugs remain cylindrical after cell wall removal, suggesting a gel-like state of the cytosol [25].

Interestingly, we observe this gelification occurs several days after proliferation arrest, while abruptly arrested cells only take a few minutes to display it. The mechanism behind that is also likely to explain the vast heterogeneity regarding the time of freezing in R+ cells during quiescence.

Overall, this method opens new opportunities to track single-cell and link their history with their phenotype, not only in the field of quiescence but also collective oscillatory behaviors, cellular cooperation, or antibiotics collective response.

## References Chapter II

- [1] J.V. Gray, G.A. Petsko, G.C. Johnston, D. Ringe, R.A. Singer, M. Werner-Washburne, "Sleeping Beauty": Quiescence in *Saccharomyces cerevisiae*, *Microbiol Mol Biol R.* 68 (2004) 187–206. <https://doi.org/10.1128/mmbr.68.2.187-206.2004>.
- [2] I. Sagot, D. Laporte, The cell biology of quiescent yeast – a diversity of individual scenarios, *J Cell Sci.* 132 (2019) jcs213025. <https://doi.org/10.1242/jcs.213025>.
- [3] A.M. Neiman, Sporulation in the Budding Yeast *Saccharomyces cerevisiae*, *Genetics.* 189 (2011) 737–765. <https://doi.org/10.1534/genetics.111.127126>.
- [4] N. Urbán, I.M. Blomfield, F. Guillemot, Quiescence of Adult Mammalian Neural Stem Cells: A Highly Regulated Rest, *Neuron.* 104 (2019) 834–848. <https://doi.org/10.1016/j.neuron.2019.09.026>.
- [5] G. Yao, Modelling mammalian cellular quiescence, *Interface Focus.* 4 (2014) 20130074. <https://doi.org/10.1098/rsfs.2013.0074>.
- [6] W. Chen, J. Dong, J. Haiech, M.-C. Kilhoffer, M. Zeniou, Cancer Stem Cell Quiescence and Plasticity as Major Challenges in Cancer Therapy, *Stem Cells Int.* 2016 (2016) 1740936. <https://doi.org/10.1155/2016/1740936>.
- [7] C.D. Virgilio, The essence of yeast quiescence, *Fems Microbiol Rev.* 36 (2012) 306–339. <https://doi.org/10.1111/j.1574-6976.2011.00287.x>.
- [8] J.R. Pringle, L.H. Hartwell, The *Saccharomyces cerevisiae* cell cycle, *Mol. Biol.* (1981) 97–142.
- [9] S. Miles, L. Li, J. Davison, L.L. Breeden, Xbp1 Directs Global Repression of Budding Yeast Transcription during the Transition to Quiescence and Is Important for the Longevity and Reversibility of the Quiescent State, *Plos Genet.* 9 (2013) e1003854. <https://doi.org/10.1371/journal.pgen.1003854>.
- [10] M. Moreno-Torres, M. Jaquenoud, C.D. Virgilio, TORC1 controls G1–S cell cycle transition in yeast via Mpkl and the greatwall kinase pathway, *Nat Commun.* 6 (2015) 8256. <https://doi.org/10.1038/ncomms9256>.
- [11] D. Laporte, A. Lebaudy, A. Sahin, B. Pinson, J. Ceschin, B. Daignan-Fornier, I. Sagot, Metabolic status rather than cell cycle signals control quiescence entry and exit, *J Cell Biology.* 192 (2011) 949–957. <https://doi.org/10.1083/jcb.201009028>.
- [12] E. Boy-Marcotte, M. Perrot, F. Bussereau, H. Boucherie, M. Jacquet, Msn2p and Msn4p Control a Large Number of Genes Induced at the Diauxic Transition Which Are Repressed by Cyclic AMP in *Saccharomyces cerevisiae*, *J Bacteriol.* 180 (1998) 1044–1052. <https://doi.org/10.1128/jb.180.5.1044-1052.1998>.
- [13] O. Kandror, N. Bretschneider, E. Kreydin, D. Cavalieri, A.L. Goldberg, Yeast Adapt to Near-Freezing Temperatures by STRE/Msn2,4-Dependent Induction of Trehalose Synthesis and Certain Molecular Chaperones, *Mol Cell.* 13 (2004) 771–781. [https://doi.org/10.1016/s1097-2765\(04\)00148-0](https://doi.org/10.1016/s1097-2765(04)00148-0).
- [14] V. Pfanzagl, W. Görner, M. Radolf, A. Parich, R. Schuhmacher, J. Strauss, W. Reiter, C. Schüller, A constitutive active allele of the transcription factor Msn2 mimicking low PKA activity dictates metabolic remodeling in yeast, *Mol Biol Cell.* 29 (2018) 2848–2862. <https://doi.org/10.1091/mbc.e18-06-0389>.

## Chapter II : References

---

- [15] D.A. Fletcher, R.D. Mullins, Cell mechanics and the cytoskeleton, *Nature*. 463 (2010) 485–492. <https://doi.org/10.1038/nature08908>.
- [16] I. Sagot, B. Pinson, B. Salin, B. Daignan-Fornier, Actin Bodies in Yeast Quiescent Cells: An Immediately Available Actin Reserve?, *Mol Biol Cell*. 17 (2006) 4645–4655. <https://doi.org/10.1091/mbc.e06-04-0282>.
- [17] D. Laporte, F. Courtout, B. Salin, J. Ceschin, I. Sagot, An array of nuclear microtubules reorganizes the budding yeast nucleus during quiescence, *J Cell Biol*. 203 (2013) 585–594. <https://doi.org/10.1083/jcb.201306075>.
- [18] C.A. Weber, K. Sekar, J.H. Tang, P. Warmer, U. Sauer, K. Weis,  $\beta$ -Oxidation and autophagy are critical energy providers during acute glucose depletion in *Saccharomyces cerevisiae*, *Proc National Acad Sci*. 117 (2020) 12239–12248. <https://doi.org/10.1073/pnas.1913370117>.
- [19] D. Laporte, L. Gouleme, L. Jimenez, I. Khemiri, I. Sagot, Mitochondria reorganization upon proliferation arrest predicts individual yeast cell fate, *Elife*. 7 (2018) e35685. <https://doi.org/10.7554/elife.35685>.
- [20] K. Tanaka, The proteasome: Overview of structure and functions, *Proc Jpn Acad Ser B*. 85 (2009) 12–36. <https://doi.org/10.2183/pjab.85.12>.
- [21] D. Laporte, B. Salin, B. Daignan-Fornier, I. Sagot, Reversible cytoplasmic localization of the proteasome in quiescent yeast cells, *J Cell Biology*. 181 (2008) 737–745. <https://doi.org/10.1083/jcb.200711154>.
- [22] R.S. Marshall, R.D. Vierstra, Proteasome storage granules protect proteasomes from autophagic degradation upon carbon starvation, *Elife*. 7 (2018) e34532. <https://doi.org/10.7554/elife.34532>.
- [23] R.P. Joyner, J.H. Tang, J. Helenius, E. Dultz, C. Brune, L.J. Holt, S. Huet, D.J. Müller, K. Weis, A glucose-starvation response regulates the diffusion of macromolecules, *Elife*. 5 (2016) e09376. <https://doi.org/10.7554/elife.09376>.
- [24] M.C. Munder, D. Midtvedt, T. Franzmann, E. Nüske, O. Otto, M. Herbig, E. Ulbricht, P. Müller, A. Taubenberger, S. Maharana, L. Malinovska, D. Richter, J. Guck, V. Zaburdaev, S. Alberti, A pH-driven transition of the cytoplasm from a fluid- to a solid-like state promotes entry into dormancy, *Elife*. 5 (2016) e09347. <https://doi.org/10.7554/elife.09347>.
- [25] M.B. Heimlicher, M. Bächler, M. Liu, C. Ibeneche-Nnewihe, E.-L. Florin, A. Hoenger, D. Brunner, Reversible solidification of fission yeast cytoplasm after prolonged nutrient starvation, *J Cell Sci*. 132 (2019) jcs231688. <https://doi.org/10.1242/jcs.231688>.
- [26] B.R. Parry, I.V. Surovtsev, M.T. Cabeen, C.S. O'Hern, E.R. Dufresne, C. Jacobs-Wagner, The Bacterial Cytoplasm Has Glass-like Properties and Is Fluidized by Metabolic Activity, *Cell*. 156 (2014) 183–194. <https://doi.org/10.1016/j.cell.2013.11.028>.
- [27] R. Orij, S. Brul, G.J. Smits, Intracellular pH is a tightly controlled signal in yeast, *Biochimica Et Biophysica Acta Bba - Gen Subj*. 1810 (2011) 933–944. <https://doi.org/10.1016/j.bbagen.2011.03.011>.
- [28] I. Petrovska, E. Nüske, M.C. Munder, G. Kulasegaran, L. Malinovska, S. Kroschwald, D. Richter, K. Fahmy, K. Gibson, J.-M. Verbavatz, S. Alberti, Filament formation by metabolic enzymes is a specific adaptation to an advanced state of cellular starvation, *Elife*. 3 (2014) e02409. <https://doi.org/10.7554/elife.02409>.

## Chapter II : References

---

- [29] L.Z. Peters, R. Hazan, M. Breker, M. Schuldiner, S. Ben-Aroya, Formation and dissociation of proteasome storage granules are regulated by cytosolic pHRegulation of PSGs by cytosolic pH, *J Cell Biology*. 201 (2013) 663–671. <https://doi.org/10.1083/jcb.201211146>.
- [30] J.A. Riback, C.D. Katanski, J.L. Kear-Scott, E.V. Pilipenko, A.E. Rojek, T.R. Sosnick, D.A. Drummond, Stress-Triggered Phase Separation Is an Adaptive, Evolutionarily Tuned Response, *Cell*. 168 (2017) 1028-1040.e19. <https://doi.org/10.1016/j.cell.2017.02.027>.
- [31] V. Ramachandran, K.H. Shah, P.K. Herman, The cAMP-Dependent Protein Kinase Signaling Pathway Is a Key Regulator of P Body Foci Formation, *Mol Cell*. 43 (2011) 973–981. <https://doi.org/10.1016/j.molcel.2011.06.032>.
- [32] D. Mateju, T.M. Franzmann, A. Patel, A. Kopach, E.E. Boczek, S. Maharana, H.O. Lee, S. Carra, A.A. Hyman, S. Alberti, An aberrant phase transition of stress granules triggered by misfolded protein and prevented by chaperone function, *Embo J.* 36 (2017) 1669–1687. <https://doi.org/10.15252/embj.201695957>.
- [33] F. Monje-Casas, C. Michan, C. Pueyo, Absolute transcript levels of thioredoxin- and glutathione-dependent redox systems in *Saccharomyces cerevisiae*: response to stress and modulation with growth, *Biochem J.* 383 (2004) 139–147. <https://doi.org/10.1042/bj20040851>.
- [34] C. Allen, S. Büttner, A.D. Aragon, J.A. Thomas, O. Meirelles, J.E. Jaetao, D. Benn, S.W. Ruby, M. Veenhuis, F. Madeo, M. Werner-Washburne, Isolation of quiescent and nonquiescent cells from yeast stationary-phase cultures, *J Cell Biology*. 174 (2006) 89–100. <https://doi.org/10.1083/jcb.200604072>.
- [35] D. Laporte, L. Jimenez, L. Gouleme, I. Sagot, Yeast quiescence exit swiftness is influenced by cell volume and chronological age, *Microb Cell*. 5 (2017) 104. <https://doi.org/10.15698/mic2018.02.615>.
- [36] A. Ocampo, J. Liu, E.A. Schroeder, G.S. Shadel, A. Barrientos, Mitochondrial Respiratory Thresholds Regulate Yeast Chronological Life Span and its Extension by Caloric Restriction, *Cell Metab*. 16 (2012) 55–67. <https://doi.org/10.1016/j.cmet.2012.05.013>.
- [37] L.E. Bagamery, Q.A. Justman, E.C. Garner, A.W. Murray, A Putative Bet-Hedging Strategy Buffers Budding Yeast against Environmental Instability, *Curr Biol*. 30 (2020) 4563-4578.e4. <https://doi.org/10.1016/j.cub.2020.08.092>.
- [38] B. Cerulus, A. Jariani, G. Perez-Samper, L. Vermeersch, J.M. Pietsch, M.M. Crane, A.M. New, B. Gallone, M. Roncoroni, M.C. Dzialo, S.K. Govers, J.O. Hendrickx, E. Galle, M. Coomans, P. Berden, S. Verbandt, P.S. Swain, K.J. Verstrepen, Transition between fermentation and respiration determines history-dependent behavior in fluctuating carbon sources, *Elife*. 7 (2018) e39234. <https://doi.org/10.7554/elife.39234>.
- [39] A. Mitchell, G.H. Romano, B. Groisman, A. Yona, E. Dekel, M. Kupiec, O. Dahan, Y. Pilpel, Adaptive prediction of environmental changes by microorganisms, *Nature*. 460 (2009) 220–224. <https://doi.org/10.1038/nature08112>.
- [40] M.M. Klosinska, C.A. Crutchfield, P.H. Bradley, J.D. Rabinowitz, J.R. Broach, Yeast cells can access distinct quiescent states, *Gene Dev*. 25 (2011) 336–349. <https://doi.org/10.1101/gad.2011311>.
- [41] L. Li, S. Miles, Z. Melville, A. Prasad, G. Bradley, L.L. Breeden, Key events during the transition from rapid growth to quiescence in budding yeast require posttranscriptional regulators, *Mol Biol Cell*. 24 (2013) 3697–3709. <https://doi.org/10.1091/mbc.e13-05-0241>.

## Chapter II : References

---

- [42] D.D. Carlo, Inertial microfluidics, *Lab Chip.* 9 (2009) 3038–3046. <https://doi.org/10.1039/b912547g>.
- [43] D.D. Carlo, D. Irimia, R.G. Tompkins, M. Toner, Continuous inertial focusing, ordering, and separation of particles in microchannels, *Proc National Acad Sci.* 104 (2007) 18892–18897. <https://doi.org/10.1073/pnas.0704958104>.
- [44] D.R. Gossett, D.D. Carlo, Particle Focusing Mechanisms in Curving Confined Flows, *Anal Chem.* 81 (2009) 8459–8465. <https://doi.org/10.1021/ac901306y>.
- [45] A.A.S. Bhagat, S.S. Kuntaegowdanahalli, I. Papautsky, Enhanced particle filtration in straight microchannels using shear-modulated inertial migration, *Phys Fluids.* 20 (2008) 101702. <https://doi.org/10.1063/1.2998844>.
- [46] S.S. Kuntaegowdanahalli, A.A.S. Bhagat, G. Kumar, I. Papautsky, Inertial microfluidics for continuous particle separation in spiral microchannels, *Lab Chip.* 9 (2009) 2973–2980. <https://doi.org/10.1039/b908271a>.
- [47] A.A.S. Bhagat, S.S. Kuntaegowdanahalli, I. Papautsky, Continuous particle separation in spiral microchannels using dean flows and differential migration, *Lab Chip.* 8 (2008) 1906–1914. <https://doi.org/10.1039/b807107a>.
- [48] M. Masaeli, E. Sollier, H. Amini, W. Mao, K. Camacho, N. Doshi, S. Mitragotri, A. Alexeev, D.D. Carlo, Continuous Inertial Focusing and Separation of Particles by Shape, *Phys Rev X.* 2 (2012) 031017. <https://doi.org/10.1103/physrevx.2.031017>.
- [49] E. Guzniczak, O. Otto, G. Whyte, T. Chandra, N.A. Robertson, N. Willoughby, M. Jimenez, H. Bridle, Purifying stem cell-derived red blood cells: a high-throughput label-free downstream processing strategy based on microfluidic spiral inertial separation and membrane filtration, *Biotechnol Bioeng.* 117 (2020) 2032–2045. <https://doi.org/10.1002/bit.27319>.
- [50] A.J. Mach, D.D. Carlo, Continuous scalable blood filtration device using inertial microfluidics, *Biotechnol Bioeng.* 107 (2010) 302–311. <https://doi.org/10.1002/bit.22833>.
- [51] H. Amini, W. Lee, D.D. Carlo, Inertial microfluidic physics, *Lab Chip.* 14 (2014) 2739–2761. <https://doi.org/10.1039/c4lc00128a>.
- [52] L. Shi, B.M. Sutter, X. Ye, B.P. Tu, Trehalose Is a Key Determinant of the Quiescent Metabolic State That Fuels Cell Cycle Progression upon Return to Growth, *Mol Biol Cell.* 21 (2010) 1982–1990. <https://doi.org/10.1091/mbc.e10-01-0056>.
- [53] S. Miles, G.T. Bradley, L.L. Breeden, The budding yeast transition to quiescence, *Yeast.* 38 (2021) 30–38. <https://doi.org/10.1002/yea.3546>.
- [54] S. Alberti, A. Gladfelter, T. Mittag, Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates, *Cell.* 176 (2019) 419–434. <https://doi.org/10.1016/j.cell.2018.12.035>.
- [55] T.M. Franzmann, M. Jahnel, A. Pozniakovskiy, J. Mahamid, A.S. Holehouse, E. Nüske, D. Richter, W. Baumeister, S.W. Grill, R.V. Pappu, A.A. Hyman, S. Alberti, Phase separation of a yeast prion protein promotes cellular fitness, *Science.* 359 (2018) eaao5654. <https://doi.org/10.1126/science.aao5654>.
- [56] O. Adame-Arana, C.A. Weber, V. Zaburdaev, J. Prost, F. Jülicher, Liquid Phase Separation Controlled by pH, *Biophys J.* 119 (2020) 1590–1605. <https://doi.org/10.1016/j.bpj.2020.07.044>.

## Appendices

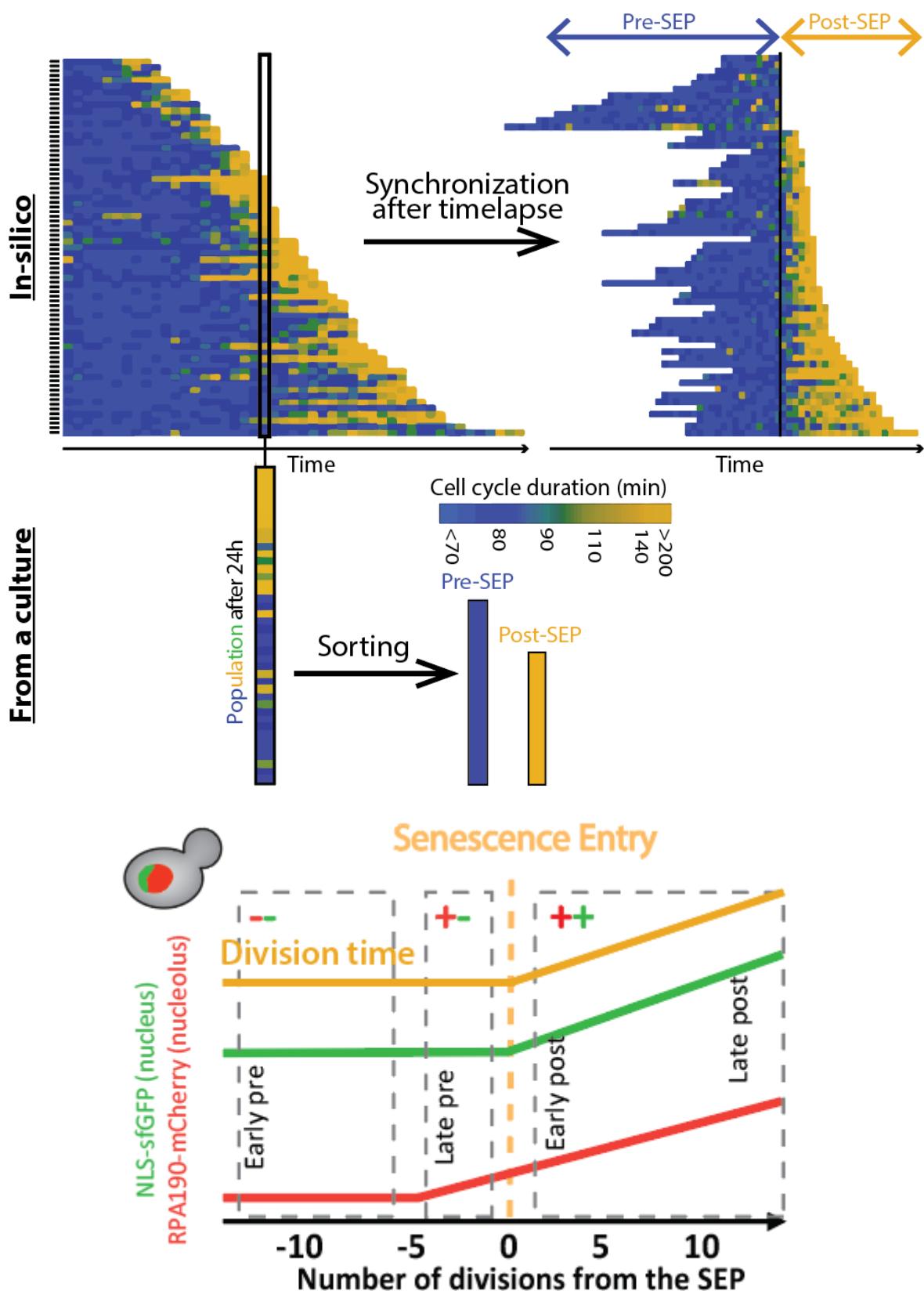
### Appendix 1: Microfluidic enrichment of old cells and sorting according to their senescent state

Single-cell timelapse approaches are powerful but limited to fluorescent reporters and can hardly probe complex signals such as chromatin state, protein-DNA interactions. Besides, they are limited to a candidate approach and do not allow genome/proteome-wide analysis.

On the other hand, when working with standard enrichment methods, the fact that cells do not age in the same way or at the same pace is not considered. Therefore, since the hallmarks of aging appear at different times from one cell to another, assaying a desynchronized population opacifies the dynamics of the hallmarks themselves as well as their in-between temporal (thus causal) links (discussed p.67) (Figure 44).. Among them, some might be triggering aging, while some *Page left blank on purpose* others might just be a byproduct (see main text).

Therefore, some kind of sorting must be done before performing population assays in order to reduce the impact of the averaging bias.

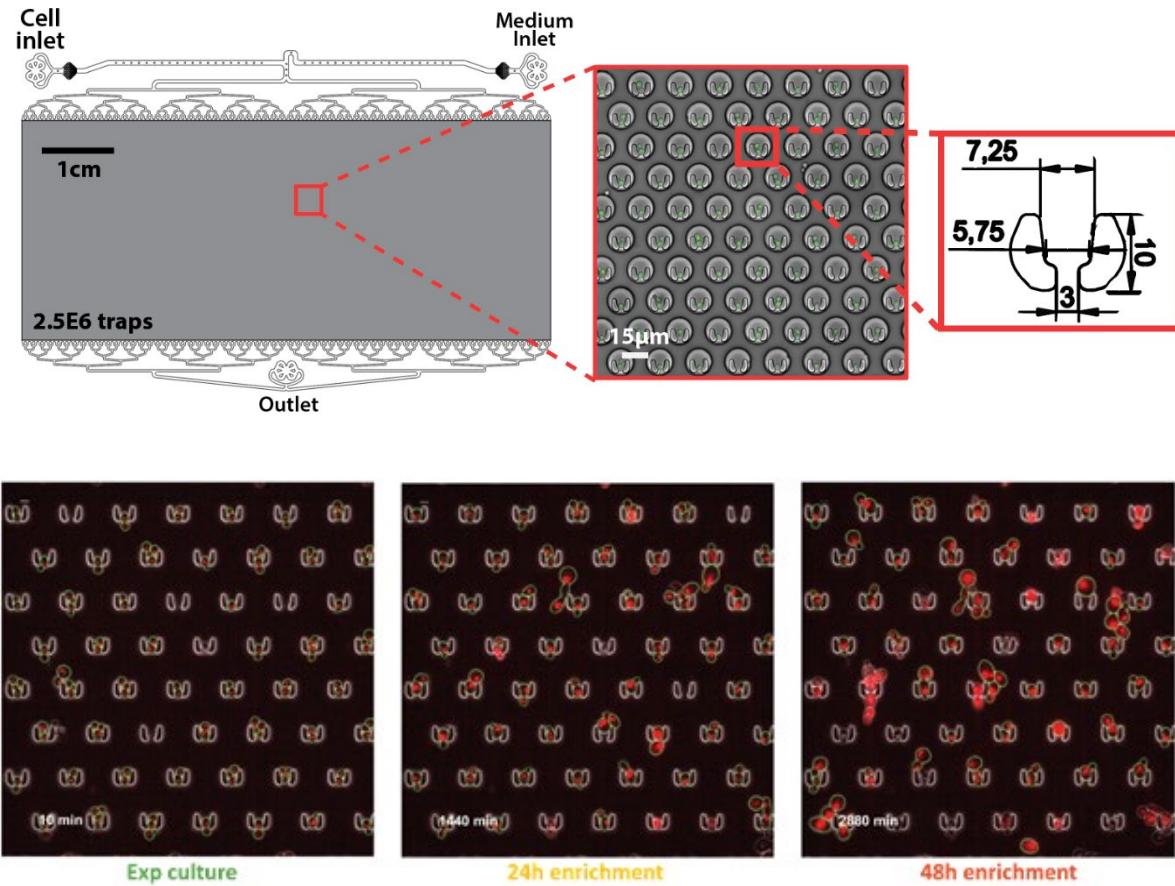
As presented in the main text (p.86), most cells follow a stereotyped path of aging in which they experience a cell-cycle slowdown (Senescence Entry Point, SEP). This slowdown strongly correlates with an increase in the karyoplasmic ratio and is preceded by an increase in nucleolus size. The SEP occurs at very different ages from one cell to another, but using these two markers could help sort the cells in pre-SEP and post-SEP categories (Appendix figure 1).



Appendix figure 1: (Top) Division duration of a population aligned to birth (left) and to the SEP (right) after a timelapse. (Middle): Harvesting a culture of old cells at a given time gives a heterogeneous population made of pre-SEP and post-SEP cells, that needs to be sorted. (Bottom): Sorting cells regarding their SEP state is possible without knowing their history, by using fluorescent markers.

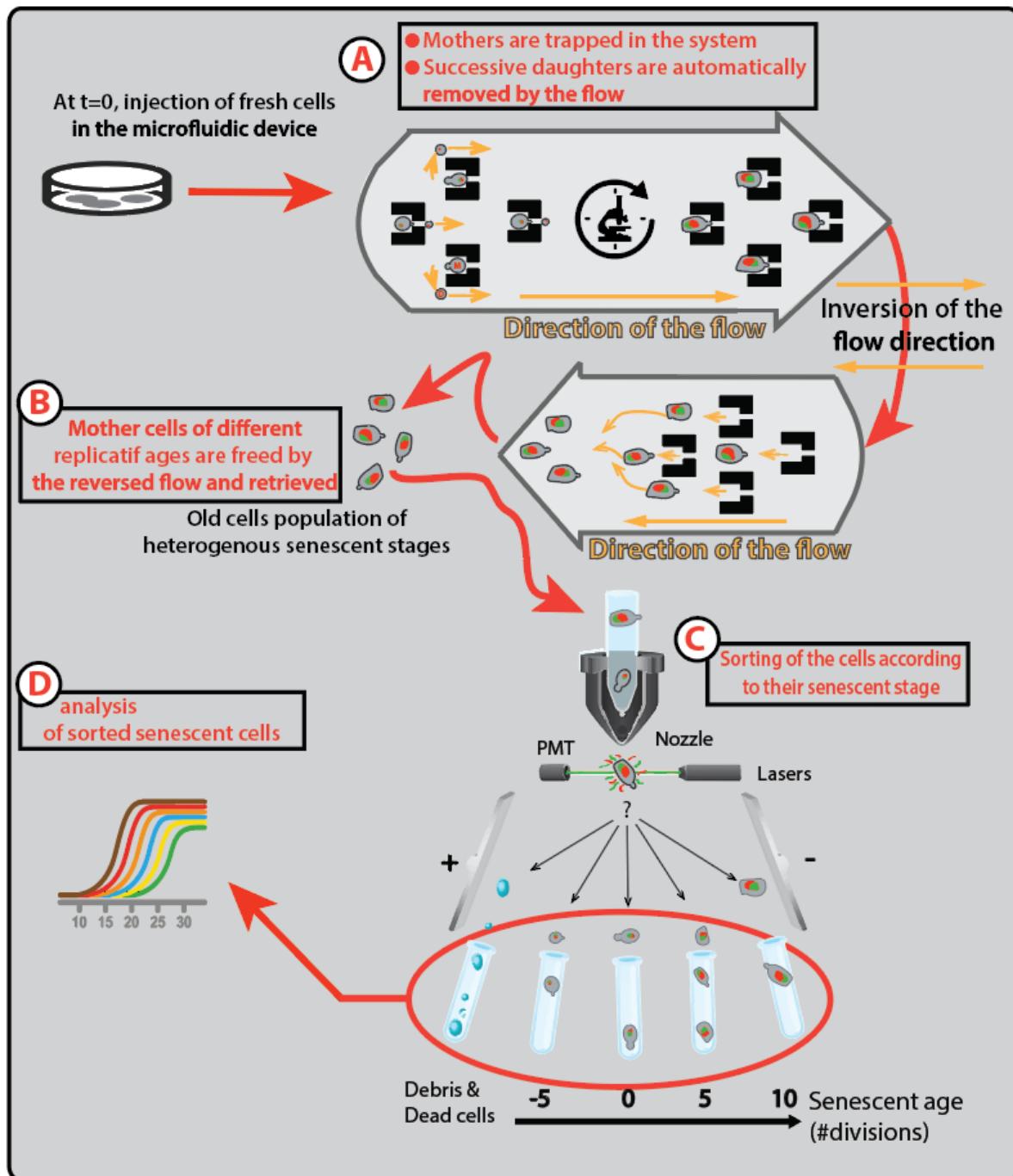
## Appendix 1

To enrich a population with old cells, I developed a microfluidic device based on microtraps similar to what is presented in the main text (p.89). Indeed, an array of 2 million U-shaped traps (improved from [3], [4]) can trap mother cells that are maintained against the structure by a constant flow of media. Hence, the progeny of the trapped mothers is automatically dissected and flushed away (Appendix figure 2).



Appendix figure 2: (Top): Schematics and image of the microfluidic device used for old mothers enrichment. (Bottom): Typical images (Phase contrast + mCherry) of the chip after injection (left), after 24h (center), or after 48h (right). Old cells are larger and their NLS-mCherry signal is stronger.

Importantly, the trapping is reversible and cells can be freed from the traps by applying a flow in the opposite direction, with an efficiency of  $97 \pm 0.5\%$  ( $N=3$  replicates of 500 cells, by counting the number of cells remaining of the traps after the retrieval). The fluorescence and size of these cells can then be measured by flow cytometry, and cells of interest can be sorted out based on the cytometric signals (Appendix figure 3).

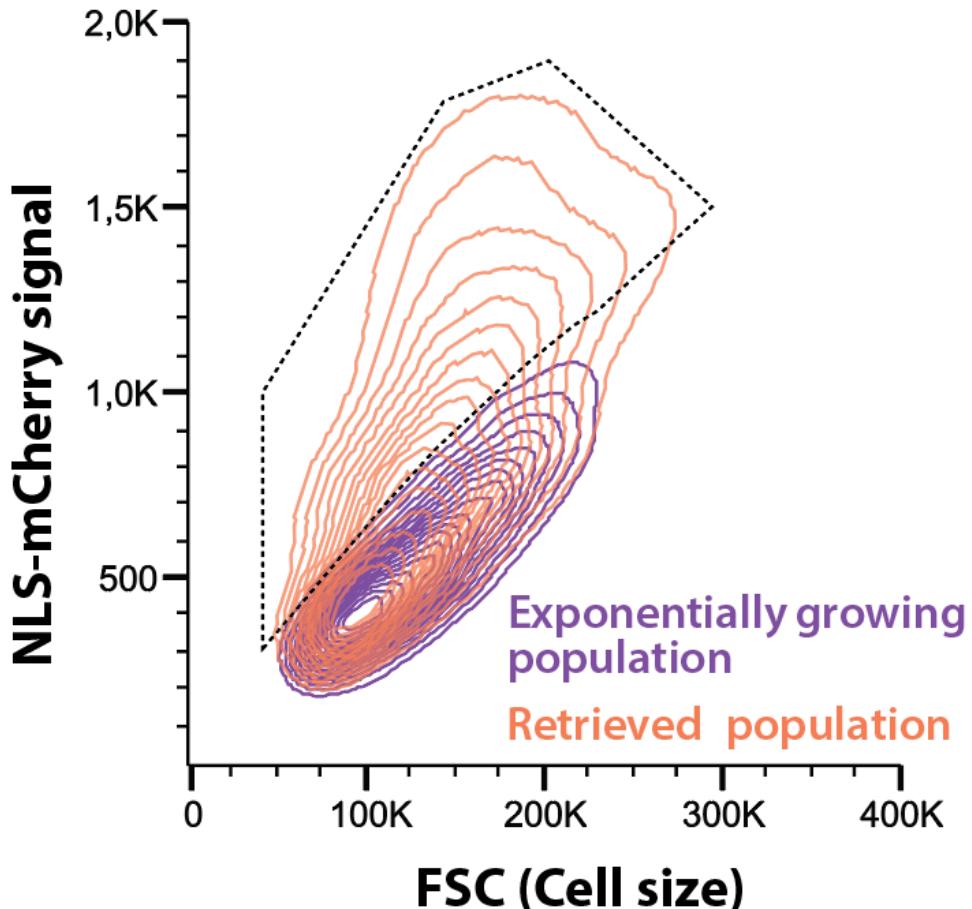


Appendix figure 3: Summary of the method to enrich a population with old individuals and sort them according to their senescence status. (A) Using a microfluidic device, one can trap and age mother cells inside specific structures that automatically remove the progeny. (B) Reverting the flow allows retrieval of the old cells. (C) FACS is used to discriminate cells according to their senescent stage. (D) Subsequent analysis can be performed on the different categories

Doing that after 24h enrichment with a strain harboring a nuclear marker (NLS-mCherry) shows that 35% of the 2 million retrieved cells have a fluorescent signal higher than the one from exponential culture (Appendix figure 4). However, we could still observe a population of small cells with a small nuclear signal, which is probably daughters born during the

## Appendix 1

harvesting. By selecting cells from different gatings relative to their size and fluorescence, we could obtain subpopulations with different post-SEP/pre-SEP ratios.



Appendix figure 4: NLS-mCherry signal against forward scatter signal from the cytometric profile of exponentially growing culture (purple) and of the retrieved population after 24h of enrichment (orange).

These preliminary results suggest that it is possible to enrich a population with old cells and sort them according to their senescence status. However, controls have to be done to validate the method. In particular, it is possible to reinject the sorted subpopulations in the microfluidic device in order to measure their cell cycle duration, size, and nucleus size, as well as their remaining replicative lifespan.

Therefore, to estimate the stringency of the sorting, measuring the age distribution of the different subpopulations (by counting the bud scars), but also the fraction of pre- and post-SEP cells will need to be done.

Why use microfluidics over already existing enrichment techniques? If the yield is two orders of magnitude lower than standard techniques, microfluidics allows the tracking of the cells

## Appendix 1

---

before harvesting them to correlate longitudinal signals with the subsequent readouts (such as transcriptomic profiles). Besides, cells can be cultured in changing environments (for instance, temporal patterns such as steps, ramps, or PWM of a given stress), which is difficult to do with the current methods.

As a perspective, the sorting could be refined using a nucleolar marker to differentiate between an “early pre-SEP” and a “late-pre-SEP” population (Appendix figure 1). Besides, the whole pipeline could be integrated into a single microfluidic device using droplet-based microfluidic sorting of single-cells [5–7].

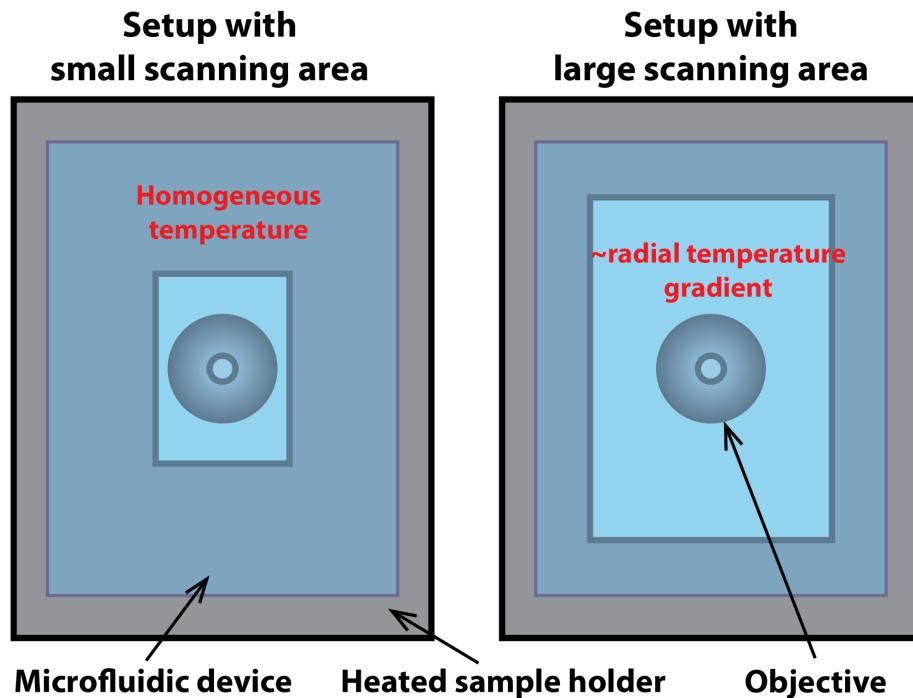
## References

- [1] S. Fehrmann, C. Paoletti, Y. Goulev, A. Ungureanu, H. Aguilaniu, and G. Charvin, “Aging Yeast Cells Undergo a Sharp Entry into Senescence Unrelated to the Loss of Mitochondrial Membrane Potential,” *Cell Reports*, vol. 5, no. 6, pp. 1589–1599, 2013, doi: 10.1016/j.celrep.2013.11.013.
- [2] S. Morlot, J. Song, I. Léger-Silvestre, A. Matifas, O. Gadal, and G. Charvin, “Excessive rDNA Transcription Drives the Disruption in Nuclear Homeostasis during Entry into Senescence in Budding Yeast,” *Cell Reports*, vol. 28, no. 2, pp. 408–422.e4, 2019, doi: 10.1016/j.celrep.2019.06.032.
- [3] M. M. Crane, I. B. N. Clark, E. Bakker, S. Smith, and P. S. Swain, “A Microfluidic System for Studying Ageing and Dynamic Single-Cell Responses in Budding Yeast,” *Plos One*, vol. 9, no. 6, p. e100042, 2014, doi: 10.1371/journal.pone.0100042.
- [4] M. C. Jo, W. Liu, L. Gu, W. Dang, and L. Qin, “High-throughput analysis of yeast replicative aging using a microfluidic system,” *Proc National Acad Sci*, vol. 112, no. 30, pp. 9364–9369, 2015, doi: 10.1073/pnas.1510328112.
- [5] J.-C. Baret *et al.*, “Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity,” *Lab Chip*, vol. 9, no. 13, pp. 1850–1858, 2009, doi: 10.1039/b902504a.
- [6] L. Mazutis, J. Gilbert, W. L. Ung, D. A. Weitz, A. D. Griffiths, and J. A. Heyman, “Single-cell analysis and sorting using droplet-based microfluidics,” *Nat Protoc*, vol. 8, no. 5, pp. 870–891, 2013, doi: 10.1038/nprot.2013.046.
- [7] A. Fallah-Araghi, J.-C. Baret, M. Ryckelynck, and A. D. Griffiths, “A completely in vitro ultrahigh-throughput droplet-based microfluidic screening system for protein engineering and directed evolution,” *Lab Chip*, vol. 12, no. 5, pp. 882–891, 2012, doi: 10.1039/c2lc21035e.

## Appendix 2: Versatile heating system for long-term microfluidic culture

Culturing cells during their replicative lifespan requires a constant temperature to avoid potential unwanted effects and ensure reproducibility. Besides, a stable temperature ensures focus stability during timelapses.

Traditional systems for temperature control in microfluidic are based on heating the plate holding the microfluidic device. This is efficient when the contact surface between the chip and the holder is high. However, for high-throughput applications, this surface has to be minimized in order to increase the area of the device that can be imaged. This leads to high-temperature heterogeneities (up to 3°K) between the device's center and edges (Appendix figure 5).



Appendix figure 5: Schematics representing the different contact area between the microfluidic device and the heated sample holder. A setup with a high contact area as a small scanning area (left) and vice-versa (right).

Other ways of controlling the temperature of a microfluidic device involve using heated fluids, which are very efficient and allow high-speed temperature switches (of the scale of the second) but require specific secondary channels for each device.

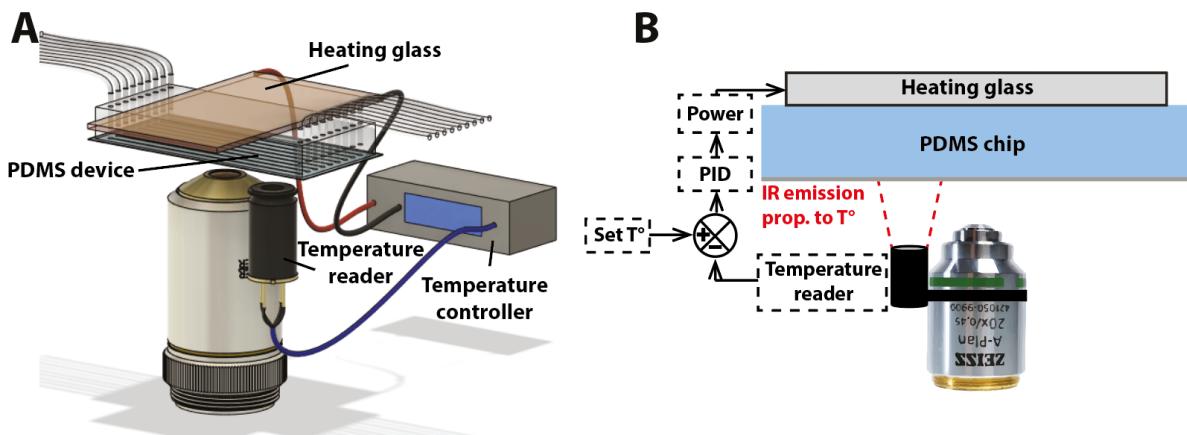
It is also possible to homogeneously control the temperature of the microfluidic device by putting the microscope in an incubator, but this solution requires a lot of space and a complex setup.

## Appendix 2

---

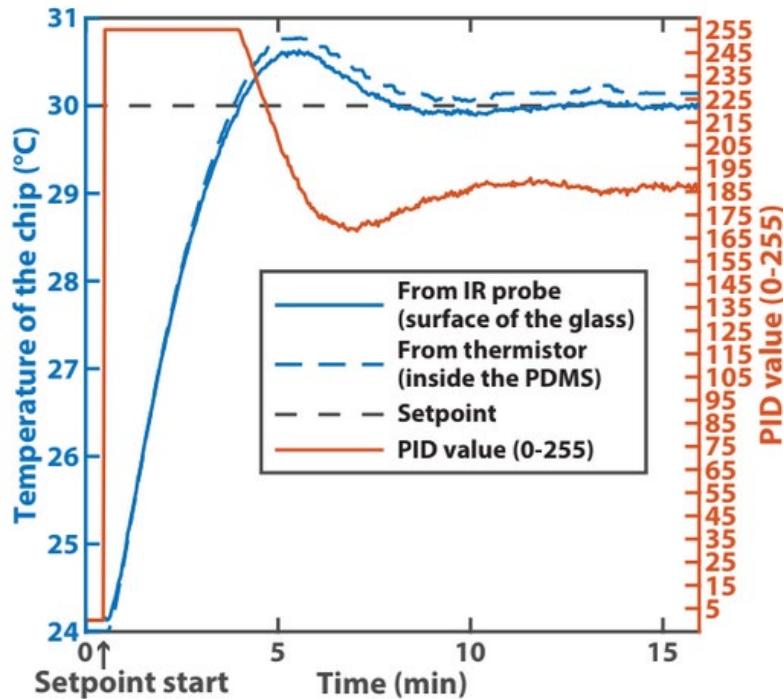
Therefore, no simple and versatile system can heat a microfluidic device on a large optical opening sample holder. Hence, I developed a portable heating system based on a heating glass in contact with the device from the top face.

With that, the heating is homogeneous and adaptable to any device. To control the temperature precisely, a probe based on the infrared emission measures the temperature of the glass slide on which the cells are. Interestingly, the PDMS and the glass slide are opaque to infrared light, so all the infrared incoming to the probe is emitted by the glass surface. Confirming that, a thermistor probe casted into the PDMS gives a very similar temperature to that of the infrared probe (Appendix figure 6). Finally, an Arduino chip with a custom program enslaves the temperature to the given setpoint using a Proportional Integral Derivative (PID) controller.



Appendix figure 6: Schematics of the heating system in 3D (A) and in 2D (B).

With this system, one can achieve stable temperature control, reaching a steady-state in 5min (Appendix figure 7) and with a maximal spatial temperature variation of 0.3°K (data not shown). Although it is not suitable to make fast temperature switches (for example, to apply a heatshock), it is applicable to any microfluidic device and costs less than 75€.



Appendix figure 7: Temperature of the chip (blue curve) measured with the infrared probe (solid line) or with a thermistor casted inside the chip (dotted line). The setpoint is represented with the black dotted line and the startpoint is indicated by the black arrow. The orange line indicates the value of the PID controller.

## **Appendix 3: (Collaboration) Specialization of chromatin-bound nuclear pore complexes promotes yeast aging**

This paper is the result of a collaboration with the Barral lab (ETH Zurich), to which I sent my microfluidic devices for them to perform aging experiments. The message of the paper is in line with the rest of the thesis since it describes how ERCs can be toxic to the cell. (See paper next page)

## Appendix 3

---

## Appendix 4: Distinct mechanisms underlie H<sub>2</sub>O<sub>2</sub> sensing in *C. elegans* head and tail

This paper is the result of an intra-lab collaboration with Sophie Quintin. Here, I developed a microfluidic device to trap *C. elegans* worms in order to observe their response to oxidative stress using timelapse microscopy.

(See paper next page)

## Appendix 4

---

## Appendix 5: (Collaboration) Self-Learning Microfluidic Platform for Single-Cell Imaging and Classification in Flow

This paper is the result of a collaboration with the Knop lab (Heidelberg). In this study, I participated in the development and fabrication of a microfluidic device for 3D focusing of cells, in order to reproduce a cytometer-like microfluidic device in which the analysis is performed by a machine-learning algorithm.

(See paper next page)

## Appendix 5

---

# Publications

[1]. DetecDiv, a deep-learning platform for automated cell division tracking and replicative lifespan analysis

Aspert Théo, Didier Hentsch, Charvin Gilles

bioRxiv, (2021) – DOI: 10.1101/2021.10.05.463175

[2]. Monitoring single-cell dynamics of entry into quiescence during an unperturbed lifecycle

Aspert Théo\*, Jacquel Basile\*, Laporte Damien, Sagot Isabelle, Charvin Gilles

\* Equally contributed to the work

eLife, 2021;10:e73186 - DOI: 10.7554/eLife.73186

[3]. Distinct mechanisms underlie H<sub>2</sub>O<sub>2</sub> sensing in *C. elegans* head and tail

Quintin Sophie, Aspert Théo, Charvin Gilles

bioRxiv, (2021) - DOI: 10.1101/2021.07.26.451501

[4]. Specialization of chromatin-bound nuclear pore complexes promotes yeast aging

Anne C. Meinema, Aspert Théo, Sung Sik Lee, Gilles Charvin, Yves Barral

bioRxiv, (2021) - DOI: 10.1101/2021.06.28.450139

[5]. Self-Learning Microfluidic Platform for Single-Cell Imaging and Classification in Flow

Constantinou I\*, Jendrusch M\*, Aspert Théo, Görlitz F, Schulze A, Charvin G, Knop M.

\* Equally contributed to the work

Micromachines, 10: 311, (2019) - DOI: 10.3390/mi10050311

# Déchiffrer les dynamiques du vieillissement à l'aide de la microfluidique et de l'apprentissage profond

## Abstract

*Aging* is a natural phenomenon defined by a loss of fitness and an increase in mortality rate. Behind this simple phenomenologic description lies a complex mechanism involving many very dynamical biological processes acting at different scales. *Saccharomyces cerevisiae* is one of the simplest model organism to study aging. Yet, despite the dozens of hallmarks of aging and longevity genes identified, no precise mechanism can describe why a cell dies after a certain number of divisions. This is partly due to a limited experimental power, with populational assays not considering the heterogeneities between cells, while the current single-cell longitudinal approaches have a too low throughput, hence preventing to grasp the complexity of the phenomenon experimentally.

Therefore, we developed a framework based on microfluidics, fast-microscopy, and deep-learning to track single-cells throughout their lifespan, with a throughput increase of two orders of magnitude compared to existing systems. With that, it is possible to track and analyse more than 30000 cells per experiment and automatically detect relevant events such as cell divisions. Thus, one can screen markers and mutants in a standardized and systematic manner in order to capture the links and temporalities of the different processes of aging.

As an application, we sought to measure the statistics of an event thought trigger an cascade of leading to death. Interestingly, we found that this event was stochastic, suggesting that aging can arise from age-independent causes.

**Keywords:** Aging, single-cell, longitudinal tracking, homeostasis, division, yeast, microfluidics, deep-learning, stochasticity

## Résumé

Le vieillissement est un phénomène naturel défini par une dégradation des fonctions et une augmentation de la probabilité de mourir. Derrière cette description phénoménologique simple se cache un mécanisme complexe impliquant de nombreux processus biologiques dynamiques agissant à différentes échelles. *Saccharomyces cerevisiae* est l'un des organismes modèles les plus simples pour étudier le vieillissement. Toutefois, malgré les dizaines de marqueurs de vieillissement et de gènes de longévité identifiés, aucun mécanisme précis ne permet de décrire pourquoi une cellule meurt après avoir effectué un certain nombre de divisions. Cela est dû en partie à une puissance expérimentale limitée, les essais populationnels ne prenant pas en compte les hétérogénéités entre les cellules, tandis que les approches longitudinales à l'échelle de la cellule unique ont un débit trop faible actuellement, rendant difficile la liaison temporelle et causale entre les processus moléculaires menant à la mort.

Par conséquent, nous avons développé un système basé sur la microfluidique, la microscopie rapide et l'apprentissage profond pour suivre des cellules uniques tout au long de leur vie, augmentant le débit de deux ordres de grandeur par rapport aux systèmes existants. Il est ainsi possible de suivre et analyser plus de 30000 cellules par expérience et de détecter automatiquement les événements pertinents tels que les divisions cellulaires. Ainsi, il est possible de cibler des marqueurs et des mutants de manière standardisée et systématique afin de saisir les liens et les temporalités des différents processus du vieillissement.

En guise d'application, nous avons cherché à mesurer les statistiques d'un événement connu pour déclencher une cascade d'événements conduisant à la mort. Nous avons constaté que cet événement avait la même probabilité quel que soit l'âge de la cellule, ce qui suggère que le vieillissement peut émerger de la stochasticité.

**Mots clefs:** Vieillissement, cellule unique, suivi longitudinal, homéostasie, division, levure, microfluidique, apprentissage profond, stochasticité