



Natural variations in yeast aging reveal genetic and environmental factors

Benjamin Barré

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THÈSE DE DOCTORAT

Natural variations in yeast aging reveal genetic and environmental factors

*Identification de facteurs génétiques et
environnementaux impliqués dans le vieillissement à
travers l'étude des variations naturelles de la levure*

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IRCAN – CNRS UMR 7284 – Inserm U1081

Population genomics and complex traits

Présentée en vue de l'obtention du grade de docteur en Sciences

De l'Université Côte d'Azur – UFR Sciences

Mention: Interactions moléculaires et cellulaires


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*“I know I do not make
everything right”*

- Emmanuel Macron



Résumé

Le vieillissement est un processus complexe déterminé par des facteurs génétiques et environnementaux qui varie d'un individu à l'autre. Bien que le vieillissement soit la cause principale de nombreuses maladies, nos connaissances sur le sujet sont relativement limitées. Tout au long de ce travail, j'ai utilisé la levure bourgeonnante *Saccharomyces cerevisiae* pour identifier les facteurs génétiques et environnementaux influant sur le vieillissement et pour comprendre les interactions qu'ils entretiennent entre eux. Jusqu'à présent, les approches classiques de génétique ont permis de découvrir un certain nombre de gènes impliqués dans la régulation du vieillissement chronologique de la levure (CLS), basé sur la longévité de celle-ci en conditions non-prolifératives. Or, ces approches se sont essentiellement centrées sur des souches de laboratoire et n'ont que très peu exploité les richesses de la biodiversité.

Dans une première partie, j'ai utilisé une large cohorte de levures composée de plus de 1000 souches naturelles de *S. cerevisiae* afin d'estimer la variabilité de longévité existant au sein de l'espèce. Leur longévité a été étudiée dans différentes conditions connues pour freiner le vieillissement: sous restriction calorique ou en présence d'un agoniste de la restriction calorique, la molécule rapamycine, qui inhibe directement la voie de signalisation TOR. Les microorganismes passent la majeure partie de leur vie dans des environnements défavorables, pauvres en ressources nutritives. Leur capacité à survivre à ces périodes de restriction (CLS) est donc primordiale. J'ai observé que les souches sauvages ont tendance à spontanément initier le programme de méiose aboutissant à la formation de spores lorsque les conditions environnementales deviennent restreintes. En revanche, les souches domestiques préfèrent entrer en quiescence, ce qui leur confère une viabilité et une résistance accrues. De plus, en ayant recours à une approche basée sur des gènes présélectionnés et à une étude d'association pangénomique, j'ai observé que la variabilité de longévité entre les différentes souches est déterminée par un large spectre de polymorphismes génétiques, tels que des mutations non-synonymes ou non-sens, et par l'absence ou la présence de certains gènes. Toutes ces composantes génétiques interagissent pleinement avec l'environnement.

Dans une deuxième partie, j'ai réalisé une analyse de liaison génétique grâce à 1056 souches descendantes de deux souches parentales. La longévité (CLS) de ces 1056 souches a été mesurée dans le but d'identifier des locus de caractères quantitatifs (QTLs). Le vieillissement chronologique a été déterminé à la fois à partir d'un milieu riche, d'un milieu restreint en calories, ou en présence de rapamycine. J'ai identifié 30 QTLs distincts, certains d'entre eux sont communs et récurrents dans plusieurs environnements, tandis que d'autres sont plus spécifiques et occasionnels. Les deux QTLs principaux, associés aux gènes *HPF1* et *FLO11*, codent tous deux des protéines du mur cellulaire, et sont jusqu'à présent non reconnus comme régulateurs du vieillissement. Étonnement, ces deux gènes contiennent des répétitions d'ADN en tandem qui s'avèrent être massivement amplifiées dans une des deux souches parentales d'origine. Alors que les allèles courts de *HPF1* et *FLO11* n'ont pas d'effet sur le vieillissement, les allèles longs sont relativement délétères, hormis en présence de rapamycine. Après investigation, il semble que la forme allongée de *HPF1* provoque la flottaison des cellules de levure au cours de la phase de croissance, les exposant à des taux plus élevés d'oxygène. Cette exposition à l'oxygène ambiant a plusieurs conséquences contribuant à un vieillissement prématuré: la perturbation de l'équilibre oxydo-réducteur des cellules, l'activation de la voie de réparation des protéines déformées, et l'altération du profil d'expression des gènes impliqués dans la biosynthèse de la méthionine, des ribosomes et des lipides.

Ces travaux apportent donc une caractérisation sans précédent de la variation naturelle du vieillissement au sein d'une espèce et révèlent plusieurs facteurs génétiques et environnementaux modulant cette diversité.

Mots-clés: Caractères quantitatifs, variations naturelles, *S. cerevisiae*, vieillissement chronologique, restriction calorique, rapamycine, répétitions en tandem, stress oxydatif

Abstract



Aging is a classical complex trait varying quantitatively among individuals and affected by both the genetic background and the environment. While aging is the highest risk factor for a large number of diseases, little is known about the underlying molecular mechanisms. Identifying the causal genetic variants underlying natural variation in longevity and understanding their interaction with the genetic background and the environment remains a major challenge. In this work, I used the budding yeast, *Saccharomyces cerevisiae*, to identify environmental and genetic factors contributing to aging. While extensive classical genetic studies discovered several genes involved in the regulation of chronological lifespan (CLS), which measures cell viability dynamic in non-dividing condition, using laboratory strains in standard conditions, there are only few studies exploiting variations in natural populations.

In the first part, I used a large cohort of more than 1000 sequenced natural *S. cerevisiae* strains to provide a species-wide overview of CLS variability. Longevity was measured in different environments, including calorie restriction (CR), a natural intervention known to increase lifespan, and in the presence of rapamycin (RM), a drug that mimics CR by downregulating the TOR pathway. Unicellular microorganisms spend most of their lifetime in harsh restricted environments interrupted by short windows of growth, making CLS an important and likely adaptive trait. I found that wild strains subjected to CLS tend to trigger the meiotic developmental process leading to the formation of gametes wrapped into a very resistant cell wall. In contrast, domesticated strains tend to enter quiescence state when starved and display a tremendous variability in their survival capacity. Moreover, using both candidate gene approach and genome-wide association studies (GWAS), I demonstrated that variability in CLS is determined by a full spectrum of genetic variant that include gene presence/absence, copy number variation, non-synonymous SNPs and loss of function. All these genetic features were strongly regulated by the environment.

In the second part, I performed linkage analysis using 1056 diploid segregants derived from a two parent advanced intercross. These 1056 diploid segregants were phenotyped for CLS to map quantitative trait loci (QTLs). The CLS was measured in complete media, CR and RM environments across multiple time points. I mapped 30 distinct QTLs, with some shared across different environments and time points, while others were unique to a specific condition. The two major effect size QTLs were linked with natural variation in the cell wall glycoproteins *FLO11* and *HPF1*, previously unknown to regulate CLS. Interestingly, both genes presented massive intragenic tandem repeat expansions in one of the founder strain used in the crossing scheme. While the short versions of *FLO11* and *HPF1* alleles did not impact CLS, tandem repeat expansions within those genes were sufficient to confer a dominant detrimental effect that was partially buffered by rapamycin treatment. Further investigation revealed that the extended form of *HPF1* makes cells floating during exponential phase, exposing them to higher oxygen rates, and leading to perturbation of redox homeostasis, activation of misfolded protein response, and alteration of multiple genes involved in methionine, ribosome and lipid biosynthesis, eventually contributing to CLS shortening.

Taken together, my work provided an unprecedented overview of natural variation in CLS in a genetic model system and revealed multiple genetic and environmental factors that shape the species phenotypic variation.

Keywords: Quantitative traits, natural variations, *S. cerevisiae*, chronological lifespan, calorie restriction, rapamycin, intragenic tandem repeats, oxidative stress

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Abbreviations

- AILs – Advanced Intercross Lines
- CFU – Colony Forming Unit
- CLS – Chronological Lifespan
- CNV – Copy Number Variation
- CR – Calorie Restriction
- FACS – Fluorescence Activated Cell Sorting
- FDR – False Discovery Rate
- GWAS – Genome Wide Association Studies
- IGF-1 – Insuline like Growth Factor 1
- LOF – Loss of Function
- MAF – Minor Allele Frequency
- NA – North American strain (YPS128)
- PBS – Phosphate Buffer Saline
- PBS – Phosphate Buffer Saline
- PI – Propidium Iodide
- POLs – Phased Outbred Lines
- QTLs – Quantitative Trait Loci
- QTNs – Quantitative Trait Nucleotide
- RHA – Reciprocal Hemizygosity Analysis
- RLS – Replicative Lifespan
- RM – Rapamycin
- ROS – Reactive Oxygen Species
- SDC – Synthetic Dextrose Complete media
- SDM – Synthetic Dextrose Minimum
- SGD – Saccharomyces Genome Database
- SIFT – Sorting Intolerant From Tolerant program
- SNP – Single Nucleotide Polymorphism
- SNV – Single Nucleotide Variant
- UPR – Unfolded Protein Response
- WA – West African strain (DBVPG6044)
- YP – YO-PRO-1 Iodide
- YPD – Yeast extract Peptone Dextrose
- Δ – Gene deletion

Preface

AGING

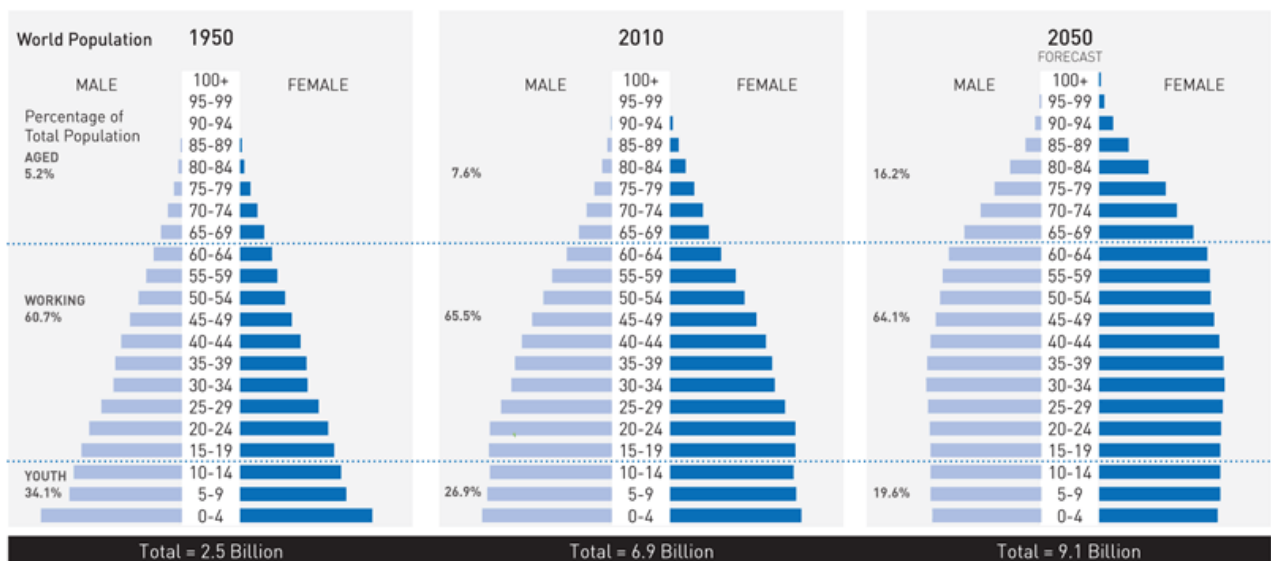
noun

“1. Phenomenons driving the transition of a living organism to death. 2. Gradual loss of strength, vitality and youth with time. 3. Acquisition of specific qualities through preservation (spirits, wines, cheese, smoked meat...).” - Le Petit Larousse

While, as a French scientist working with yeasts, I certainly appreciate the virtues of aging on wine and cheese, rare are those appreciating the tides of Time on themselves. Unlike alcohols, most living organisms, including humans, do not get better with time. Increased wisdom and knowledge are at a minimum accompanied with wrinkles, frailty and abdominal (or rear) adiposity. This constitutes the basic package. More generally though, aging comes with a plethora of undesirable diseases, such as Alzheimer, diabetes, cancer, ischemia, pneumonia, atherosclerosis, osteoporosis, and others, leading somehow to death.

From a scientific point of view, aging is a fascinating process encompassing pretty much all fields of biology. Studying aging can be approached under the scope of genetics, cellular biology, metabolism, chemistry, physiology, medicine, psychology, or even philosophy. From a societal point of view, because of its implications and its intrinsic link with death, aging has fascinated mankind since immemorial time. The imprinting of aging on humanity is endless; it has fuelled the essence of religions, orchestrated lifestyles, shaped family models, drove throne inheritance, or even created generation gaps.

In today's world, aging has become closely



The aging global population: 1950-2050

(U.N. Population Division, "World Population Prospects: The 2010 Revision")

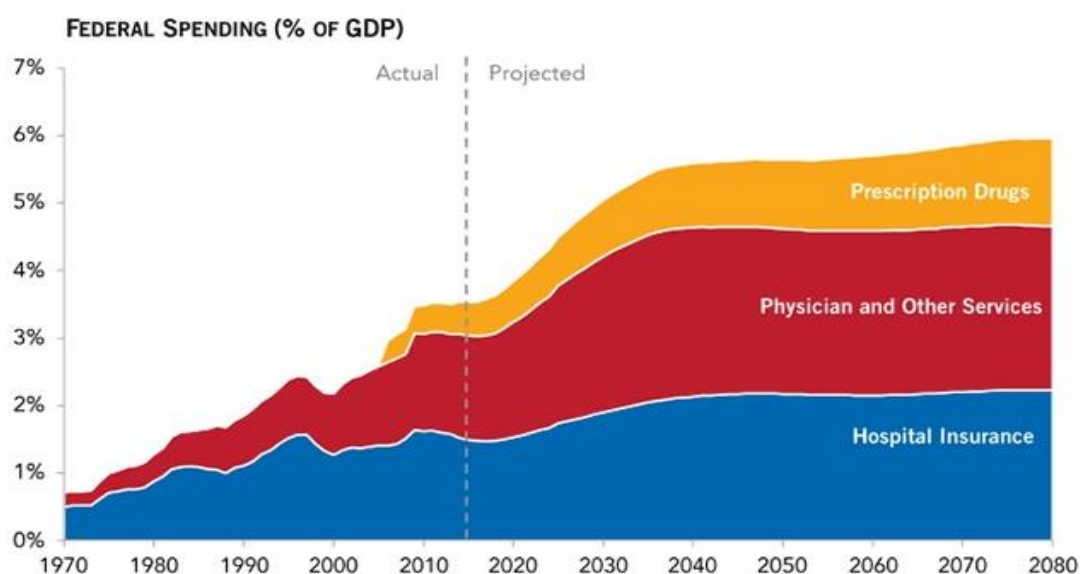
tied with economic concerns. The consumer society, helped by media and social networks, has imposed the diktat of youth and perfection, leading to exponential growth of cosmetic expenses and all other sort of anti-aging interventions. More importantly than beauty cream business, the frequency of elders within the population is strikingly increasing thanks to improved health conditions and remarkable progresses of medicine, associated with fertility decline. It is estimated that the amount of humans above the age of 65 will triple by 2050, reaching a total number of 1.5 billion souls. This phenomenon has two main drawbacks. First, it will substantially increase public health care spending. Second, it will unbalance the ratio between active and retired people. Altogether these issues will become an unsustainable burden for governments that will be forced to adjust their future policies.

In this context, the main goal of aging research, beyond global human care, is to promote healthspan, thus decreasing the incidence of age related diseases and allowing people to work longer. Nonetheless, it is likely that the whole professional world will need a revamp in the upcoming decades.

During my PhD, I have had the motivation and interest to work on aging in a team whose focus is about population genomics and genome evolution in the budding yeast *Saccharomyces cerevisiae*. This mixture gave birth to two projects at the crossroad of both disciplines and will constitute the result part of this manuscript.

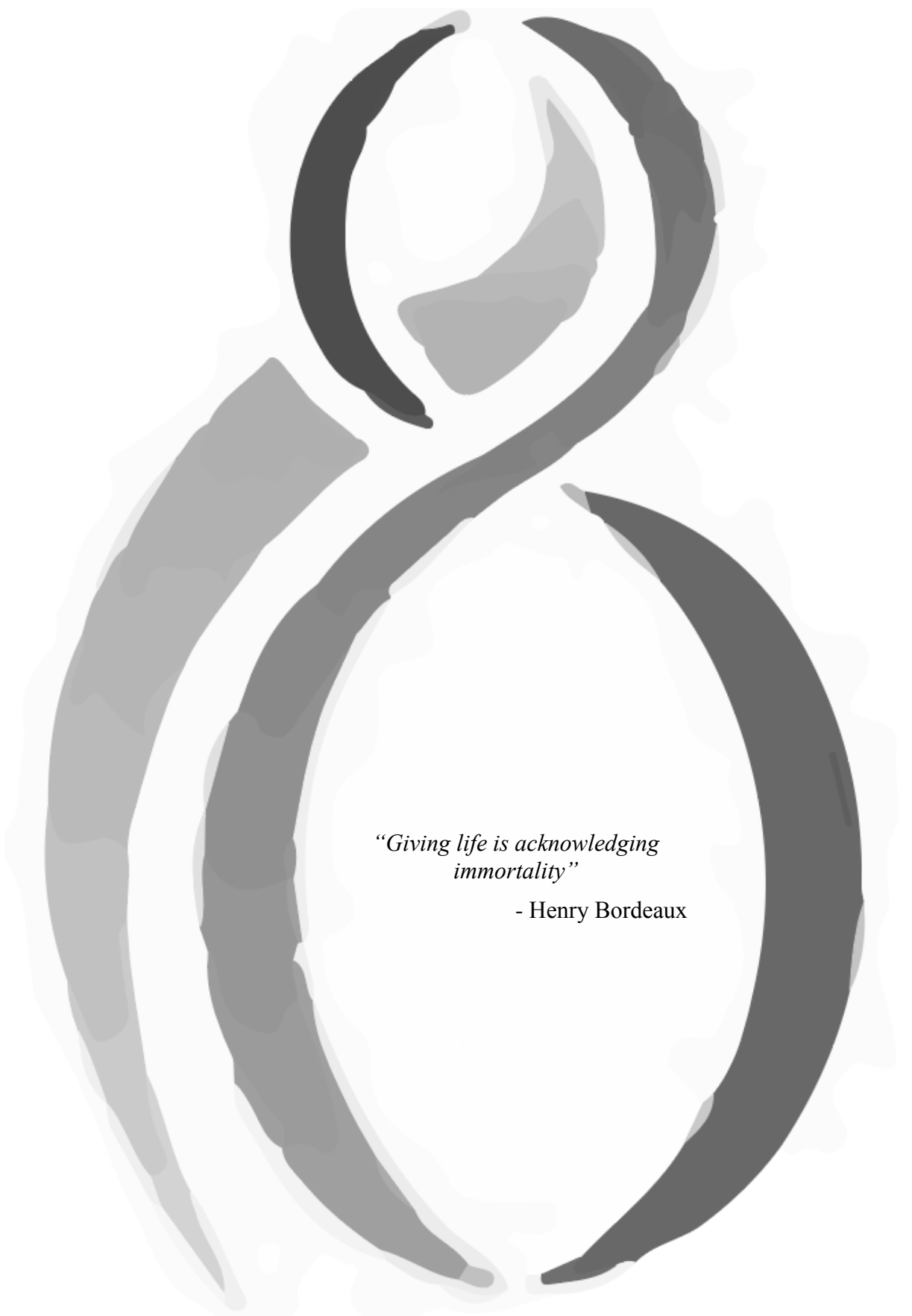
First, a collaborative project led by the team has brought an unprecedented species-wide description of *S. cerevisiae* through the sequencing and phenotypic analysis of 1011 isolates (Peter & De Chiara et al., 2018). Taking the advantage of this resource, I have studied natural aging variations at the population level, and tried to explain it under the scope of evolution.

Second, I have used state-of-the-art forward genetic strategies developed in the lab to dissect, at high-resolution, the genetic architecture of aging variation within a two parental outbred cross. This work was performed in different environmental conditions relevant to aging, in an attempt to unravel the complex gene-environment interactions.



Spending projection of the American health insurance Medicare as a share of GDP (Centers for Medicare and Medicaid services, 2015)

INTRODUCTION



*“Giving life is acknowledging
immortality”*

- Henry Bordeaux

Chapter 1

The plasticity of aging

According to the Bible, the record man of longevity is the patriarch Methuselah who died at the age of 969 years old. While his grandson Noah enjoyed a life of 950 years -in virtue of the sea air-, further descendants were less fortunate and had gradually decreasing life expectancies, until a threshold of 120 years officially set by God. This was before the French lady Jeanne Calment dismantled divine predictions by reaching the age of 122 years old in 1997 ([Robine & Vaupel, 2001](#)).

In the meantime, the 4850 years old bristlecone pine Methuselah has performed better than the patriarch of the same name, and witnessed both pyramid construction and the onset of spatial conquest.

In this first chapter, I will describe how broad and diverse aging is across the tree of life.



Negligible senescence. The elves from *The Lord of the Rings* can live more than a thousand years without showing any sign of dementia.

A) Natural variations of human aging

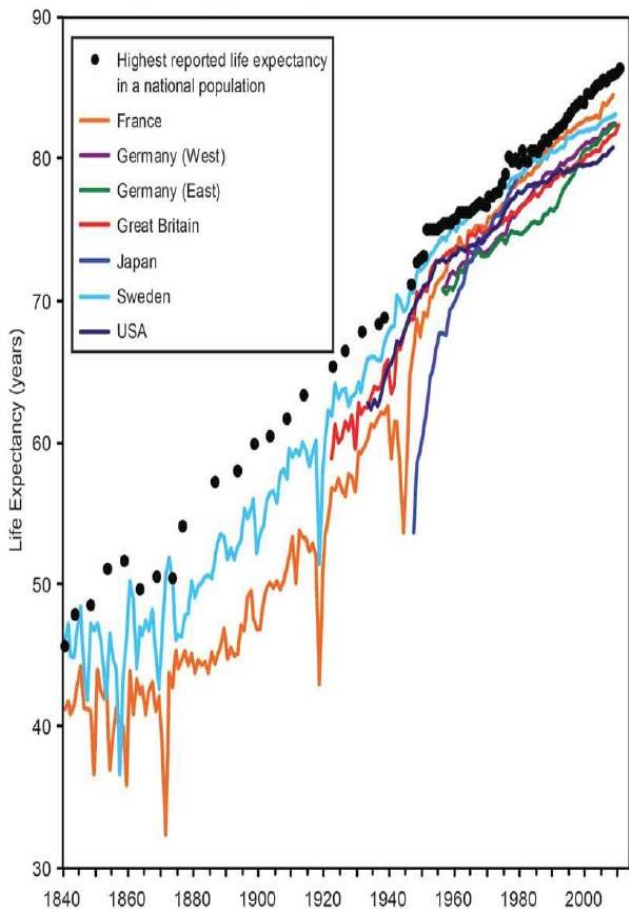
A1. Socio-economic and environmental factors

Human life expectancy has been steadily increasing over the past 150 years, ranging from an average of 45 years old in 1850, to a bit more than 80 years old nowadays, which represents an average increase of 3 months per year (Oeppen & Vaupel, 2002; Christensen et al., 2009). This remarkable achievement is mainly the result of prosperity and improved health conditions (Vaupel, 2010). On the one hand, elderly care has been considerably developed in our modern societies, while on the other hand, amelioration of sanitary conditions and medicine have greatly pushed back the occurrence and lethality of infectious and parasitic diseases which used to be leading causes of death. Today, lifespan expectancy is still increasing, and developing countries are little by little catching up.

However, despite ever increasing life expectancy, no human being has lived longer than Jeanne Calment the past 21 years. In fact, while it is estimated that maximum lifespan has doubled from 57 to 115 years between the first hominids 4 million years ago and the *Homo sapiens* 100 000 years ago, it is thought that this capacity has not evolved since then (Hayflick, 1996). Tracking human longevity has been particularly difficult until birth and death registers started to be systematically recorded in the 19th century. Although no prehistoric human remains were dated to exceed 50 years old, Ancient Egypt papyruses witness maximum ages relatively

comparable to those of today (Jeune, 2002). Nevertheless, reaching very old age in the past was a rare achievement, and it is thought that turning centenarian before the 1800's was nearly impossible (Jeune, 2002). All in all, it is plausible that life expectancy will keep increasing as we eradicate 'accidental' causes of death, until it reaches a plateau defined by intrinsic human genetics at which time life will be principally hindered by cardiovascular failure, pneumonia, or cancer (Kirkwood, 2017).

Beyond social and economic factors, lifestyle is a major driver of human lifespan variations. For instance, mortality predictions in Sweden in 2008, suggested that even though most people would reach 84 years old, one out of six would die before

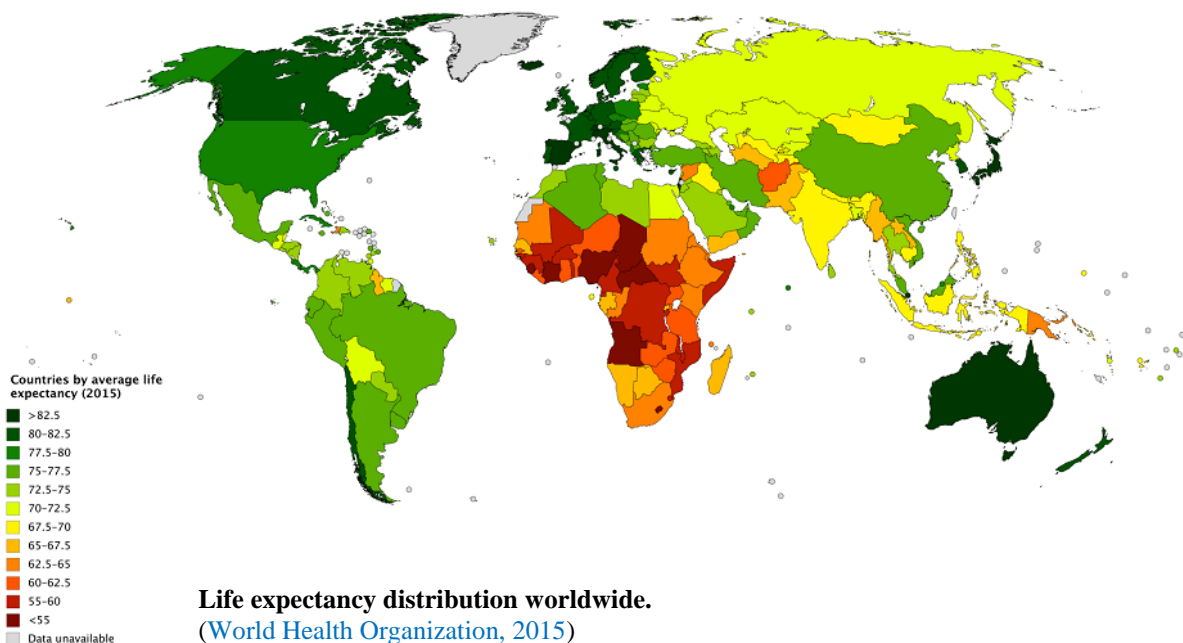


Female life expectancy in developed countries: 1840-2009 (Christensen et al., 2009)

the age of 70 (Vaupel, 2010). Main risk behaviours include smoking, sedentary lifestyle, obesity and alcohol consumption. The incidence of smoking on life expectancy is striking. In the 1980s, while life expectancy in USA and Denmark were among the highest worldwide, a sudden stagnation occurred, which was later attributed to smoking trends (Wang & Preston, 2009; Juel et al., 2008). Not later than this summer (2018), a comprehensive study about alcohol consumption found that even a daily drink has health repercussions (Gakidou et al., 2018). Although these results have probably lowered the appreciation of the public opinion for the scientific community, they reemphasized the detrimental effect of alcohol on health (7th death and disabilities risk factor). In addition to increasing the incidence of premature death and age-related diseases, these risky lifestyle behaviours have been associated with signs of accelerated aging such as telomere shortening or neurodegeneration (Valdes et al., 2005;

Ronan et al., 2016).

Along with a balanced lifestyle, it is now well established that proper nutrition is the key to living old. In the 16th century, Luigi Cornaro, a Venetian noble particularly active in the local festivities, embraced a sober lifestyle after getting close to death. According to his physician's recommendations, he restricted himself to a daily dose of 350g of food. After some time, he reported health benefits obtained from this diet in several books pioneering the concept of calorie restriction (Cornaro, 1558). More recently, the well-known Mediterranean diet was correlated with health enhancement and lower death rate, especially regarding coronary diseases (Singh et al., 2002; Trichopoulou et al., 2005). The Mediterranean diet relies on food quality rather than calorie input, it is mainly based on vegetables, fruits, cereals, dairy products, unsaturated fatty acids (from the famous olive oil), and fish intake instead of meat (Bach-Faig et al., 2011). A somewhat similar regimen can be found in Japan and



can be correlated to high life expectancy observed in this country. This may be particularly well illustrated by inhabitants of the Okinawa island in Japan. The number of centenarians on this island is five times higher than worldwide frequency and is associated with lower disease risk and mortality rate (Willcox et al., 2006). In addition with regular physical activity, this phenomenon results from the low-calorie diet of Okinawans which are consuming on average 17% and 40% fewer calories compared to mainland adult Japanese, and Americans, respectively (Suzuki et al., 2001). Interestingly, this trend is now disappearing as the possible result of food westernization brought by US soldiers in the 1960 (Kagawa, 1978; Most et al., 2017).

Independent of culinary habits, historical events have sometimes compelled entire populations to calorie restriction. Such events happened recently during the first and second World Wars, which, somewhat paradoxically, were found to decrease mortality rate by 30% (Strom & Jensen, 1951, Most et al., 2017). A 3rd thermonuclear World War might not be a wise approach to promote human lifespan though. Finally, it seems that even the season of birth might impact longevity. Indeed, Austrian and Danish babies born between autumn and winter were found to live slightly longer, possibly due to higher availability of fruits and vegetables throughout pregnancy, although these results must be considered with precaution (Doblhammer, 1999; Kannisto et al., 1997). To conclude, if you want to enjoy a long and vibrant life, remember to eat five fruits and vegetables per day.

A2. Genetic factors

Despite the preponderance of environmental factors in lifespan regulation, the influence of genetics must not be forgotten nor underestimated. Genetics certainly regulate human longevity similarly to trait diversity or disease susceptibility within the population. The way humans age and the symptoms they develop can be highly different from one person to another. A study on Danish centenarians revealed that three quarters suffer cardiovascular diseases, half suffer osteoarthritis and dementia, and a third suffer ischemic heart disease (Andersen-Ranberg et al., 2001). On the other hand, the famous Armenian singer-songwriter Charles Aznavour was still giving live concerts in Japan at the age of 94, ten days before he passed away due to a pulmonary oedema. Jeanne Calment, dean of humanity, who was still biking at the age of 100, was eating a kilogram of chocolate per week, drinking a daily glass of port-wine and smoking cigarillos almost until her death. Saying this after writing multiple paragraphs on the importance of sober lifestyle probably ruined all my credibility. Even though there may be a part of chance, there is surely a part of genetics. Jeanne Calment's brother died at the age of 97 while her parents died at the age of 93 and 86 years old, which is quite remarkable for the beginning of the 20th century.

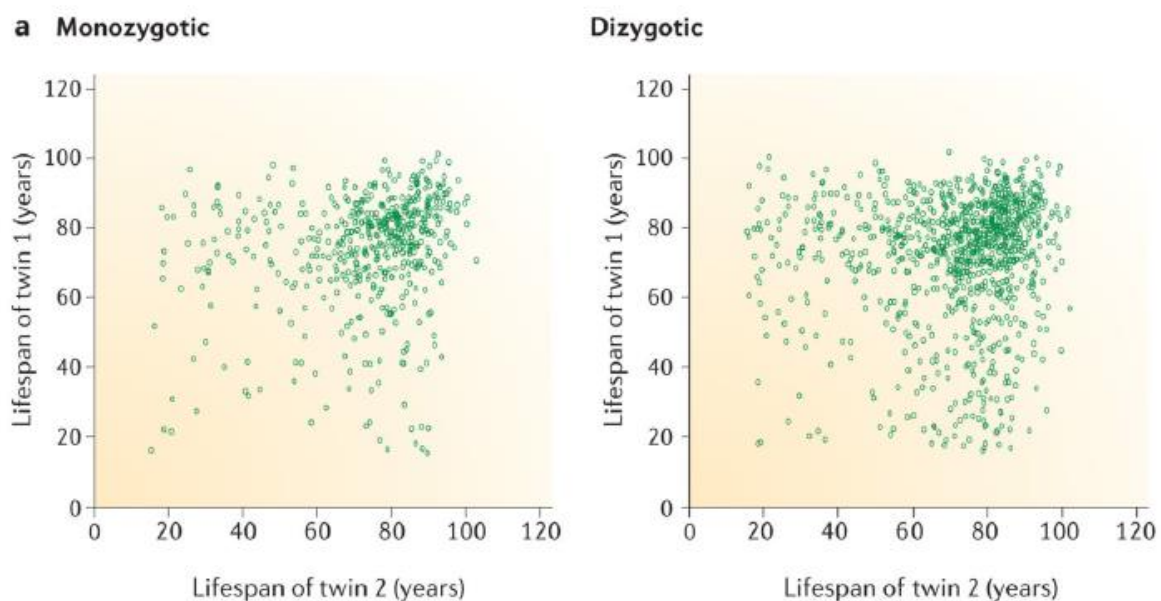
Studies to determine longevity inheritance began in the past century with the observation that parents of long lived had higher lifespans compared to parents of short lived (Pearl & Pearl, 1934). While suffering methodological sampling issues, these results were later confirmed by

another study paying attention to pick and compare cohort of individuals from the same sex and the same localisation (Poulain et al., 1999). By extension, lifespan inheritance was also found for siblings with the striking observation that siblings of centenarians have four times more chance to reach 80 years old compared to siblings of young lives (Perls et al., 1998). According to studies performed on large cohorts of twins, the average lifespan difference between monozygotic twins is about 14 years compared to 19 years for dizygotic twins, setting at 25% the contribution of genetics to human lifespan variation (Herskind et al., 1996).

Another noticeable genetically driven longevity discrepancy is seen for genders. It is well established that women live longer than men. The reasons for this phenomenon are still misunderstood though. Several hypothesis point to the effect of gene-sex interactions, or to hormonal differences. Another hypothesis suggests that harbouring two copies of the X chromosome protects

against mutations in mitotically active tissues and gives women an advantage over men. In favour of this argument, the fact that this trend is reversed for birds where females are heterogametic and shorter living (Jeune, 2002).

Although longevity inheritance is now well recognized, identification of causative genes and polymorphisms is barely uncovered. Further progress is mainly limited by the logistics and technical challenges associated with the use of humans as a model system. Indeed, human lifespan is extremely long, DNA sequencing expensive, sampling is size limited and biased by socio-economic environments and lifestyle, and genetic engineering is tedious and restricted to in vitro cell lines, among others. Moreover, despite the discovery of genes able to double lifespan of model systems, there is still no evidence of such strong lifespan regulators in mammals. Human lifespan is thus presumably regulated by a multitude of genetic variants with a very modest effect essentially masked



Lifespan of monozygotic and dizygotic twins. Each dot represents a pair of Danish twins of the same sex born between 1870 and 1900. (Christensen et al, 2006)

by environmental factors.

Despite these difficulties, few human allelic variants have been associated with longevity. As of today, only two variants have been strongly and repeatedly identified (Murabito et al., 2012; Christensen et al., 2006). The first one is found in the gene *APOE* which is involved in lipoprotein regulation. The *APOE4* isoform is linked to increased risk of cardiovascular disease and Alzheimer and its frequency is halved in centenarians (Schachter et al., 1994). The second one is the forkhead transcription factor *FOXO* which acts downstream of the insulin/IGF-1 signalling and mediates stress response (see chapter 2). Its variant *FOXO3a* has been associated with longer survival in several cohorts of elders (Willcox et al., 2008). Apart of these two, a dozen of additional gene polymorphisms have been narrowed down, with involvement in various mechanisms such as metabolism, cholesterol homeostasis, immune response, or oxidative phosphorylation. However, multiple candidate genes could not be replicated across studies and consequently the

literature lacks a clear consensus (Jeune, 2002; De Benedictis et al., 2001; Christensen et al., 2006, Murabito et al., 2012). Moreover, these studies do not systematically differentiate healthspan from lifespan. For instance, using a cohort of medically assisted surviving centenarians with poor health condition might give a different output compared to healthy individuals.

While most human polymorphisms have little influence on longevity, extreme cases exist and result in progeroid syndromes. The two best known examples are the Werner and Hutchinson-Gilford syndromes which are triggered by the alteration of genes involved in genome stability. The former is due to a mutation in the *WRN* gene that is encoding a helicase protein involved in DNA repair, and the latter is due to a mutation in the *LMNA* gene encoding lamin A, a component of the nuclear envelope (Yu et al., 1996; Eriksson et al., 2003). In both cases, patients suffer premature signs of aging and eventually die at young ages. Less known, the reverse case also exists under the



Neotenic complex syndrome. Photographs of Brooke Greenberg at the age of 5 (left) and 15 (right) years old (Walker et al., 2009).

name of neotenic complex syndrome (Walker et al., 2009). Neotenic complex syndrome is an extremely rare disease (only diagnosed in 7 humans so far), causing an extreme form of developmental delay. Patients never grow up and look frozen in their young age, although their life expectancy does not exceed 20 years old because of physiological failures associated with developmental troubles. A couple of mutations in different genes involved in transcription regulation and chromatin modification have been identified, but the overall mechanisms of this disease remain elusive (Walker et al., 2018).

B) Natural variations of aging in the wild

B1. Metazoans

Monitoring animal aging constitutes by essence a fastidious task since it is very unlikely to reach old age in the wild. Hayflick estimated that humans had a life expectancy inferior to 30 years old during 99,9% of their existence (Hayflick, 1996). In practice, mice can enjoy a 3-year lifespan in laboratory conditions, but, in the wild, more than 90% would not pass the first year (Phelan & Austad, 1989). Unsurprisingly, the best characterized animals are those used as models for research along with domestic pets. The roundworm *caenorhabditis elegans* and the fruit fly *drosophila melanogaster* are among the most popular systems to study aging, in virtue of their tractability and their short lifespans (roughly 25 and 50 days, respectively). Despite the impediments, efforts were made

to obtain a comprehensive view of longevity across the tree of life, and the least we can say is that aging diversity is stunning (Finch, 1990; De Magalhaes, 2011; Jones et al., 2014). Lifespan variation between the shortest living metazoan, the mayfly (24 hours), and the longest living one, the ocean quahog clam (500 years), is equal to an outstanding 182500-fold lifespan difference (Abele et al., 2008).

The extreme longevity variability in the wild does not follow clear rules or logic. One of the only trends that has been consistently noticed, is the relationship between body mass and maximal life expectancy (Promislaw, 1993; De Magalhaes et al., 2007). While insects are generally among the shortest-living metazoans, larger animals such as whales or elephants enjoy much longer lives. The most likely explanation stems from mortality rates in the wild. Indeed, mainly due to predation, large animals usually survive much longer than the small ones, favouring the evolution of pro-longevity mechanisms. Interestingly, this trend is true when comparing inter-species but not intra-species lifespan differences, which is particularly well exemplified by dog breeds (Rollo, 2002). The reason could be explained by trade-offs between growth rate



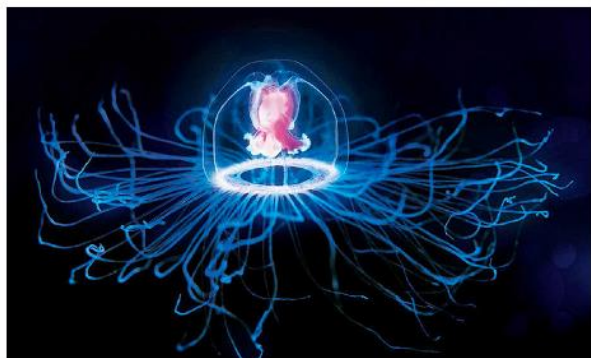
The mayfly, also known as « Ephemeral » in French, is the shortest living metazoan

and maintenance (see chapter 2).

Correlation between body mass and lifespan suffers a vast amount of exceptions though. Birds usually display significantly long-life expectancies compared to their size (Holmes & Ottinger, 2003). Parrots can potentially reach 100 years old, while bats, the only flying mammals, get above the age of 30. Similarly to large animals, the ability to fly could protect against predation and contribute to improved longevity (De Megalhaes, 2011). Another nice counter example comes from the mysterious naked mole rat. Despite being naturally ugly, this eusocial mammal displays negligible signs of senescence and lives ten times longer than other rodents (28 years) (Buffenstein, 2008). Until very old age, naked mole rats do not show increased mortality rate, nor loss of reproduction capacity, nor physiological changes (Finch, 2009). Even more remarkably, naked mole rats do not develop cancer (Buffenstein, 2008).

It is usually thought that aging is a universal unavoidable process, however we have now observed many animals challenging the tides of time. Several species of turtles show improved fertility with age, without mortality rate increase nor loss of vigor (considering that a turtle's vigor can possibly drop...) (Congdon et al., 2001,

Finch, 2009). The Galapagos, and the Aldabra giant tortoises are among the longest living animals and can reach ages close to 200 years old, if not more. The ultimate champions of longevity might actually be hidden in the depths of the ocean. The rockfish *Sebastes aleutianus* can live more than 200 years without showing any sign of aging (Cailliet et al., 2001). Red sea urchins, and extremely slow growing tubeworms *Lamellibranchia* can also exceed 200 years old (Bergquist et al., 2000). Molluscs are also top ranked with the ocean quahog clams and the deep-sea oysters overcoming 400 years old (Abele et al., 2008). Closer to us, old Greenland shark specimens were recently estimated to be at least 272 years old (Nielsen et al., 2016), making them the longest living vertebrates, while bowhead whales are the longest living mammals with a life duration of 211 years (George et al., 1999). Last but not least, some marine species might have broken the code of immortality. This is likely the case of coral and of the highly regenerative hydra (Bythell et al., 2018; Martinez, 1998). Another fascinating candidate for immortality is the jellyfish *Turritopsis dohrnii*. Similarly to Benjamin Button, this intriguing animal has the capacity to reverse time and to go back into its juvenile form in case of stress, sickness, or senescence, before



Champions of longevity. The naked mole rat (left) lives in average 10 times longer than other rodents, while the jellyfish *Turritopsis dohrnii* (right) is considered immortal.

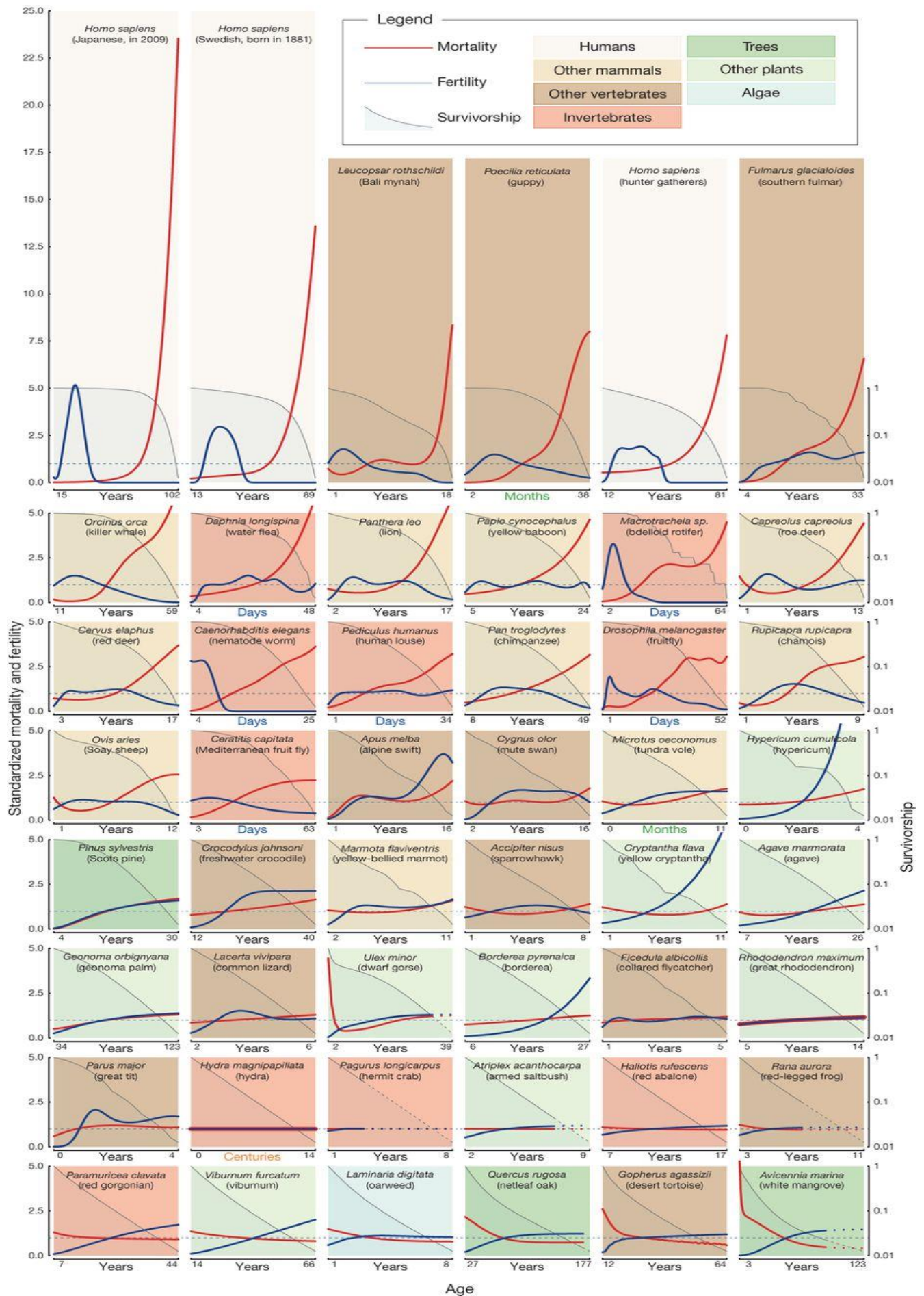
turning adult again (Piraino et al., 1996). It is important to keep in mind that all these extremely long living animals are rare specimens and that in most cases their sacrifice is required to determine their age. Therefore, their real maximum lifespan remains undetermined. They lived happily more than 200 years without human intervention.

Life expectancy of each species is intrinsically defined by genetics, however environmental triggers can sometimes have surprising outcomes. A striking example can be observed with ants. While they share the same genome, ant workers live one hundred times less compared to queens. Such a difference is simply the result of pheromone exposure and diet composition received during their larvae stage (Finch, 1990; Kirkwood & Austad, 2000). Also worthy of interest, *Sander vitreus*, a fish found in North American waters, can experience five-fold lifespan differences according to the temperature (Mangel & Abraham, 2001). Similarly, when nutrition conditions are harsh, the round worm *C. elegans* juveniles can enter a special state called *dauer* which substantially delays development and increases their lifespan (Klass & Hirsh, 1976). On the other hand, some animal species experience sudden death after reproducing, a process called semelparity. The most well-known example is salmon. After returning to rivers and spawning, salmon stop feeding themselves and develop several pathologies in response to hormonal cues (Finch, 1990; De Magalhaes, 2011).

B2. Plants and microorganisms

The animal kingdom shows a broad plasticity of aging, but this might be nothing compared to the vegetal kingdom. Everyone has heard about magnificent centenarian oak or olive trees, but plant longevity can actually reach thousands of years, although, like animals, rare are those which had this chance. Several millenary trees have been listed throughout the world, from different species (cypress, yew, cedar, olive, sequoia, oak, bristlecone pine) with the oldest, namely Methuselah, estimated close to 5000 years old. In fact, mortality rate in plants is often stable, as if they do not experience aging (Jones et al., 2014). However, millenary life expectancy is not common. Signs of senescence associated with functional decline were found in some species such as apple and orange trees. Also, multiple plant species experience semelparity similarly to salmon. For instance this is the case of bamboo which reproduces only once before decaying (Finch, 1990).

More generally, studying aging in plants raises a fundamental philosophical question regarding the nature of individuality. Indeed, plants are organisms with indefinite growth which can be grafted and clonally replicated endlessly (Finch, 1990). Should the grafts be considered as the same individual? My feeling is no, only the originating tree should be seen as a unique entity. However, the inner part of most trees, the so-called heartwood, is made of dead tissues, and sometimes, due to diseases, half of the tree is dead while the other part is still thriving. In the case of millenary trees, it is unlikely that the same cells



Diversity of aging across the tree of life. Fertility, mortality rate and survivorship of multiple species from the animal and the plant kingdoms (Jones et al., 2014).

survived several thousand years: old cells keep being clonally replaced by new cells growing on the outer layers. A puzzling example is the Pando tree, which is at some point considered to be the oldest and biggest organism of life history with an estimated age of 80000 years, and a size of 43 hectares (Dewoody et al., 2008). In fact, Pando is a giant clonal population made of thousands of trees born from the same root system. All trees are genetically identical and connected through their roots, even though they are independent since the death of one tree does not affect the others. Defining in this case the limits of individuality becomes blurred. If Pando is seen as a single individual, then we could also consider human lineages to be 100000 years old since the appearance of *Homo sapiens*, from my point of view.

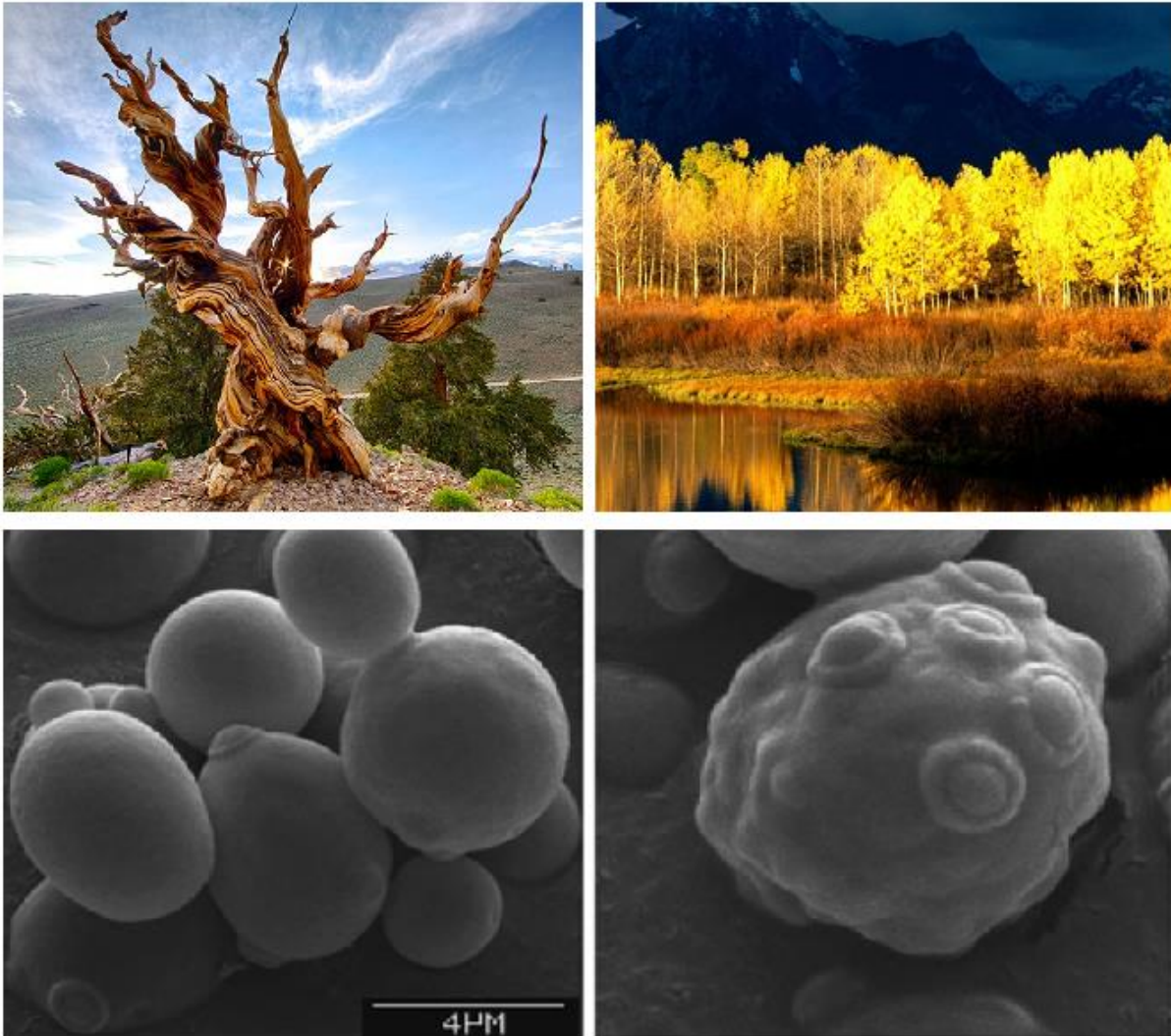
For quite a long time, unicellular organisms were considered immortal. Evidence proving the contrary has now been well described. In the kingdom of unicellular organisms, the baker's yeast *Saccharomyces cerevisiae* has the property to divide asymmetrically by budding, allowing the tracking of mother and daughter cells. While *S. cerevisiae* has a vegetative growth and can be propagated endlessly, Barton found in 1950 that a single mother cell can only divide a finite amount of time (Barton, 1950). Later on, Mortimer and Johnston quantified this process more in detail and found that mortality of mother cells increases with the number of divisions as a typical hallmark of aging (Mortimer & Johnston, 1959). Replicative aging in yeast is also accompanied by physiological decline such as slower growth rate, insensitivity to sexual pheromones and inability to produce gametes (Lee et al., 2012; Muller, 1985,

Boselli et al., 2009). Remarkably, every produced daughter is entirely rejuvenated, thus preventing the senescence of the whole species (Kennedy et al., 1994). Yeast replicative lifespan has become a pivotal model in aging research, however its occurrence in the wild remains doubtful. Because yeasts grow exponentially, mother cells rapidly get outnumbered by daughters and never reach old age due to nutrient exhaustion. In fact, yeasts, like most unicellular organisms, spend most of their lifetime in non-proliferating conditions interspersed by rare opportunities of replication (Liti, 2015). To sustain these potentially long starvation periods, yeasts have evolved sophisticated developmental mechanisms such as quiescence and sporulation. In the absence of rich carbon source and nitrogen, diploid yeasts trigger the sporulation program which is associated with meiosis and leads to the formation of highly resistant immortal gametes wrapped into robust spore walls (Neiman, 2011). Alternatively, nutrient scarcity can promote entry into quiescence, a state of arrested growth associated with cell wall thickening and much improved stress resistance properties (Gray et al., 2004). The study of yeast survival in non-proliferative conditions has become another popular model for aging research (Longo et al., 1996; Longo & Fabrizio, 2012) (see chapter 3).


Determining whether symmetrically dividing microorganisms, such as the fission yeast *Schizosaccharomyces pombe* or the bacteria *Escherichia coli*, experience aging has been strongly debated and essentially dismissed. Because cells divide by fission and produce two apparently identical daughters, it was impossible to track any possible aging mother.

However, it was recently found that cytokinesis is in fact asymmetrical and that daughters inherit either a young or an old pole. By following cells inheriting the old pole, it has been possible to demonstrate

signs of senescence for both *E. coli* and *S. pombe* (Stewart et al., 2005; Barker & Walmsley, 1999).



Vegetal and fungal lifestyles. The 4850 years old tree Methuselah (top left). The Pando tree, oldest and biggest living organism (top right). Young (bottom left) and aged yeast (bottom right) *S. cerevisiae* (Laun et al., 2001).



*“Nothing in biology can be
understood except in the light of
evolution”*

- Theodosius Dobzhansky

Chapter 2

Why do we age?



Aging is a highly multifactorial process that affects a myriad of cellular and physiological functions. Countless theories aimed to explain the essence of aging have emerged in the past decades, stimulated by the latest discoveries and improved understanding of biological systems. In an attempt to rationalise aging theories, Medvedev listed more than 300 of them and came to the conclusion that the existence of a single unified theory is not realistic ([Medvedev, 1990](#)). Despite this lack of consensus, it is now well accepted that aging organisms accumulate various kind of damages and dysfunctions which altogether lead to senescence and death. In this regard, I particularly like the definition of aging proposed by Gladyshev which may reconcile most hypotheses:

“Aging: progressive decline in fitness due to the rising deleteriome adjusted by genetic, environmental, and stochastic processes”

Gladyshev, 2016.

In this chapter, I will explain the evolutionary origins of aging before summarizing its main biological causes.



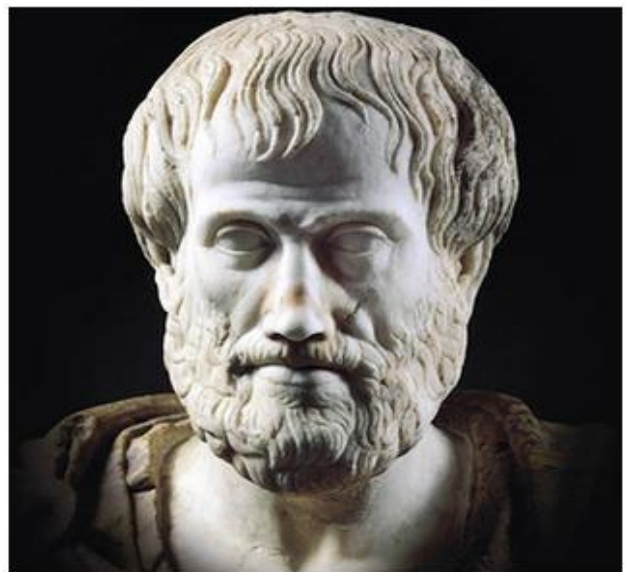
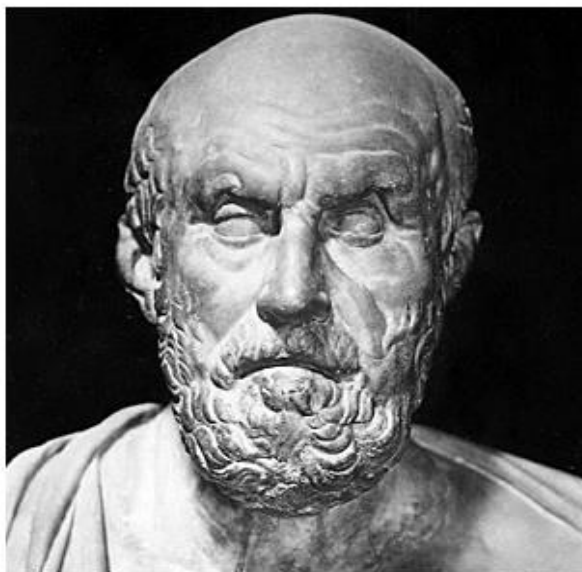
Very programmed aging. In *Time Out*, human lifespan is regulated by an accurate stopwatch.

A) Evolutionary theories of aging

A1. From fatalistic theories to Darwin

First theories and attempts to rationalise aging can be traced back to the Ancient Greece. At that time, Hippocrates mainly considered aging as a disease resulting from the progressive loss of inner moisture and heat, making the body colder and drier, similar to earth. Theories of Hippocrates were later continued by Aristotle who was seeing life as the flame of an oil lamp running out of fuel. He thought that inner heat was produced in the heart and then spread throughout the body (Jeune, 2002; Ritch, 2012). Aristotle was also the first to distinguish premature from senescent death, introducing the notion of species specific maximal longevity (Vaupel, 2010). This initial concept did not evolve much during the upcoming millennium. A common point of view emerged considering aging as a wear and tear process. Aging was seen as the result of a progressive irreversible degradation, similarly to inanimate objects or minerals (De

Magalhaes, 2011). More concretely, this can be exemplified by tooth erosion which can lead to nutrition issues in aged mammals such as horses or humans (Finch, 1990). In accordance with the second law of thermodynamic, wear and tear theory could also be the consequence of ever increasing entropy, explained by the spontaneous trend of any ordered system to tend toward chaos. As of today, the overall accumulation of damages during aging is well accepted, however the wear and tear hypotheses face multiple limitations. While it is true that entropy naturally increases in any ordered system, this can be reversed by the application of energy. It is now well established that all biological systems have developed sophisticated maintenance and repair mechanisms to sustain. For instance, tooth erosion can be overcome by replacing them, as sharks do, or by continuous growth like rodents. Also, wear and tear theories fail to explain the broad diversity of aging between species. Since we are made of similar molecules, how come mice can only live 3 years while humans can make it to 80? Lastly, wear and tear theories cannot



Pioneers of the aging field. Hippocrates (left) and Aristotle (right) were convinced that life was similar to an inner flame that progressively extinguishes.

explain the existence of quasi immortal animals as described in the previous chapter (De Magalhaes, 2011; Vijg & Kennedy, 2016).

Thus, until Darwin's seminal work on species evolution, the perception of aging was mainly fatalistic. It was thought that lifespan was merely an intrinsic species characteristic and that longevity was designed similarly to observable traits such as the long tail of rats or animal's fur (Goldsmith, 2004). After the publication of the *Origin of Species* in 1859, obvious discrepancies emerged between Darwin's survival of the fittest and the core existence of aging. If living organisms have systematically evolved to optimise their reproduction and survival, how is it possible that a mechanism which decreases fertility and promotes frailty exists? Darwin himself could not find a proper answer to this question and acknowledged that if a process like aging exists, it must be somehow compensated by some undisclosed benefits. Nevertheless, he was unable to explain the nature of these benefits, nor the reason why lifespan was so variable among species. On this point, the biologist George C. Williams stated:

"It is 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".

From here, two schools of thought arose and gave birth to programmed and non-programmed theories of aging (Kirkwood & Melov, 2011; Goldsmith et al., 2004). Proponents of programmed theories suggest that aging is an active adaptive mechanism genetically determined with

evolutionary advantages, while proponents of non-programmed theories propose that aging is a passive not adaptive adverse mechanism.

A2. Programmed theories of aging

A few years after Darwin, in 1882, the evolutionary biologist August Weismann, famous for distinguishing the germ line from the soma, attempted to explain aging as a programmed process. Unlike Darwin's original conception, Weismann considered that natural selection could be driven by population rather than individual benefit, a theory that is now referred as group selection (Wynne-Edwards, 1986). He suggested that since older individuals progressively lose their fitness, they become less competitive than younger ones and in turn become a burden for the community. Consequently, Weismann proposed that aging could help to clear out worn individuals, thereby leaving more resources for younger generations (Goldsmith, 2004; Kirkwood, 2005). It has also been proposed that aging could be beneficial for the group by accelerating generation turnovers and promoting genetic diversity through elimination of older individuals and prevention of elder supremacy (Goldsmith, 2008). Additionally, Mitteldorf suggested that aging could help to maintain demographic homeostasis. He estimated that if an animal becomes too fit in its environment it could destabilize the whole ecosystem and lead to extinction (by excessive predation followed by famine for instance) (Mitteldorf, 2006).

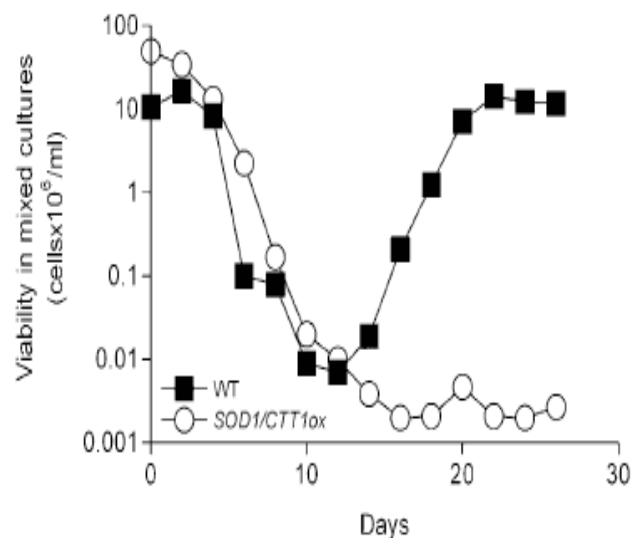
So far, the main results supporting programmed aging theories come from the budding yeast. Surprisingly, in 1997, Madeo

and colleagues found that this unicellular organism is able to commit suicide by triggering apoptosis (Madeo et al., 1997). While apoptosis is now recognized to substantially contribute to metazoan development and homeostasis, the idea that a single celled organism could commit suicide sounded irrational in regard to Darwinian predictions. Yeast apoptosis is triggered by cellular oxidative damages or environmental cues such as low pH or acetic acid which are usually encountered during chronological aging in nutrient restricted stationary phase (Herker et al., 2004; Fabrizio et al., 2004; Burtner et al., 2009). Interestingly, nutrients released by lysis of apoptotic cells during chronological aging promote regrowth of a small subset of mutants during long term cultures, similarly to previous observations with bacteria (Zambrano et al., 1993; Fabrizio et al., 2003; Fabrizio et al., 2004). This phenomenon is bolstered by oxidative stress sensitivity: strains deleted for superoxide dismutase exhibit accelerated aging and higher mutation rates, while increasing death rate through apoptosis, nutrient release and regrowth frequency (Fabrizio et al., 2004). Even though superoxide dismutase deprived strains died earlier, they outcompeted oxidative stress resistant cells in long term stationary cultures (Fabrizio et al., 2004). Accelerated aging induced by oxidative stress thereby appears as an adaptive mechanism (Longo et al., 2005).

Moreover, it was found that stationary phase cultures are not homogeneous. In fact, upon entry into stationary phase, only a fraction of cells become resilient quiescent cells, while the rest suffer high genetic instability and keep

dividing occasionally before dying (Allen et al., 2006; Aragon et al., 2008). This suicidal behaviour of non-quiescent cells is thought to increase the probability to acquire adaptive mutations and to feed quiescence cells which are guaranteeing in any case the survival of the lineage.

Despite convincing arguments of programmed aging in yeasts, no comparable evidence has been uncovered in higher eukaryotes so far. Discovery of the first genes regulating longevity in the 1990s reinforced programmed aging proponents' convictions (Johnson, 1990; Kenyon et al., 1993). Nevertheless, the only concrete example of programmed aging in metazoans relies on semelparous species. As described in the first chapter, some species of salmon, marsupial, octopus, or bamboo, among others, suddenly die after reproduction, presumably to increase the chances of survival of their offspring by freeing up resources (Finch, 1990).



Altruistic aging program in yeasts. The strain DBY746 (black) was competed in liquid cultures with its long-living counterpart (white) over-expressing the anti-oxidant enzymes SOD1 (dismutase) and CTT1 (catalase) (Fabrizio et al., 2004).

A3. Non-programmed theories of aging

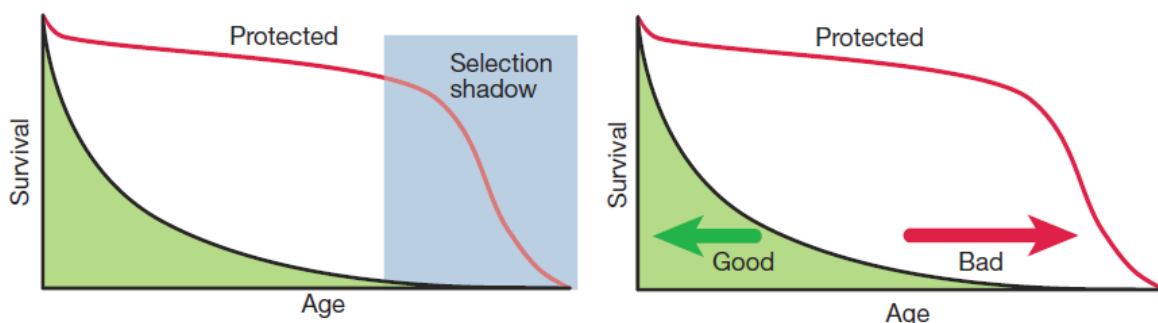
Programmed theories of aging suffer multiple flaws which have been addressed by opponents and constitute the foundations of non-programmed theories. First, Weismann suggested that an active program of aging could benefit the group by removing old uncompetitive individuals. However this argument is circular: the point of aging would be to get rid of aged beings. Second, according to programmed theories, aging would promote generational turnovers and genetic diversity. However, in the wild, it is extremely unlikely to grow old due to the predominance of natural death (predation, famine, disease, cold...): aging is not a force of mortality in the wild. Third, the finding of genes regulating lifespan is considered as proof of an aging program. However, thousands of longevity genes have been identified so far and none can completely prevent senescence: aging cannot be a simple developmental program (Kirkwood, 2005; Kirkwood & Melov, 2011).

Since aging is a passive non adaptive process unlikely to happen in the wild, the former Nobel Prize Peter Medawar proposed that allelic variants with deleterious effects

at late age could randomly evolve in the genome due to the absence of selection pressure against them (Medawar, 1952). This hypothesis called ‘the mutation accumulation theory’ stems from the fact that forces of natural selection decline after reproduction. By extension, it implies that even if de novo mutations promoting lifespan arise in the genome, they would not be retained because of selective weaknesses post-replication.

A bit later, in 1957, in the continuity of previous assertions, George Williams introduced the idea of ‘antagonistic pleiotropy’ according to which genes having beneficial effect in early life could become detrimental in the long term (Williams, 1957). To illustrate his hypothesis, he used to refer to bone calcification, which could be beneficial during growth but would generate arterial calcification at later age. Nevertheless, Williams himself admitted that his proposal lacked clear validations and, as of now, only few convincing examples have been listed (Leroi et al., 2005).

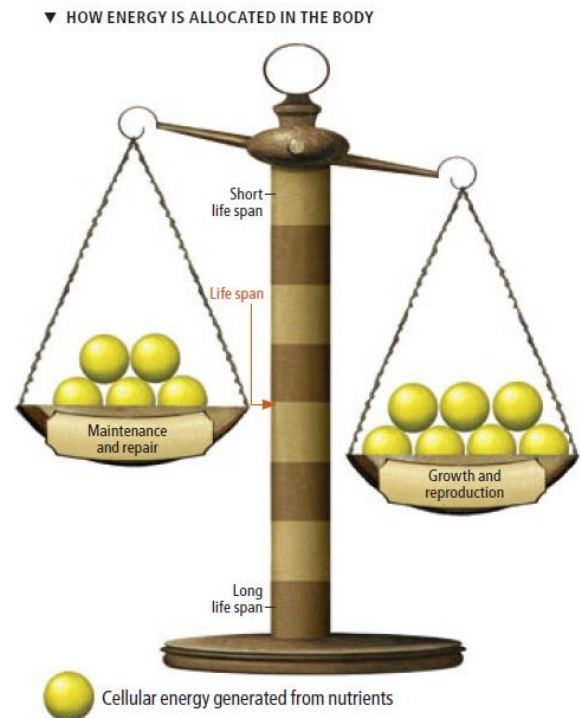
Currently, the most accepted aging theory was elaborated by Thomas Kirkwood



Non-programmed theories of aging. The mutation accumulation theory (left) suggests that the force of selection decline with age (selection shadow), allowing accumulation of late-acting deleterious mutations. The antagonistic pleiotropy (right) suggests that alleles with beneficial effect early in life could become detrimental on the long run. Red curve corresponds to the period protected from age related effects (Kirkwood & Austad., 2000).

in 1977 (Kirkwood, 1977). Following the idea of trade-offs introduced by Williams, Kirkwood proposed the ‘disposable soma theory’ according to which energetic resources are optimally allocated between somatic maintenance and reproduction (Kirkwood & Austad, 2000). Kirkwood estimated that since nutrients are scarce in the wild, the limited available energy must be tuned toward fast and efficient reproductive success, which is the most important task of any living organism to avoid extinction. Investing energy in maintenance and regeneration to prevent senescence would have a cost on reproductive capacity and would be futile since mortality rates in the wild are extremely high. For instance, more than 90% of wild mice die during their first year, mainly due to failure to maintain thermogenesis (Phelan & Austad, 1989; Berry & Bronson, 1992). Even though repair mechanisms exist and are essential, they must be balanced to maintain individual’s health until sexual maturity. Once reproductive duty has been accomplished, the soma loses its evolutionary force and decay happens since investment in maintenance does not counter progressive damage accumulation. On the other hand, germ cells must be immortal to prevent species extinction.

According to Kirkwood, lifespan variability among species has been genetically adjusted based on mortality rate and development time. Larger animals with long development and late maturity must stay healthy until they become able to reproduce. Inversely, smaller animals with low survival expectancy must spend all their efforts to grow and spawn as quickly as possible. This



The disposable soma theory. According to Kirkwood’s theory, there is an energetic trade-off between growth and maintenance (www.ScientificAmerican.com).

rule is then adjusted with the extrinsic mortality of each species: turtles, which are protected by their shells, or birds, which are out of range of predators, have evolved longer lifespans.

Kirkwood’s theory is supported by multiple founded examples. Several genes enhancing longevity while impairing growth or reproduction have been identified in mice, fruit flies, nematodes, and yeasts (Jenkins et al., 2004; Delaney et al., 2011). Evolution experiments selecting long living *Drosophila melanogaster* succeeded in isolating individuals with better longevity at the cost of reduced fertility and increased development time (Zwaan et al., 1995; Buck et al., 2000). Also, it was observed that dwarf mutant mice or fruit flies were living

longer (Brown-Borg et al., 1996; Clancy et al., 2001).

Opponents of the disposable soma theory usually consider that it fails to explain the well-known benefits of calorie restriction on lifespan: according to the theory, living organisms fail to maintain themselves due to limited energy supply, therefore, when nutrients are further restricted lifespan should be shortened, which is not the case. In fact, seen from another perspective, the disposable soma theory fits rather well with the concept of calorie restriction. During periods of starvation, reproduction is not optimal because available resources are insufficient to sustain pregnancy and to assure offspring development. Thus, until conditions become favourable again for reproduction, limited resources must be oriented toward survival of the soma (Kirkwood, 2017).

While Kirkwood's theory is widely accepted within the community, some inconsistencies remain. For instance, a certain amount of genetic interventions can extend lifespan without affecting reproduction or growth (Clancy et al., 2001; Dillin et al., 2002).

B) Hallmarks of aging

Independently of its fundamental evolutionary causes, aging is a highly multifactorial process characterised by the onset of multiple age-associated dysfunctions leading to loss of physiological integrity, as per Gladyshev's definition. In an effort to classify drivers of aging, major molecular and cellular processes involved were gathered under 9 distinct hallmarks that I

will briefly describe (Lopez-Otin et al., 2013).

B1. Genetic instability: The DNA molecule is permanently attacked by exogenous (chemicals, UV) and endogenous (oxidative stress, replication errors) stress. Despite sophisticated repair mechanisms, various kinds of DNA damages irrepressibly accumulate throughout life. Altogether, these damages induce mutations, dysregulate genetic networks and generate replicative stress, eventually culminating in cellular dysfunction and potentially cancer (Promislow, 1994; Hoeijmakers, 2009). This is the core of the so-called 'somatic mutation' theory (which must not be mistaken with the 'mutation accumulation' of Medawar) (Kirkwood, 2005). The role of DNA integrity in aging is further emphasised by human progerias, such as Werner and Hutchinson-Gilford syndromes, which are caused by genome instability (Yu et al., 1996; Eriksson et al., 2003).

B2. Telomere attrition: In 1961, Hayflick discovered that human somatic cells cannot divide endlessly, a process called replicative senescence (Hayflick, 1961). This limited replicative capacity was later attributed to telomere shortening (Olovnikov, 1973). Due to the end replication problem, telomeres gradually shrink after each cell division, until they reach a critical length triggering cellular senescence and the irreversible exit of cell cycle (Kim et al., 2002). Telomere shortening can be prevented by the enzyme telomerase which is mainly expressed in the germ line and in some stem cells (Greider & Blackburn, 1985; Chiu et al., 1996). Human somatic tissues do not express telomerase and consequently turn senescent with time, contributing to aging caused by lack of



The 9 hallmarks of aging. (Lopez-Otin et al., 2013).

tissue renewal and release of proinflammatory cytokines perturbing the microenvironment (Campisi, 2005). Nevertheless, the role of telomere shortening in cellular senescence and aging remains hotly debated (De Magalhaes & Passos, 2018). For instance, mice are short living while having extremely long telomeres and high telomerase activity (Prowse & Greider, 1995). Additionally, over-expression of telomerase in mice does not improve lifespan and increases cancer incidence (Artandi et

al., 2002; De Jesus, 2011). Lastly, it seems that telomere shortening is mainly provoked by oxidative stress rather than cellular division (Von Zglinicki, 2002).

B3. Epigenetic alterations: DNA is meticulously wrapped around proteins, namely histones, in complex structures called chromatin. Chromatin organisation is of key importance in regulating genetic expression by modulating the accessibility of transcription machinery to DNA. This

mechanism is orchestrated by post-translational modifications of histones, mainly acetylation and methylation (Sen et al., 2016). During aging, a general loss of histones is observed along with acetylation and methylation rearrangements, leading to chromatin remodelling and altered genetic expression (O'Sullivan et al., 2010; Hu et al., 2014; Fraga & Esteller, 2007). In addition of histone modification, the DNA molecule itself can be methylated on cytosine residues in CpG dinucleotides, which is an important process involved in transcriptional repression (Sen et al., 2016). During aging, it has been observed that DNA exhibits global demethylation, and localised hypermethylation (Cruickshanks et al., 2013). Interestingly, the age-associated methylome is quite reproducible, and it was found that the methylation profile at 353 distinct sites can be accurately used as an epigenetic clock to predict age (Horvath, 2013).

B4. Loss of proteostasis: Protein folding is an essential biological process required to assure their functionality. Because of various kinds of stresses, or simply due to stochastic errors, proteins may lose their native conformation, making them dysfunctional and aggregating. In most cases, specialised proteins called chaperones will be able to refold them back to their initial effective state (Hartl et al., 2011). With aging, oxidative stress increases and particularly alters proteins due to reaction between amino acid residues and oxygen species (Stadtman, 1992; Labbadia & Morimoto, 2015). Oxidised and carbonylated proteins tend to aggregate and initiate chain reactions with other cellular components, disrupting homeostasis and eventually

causing cell death and diseases such as Alzheimer (Fedorova et al., 2013; Powers et al., 2009). Repair mechanisms exist for methionine and cysteine residues which are particularly sensitive to oxidation because of their thiol group. However, oxidative damages on other residues are irreversible (Stadtman et al., 2005). To prevent accumulation of aggregating proteins, cells have evolved essential recycling mechanisms such as the proteasome and autophagy, leading to complete protein destruction. While activation of these recycling mechanisms has been consistently shown to increase lifespan, oxidative damages keep increasing during aging and eventually overwhelm repair systems (Morrow et al., 2004; Eisenberg et al., 2009; Liu et al., 2011).

B5. Deregulated nutrient sensing: Perception of nutrient availability is of vital importance for organisms to adjust and respond appropriately to their environment. In mammals, the protein hormones insulin and insulin-like growth factor 1 (IGF-1), and the target of rapamycin (TOR) pathway, are pillars of the nutrient sensing signalling (Efeyan et al., 2015). In echoes with the disposable soma theory, activation of insulin/IGF-1 and TOR signalling stimulates anabolic metabolism, promoting growth and replication, while inhibiting protective processes like autophagy and DNA repair (Li et al., 2014; Efeyan et al., 2015; Vermeij et al., 2016). Interestingly, insulin/IGF-1 and TOR pathways are highly conserved through evolution and their orthologues strongly regulate lifespan in model organisms (Kenyon, 2001). During the course of aging, mammals develop hyperinsulinemia and insulin resistance

causing hypertension, dyslipidemia, obesity, diabetes and cancer (Bartke, 2008). Conversely, calorie restriction, whose pro-longevity effect is mainly mediated by down-regulation of nutrient sensing pathways, reduces circulating insulin while enhancing its sensitivity (Fontana et al., 2004).

B6. Mitochondrial dysfunction: Mitochondria are mainly known for being cellular energy factories due to their role in ATP synthesis by respiration. In the 1970s, progressive elucidation of the respiratory chain mechanisms led to the discovery that electron leakage could occur during the process and constitutes the main source of reactive oxygen species (ROS) (Chance et al., 1979; Cadenas & Davies, 2000). Due to their capacity to damage biomolecules (DNA, proteins, lipids), Harman proposed that aging is caused by ROS accumulation driven by mitochondria (Harman, 1956; Harman, 2003). Mitochondria themselves are the first victims of ROS production. During aging, mitochondrial DNA mutations and mitochondrial dysfunction increase, leading to impaired ATP synthesis and cell death (Kujoth et al., 2007; Cottrell et al., 2001). While the original theory of Harman holds true, it is now established that mitochondria are involved in key metabolic processes, such as amino acid, lipid and iron/sulfur cluster synthesis, which could have stronger implication than ROS production in aging (Breitenbach et al., 2014). This idea is supported by experiments showing that mitochondrial dysfunction can accelerate aging without increasing ROS levels (Edgar et al., 2009; Ristow & Schmeisser, 2011).

B7. Cellular senescence: As described previously, Hayflick found several decades ago that human somatic cells can only

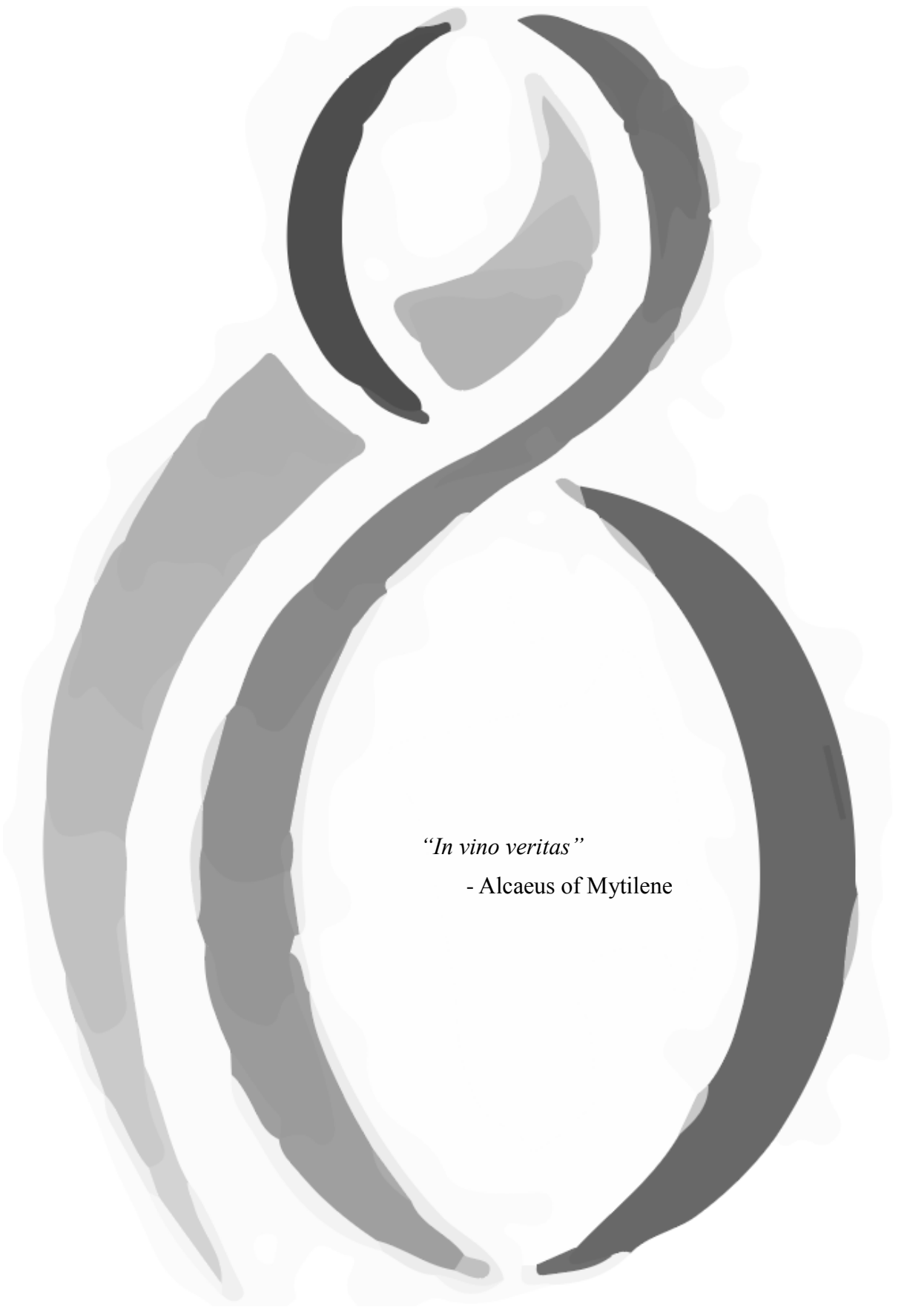
divide a finite amount of times before irreversibly exiting cell cycle and becoming senescent (Hayflick, 1961). Senescent cells are flattened and enlarged, they remain metabolically active and exhibit significant changes in gene expression and chromatin organization (Jeyapalan & Sedivy, 2008). Originally it was thought that senescence is only triggered by telomere shortening. However, there are now multiple evidences showing that senescence can be induced by DNA damage and oxidative stress, independently of telomere length and replication (VonZglinicki, 2002; Melk et al., 2003; Collado et al., 2007). During aging, the number of senescent cells increases in response to cellular stress (Dimri et al., 1995). On the one hand, senescence induction is related to oncogene activation and constitutes a major protection against cancer (Wynfor-Thomas, 1999). On the other hand, senescent cells are secreting proinflammatory cytokines and others extracellular factors disrupting tissue homeostasis and contributing to aging (Rodier & Campisi, 2011).

B8. Stem cell exhaustion: Stem cells are undifferentiated cells with high proliferative potential involved in tissue homeostasis by replacing dying somatic cells. With age, tissue regenerative capacity declines, as witnessed by low repair of muscle fibers, impaired intestinal function, or immunosenescence (Lopez-Otin et al., 2013; Rando, 2006). Unlike somatic cells, stem cells express telomerase and consequently should not have a limited replicative capacity (Chiu et al., 1996). Nevertheless, during aging, stem cells become senescent and display impaired functions (Martin et al., 2000; Molofsky et al., 2006). While stem

cells may experience age related oxidative stress and DNA damage similarly to somatic cells (Rossi et al., 2007), some results suggest that alteration of the surrounding environment is the cause of stem cell attrition (Rando, 2006). This idea is supported by parabiosis experiments in which exposition of declining stem cells from old mice was rescued by exposition to young systemic milieu (Conboy et al., 2005). Lastly, even though stem cell decay somehow contributes to the aging of tissues with high cellular turnovers, their implication in maintenance of non-regenerative organs such as brain or heart is doubtful (Rando, 2006).

B9. Altered intercellular communication:

Multicellular organisms are made of interdependent organs and tissues that require a close degree of interaction and communication to be functional. With aging, endocrine signalling becomes dysregulated, impairing intercellular communication (Russell & Kahn, 2007). Because of the involvement of the brain in regulating hormonal levels, the neuroendocrine theory of aging proposed that the brain could act as a master clock through hormonal regulation during life (Everitt, 1980). However, although hormonal anti-aging therapies might slightly improve elder's health, they have undesirable side effects and fail to increase lifespan (Blackman et al., 2002; Dominguez et al., 2009). Beyond the endocrine system, local cellular communication also impacts aging. This is the case of the secretions released by senescent cells, namely “senescence secretory associated phenotype” (SASP), which are contributing to stem cell decline (Rando, 2006; Drummond-Barbosa, 2008).



“In vino veritas”

- Alcaeus of Mytilene

Chapter 3

Saccharomyces cerevisiae, a budding aging model

Aging has historically been seen from a fatalistic point of view. Before Darwin, aging was contemplated as an irrepressible loss of vital energy, when not considered to be limited by divine laws. After Darwin, aging was thought to be either a sophisticated developmental program, or a non-adaptive passive and entropic mechanism. In any case, rare were those believing that anything could be done against it. Nevertheless, during the Renaissance a wave of pro-longevity emerged, driven by the strong conviction that sober and balanced lifestyles could lead to century old ages (which was not fundamentally wrong, albeit exaggerated). Later, at the end of the 19th century, in a new surge of optimism, the physiologist Brown-Sequard thought he could rejuvenate by injecting himself extracts of animal testicles (Jeune, 2002). An attempt that remained sterile and did not encourage further research.

In fact, the interest for aging research really boomed at the beginning of the 1990s, when Johnson and Kenyon discovered the first genes extending lifespan (Johnson, 1990; Kenyon et al., 1993). For the first time, it became evident that aging was not genetically immutable. Today, aging has become a major axis of research. Several hundred genes regulating lifespan have been uncovered across multiple model organisms and led to the fundamental observation that the underlying mechanisms of aging are strongly conserved from yeasts to humans.

In this chapter, I will review the use and the relevance of the budding yeast *S. cerevisiae* in aging research.



A pleiotropic organism. From rum industry to aging research, yeasts can do everything. For sure, *Jack Sparrow* won't complain about it.

A) Unravelling the genetics of aging using yeasts

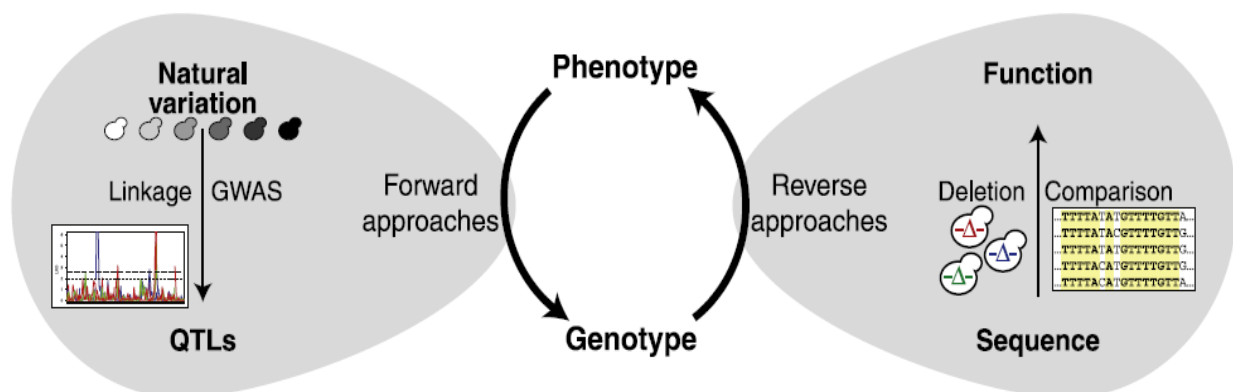
A1. Dissecting the architecture of complex traits

Seventeen years. It is the time elapsed since the sequencing of the first human genome (Venter et al., 2001). Although it was, and still is, a major scientific milestone, interpreting the ensuing dataset probably felt like reading a book written in Ancient Greek: you can gauge the overall structure, guess where the chapters are, but it does not make much sense. Thus, the next major endeavour of the scientific community was to give a meaning to these billions of DNA base pairs. While this task is still ongoing, substantial progress was made in delimiting the genes and attributing them a core function. However knowing that a gene encodes a kinase protein, a transcription factor, or a transmembrane component is only the first step. The key underlying challenge is to link genotype and phenotype with each other.

The only common point between tomato size and aging (that I am aware of) is that they are both complex traits. When I speak

with non-scientific people, they often assume that each single gene entirely defines a trait. Hence, there should be a gene determining the size of a tomato and another one setting people's life duration. Even though I wish genetic was that straightforward, in practice most observable traits are defined by complex interactions between hundreds, if not thousands, of genes with themselves and with the environment. Consequently, unlike Mendelian traits that are monogenic and end up in a binary phenotypic distribution (round or wrinkled pea seed), complex traits result in a continuous phenotypic distribution with an almost infinite number of shades. For this reason, complex traits are commonly referred to as quantitative traits and are the roots of population diversity.

Dissecting the genetic architecture of a complex trait is a tedious task that can be tackled by two different approaches: reverse or forward genetics (Nieduszynski & Liti, 2011). The core principle of reverse genetics consists in identifying sequence divergences or creating them manually by mutagenesis or genetic engineering before observing the subsequent effect on a given phenotype. Reversely, forward genetics stems from



Forward and reverse genetics. Reverse genetics tries to infer a phenotype from the genotype, while forward genetics tries to infer a genotype from the phenotype (Nieduszynski & Liti, 2011).

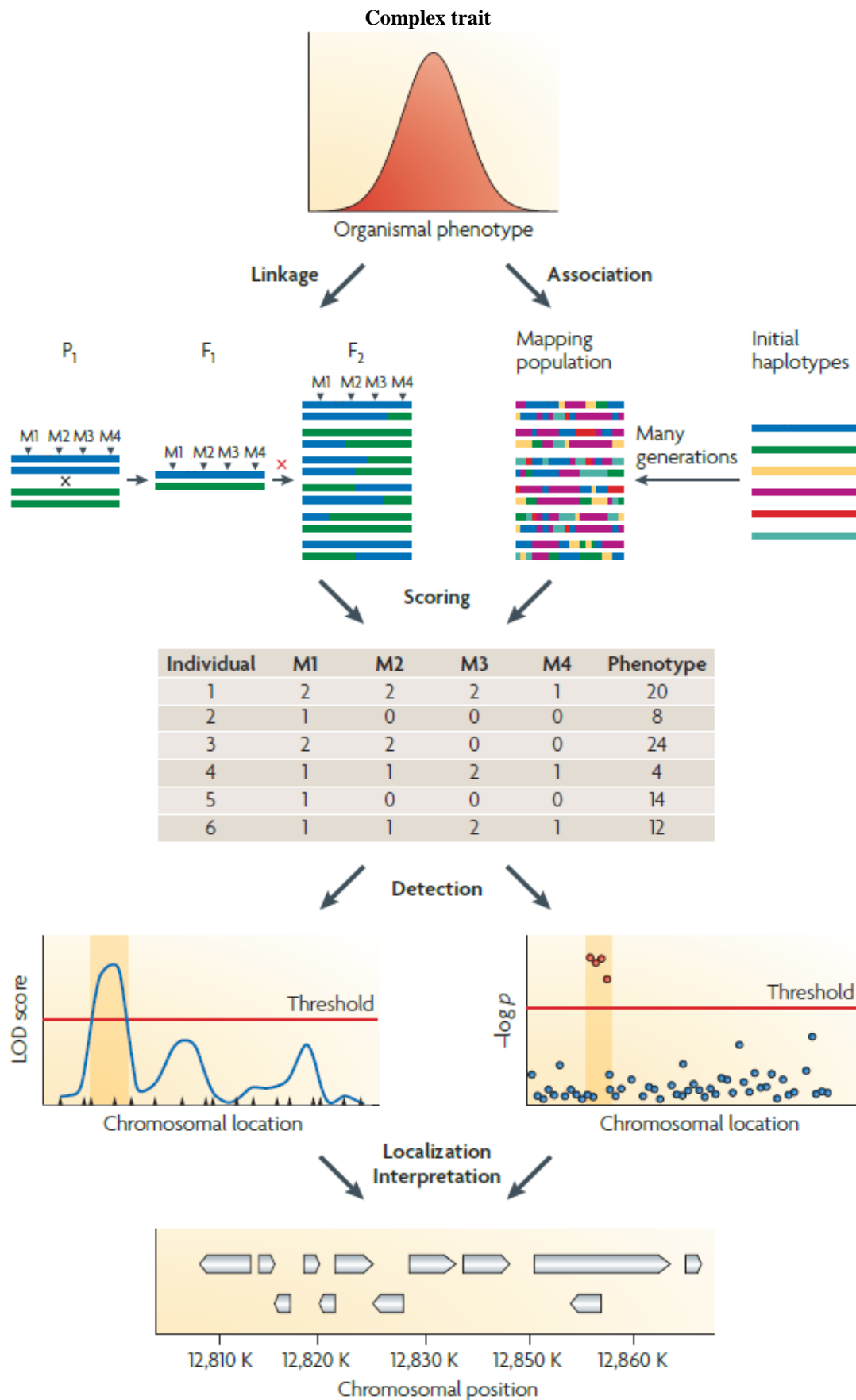
phenotypic variability and tries to associate it with genomic data to identify causal genetic locations, namely Quantitative Trait Loci (QTLs), or possibly causal polymorphisms, namely Quantitative Trait Nucleotide (QTN).

More concretely, forward genetic studies can be performed according to two distinct strategies: genome-wide association studies or linkage analysis (Mackay, 2009; Liti & Louis, 2012). On the one hand, genome-wide association studies (GWAS) make use of historical genetic recombinations occurring within natural populations, while on the other hand linkage analyses make use of artificial outbred crosses. Both methods rely on the segregation of genetic markers in between individuals fostered by recombination events. Markers co-segregate with causative variants due to genetic linkage and thereby help to locate genes regulating the studied phenotype. Based on this principle, the more the genome is shuffled, the better the resolution is. Thus, taking advantage of the extremely high amount of recombinations which occurred throughout evolution, GWAS has a higher sensitivity compared to linkage analysis. However, GWAS fail to detect rare variants and are biased by population structures (Mackay, 2009; Nieduszynski & Liti, 2011). Conversely, because they are based on experimental crosses, linkage analyses do not suffer these caveats. In the meantime, improved strategies were developed to overcome the resolution issues of linkage analysis. For instance, the X-QTLs approach uses large pool of segregants submitted to selection before analysing allelic variation by sequencing (Ehrenreich et al., 2010). The iQTL pushes further the

concept of X-QTLs by using advanced intercross lines instead of F1 hybrid, thus increasing the recombination landscape (Parts et al., 2011; Cubillos et al., 2013).

S. cerevisiae is a unicellular fungus, wrapped into a rigid proteo-glycan cell wall. It has a small genome size (12Mb) made of 16 chromosomes and was the first eukaryote organism fully sequenced (Goffeau et al., 1996). Owing to its intrinsic aptitudes it has been excessively successful at dissecting complex traits and has arguably the best annotated genome (SGD: www.yeastgenome.org).

Yeasts mainly reproduce quickly by vegetative reproduction (approximately 90 minutes per generation), but can also be easily engaged in sexual cycles permitting outbreeding. Its life cycle is perfectly understood and mastered: the field of yeast genetics was pioneered in the 1930s, at the Carlsberg laboratory, by Ojvind Winge whose breeding experiments were aimed at improving brewing characteristics (Barnett, 2007). Business-making and beer have always been strong drivers of scientific progress, especially in the field of yeast genetics. This facility makes yeast an ideal candidate for forward genetics. However, while the art of QTL mapping is practiced since the beginning of the 20th century (Sax, 1923), it has long been dismissed in yeasts for the sake of reverse genetics. The rise of yeast quantitative genetics really started in 2002 with two groundbreaking publications studying the architecture of heat resistance and transcript abundance using linkage analysis (Steinmetz et al., 2002; Brem et al., 2002). Since then, multiple linkage-based studies have contributed to decipher the genetic basis of a plethora of traits such as



Quantitative trait locus mapping. Experimental procedure of linkage analysis (left side) and GWAS (right side). M_x: genetic polymorphisms. P_x: parental strains. F_x: generation number (Mackay, 2009).

ethanol tolerance, acetic acid production, telomere length or mitochondrial instability (Hu et al., 2007; Marullo et al., 2007, Gatbonton et al., 2006, Dimitrov et al., 2009). On the other hand, yeast GWAS remain largely unexplored because of limited sampling and high population structures (Nieduszynski & Liti, 2011). In fact, the first successful yeast GWAS was performed this year (2018) thanks to the whole-genome sequencing and phenotyping of an unprecedented wide yeast collection (Peter & De Chiara et al., 2018).

Since the 1980s, we know how to accurately genetically engineer yeasts by taking advantage of its proficient homologous recombination repair machinery (Ito et al., 1983). Thanks to this know-how, researchers have the possibility to alter the genome in any way they desire within a very short time-lapse. This property makes yeasts also very powerful to perform reverse genetic approaches. Besides generating targeted mutants, this technology made possible the creation of invaluable strain libraries such as the ORF deletion, the over-expression, and the GFP-tag collections which are available to the whole community (Winzeler et al., 1999, Giaever et al., 2002, Huh et al., 2003, Sopko et al., 2006). Using these collections, systematic genome-wide screens were run to study the effect of each single gene on a given phenotype. In 2004, after only 5 years of exploitation, the yeast deletion collection already substantially contributed to unravel the genetic basis of complex traits by assigning more than 5000 genes to over 100 different phenotypes tested (Scherens & Goffeaur, 2004).

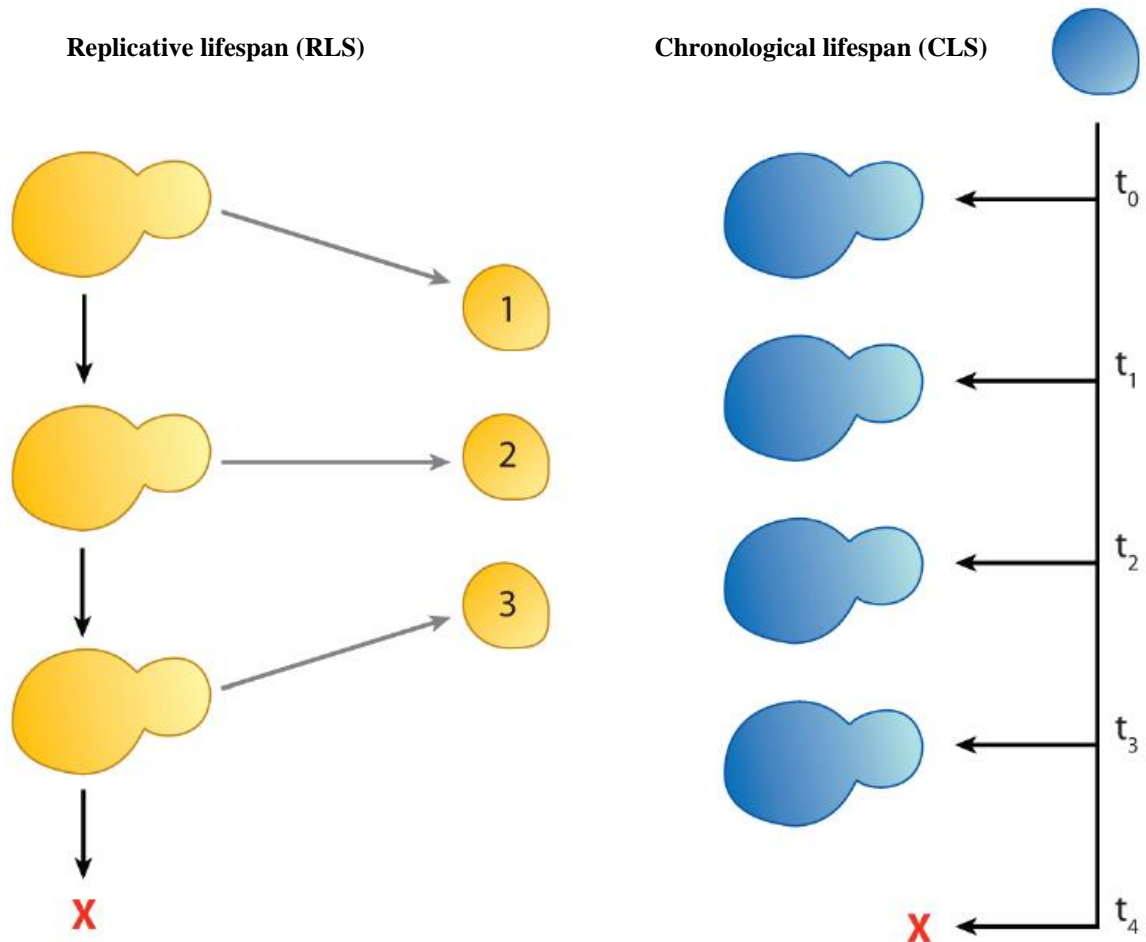
Altogether, forward and reverse genetics in yeasts have allowed researchers to define

genetic and protein interaction networks at an unparalleled level compared to other model systems (Botstein & Fink, 2011).

A2. One organism, two aging paradigms

While it may sound surprising at first, the budding yeast has become a pivotal model in aging research. In addition to the conservation of aging mechanisms with higher eukaryotes, the unprecedented knowledge and control we have of this organism, coupled with its inherent cost-efficient tractability, makes yeast a very powerful model system. When working on aging, *S. cerevisiae* offers two different approaches.

First, the yeast replicative lifespan (RLS) is defined as the number of divisions a single cell can perform throughout its life. It is proposed as a paradigm to study the aging of proliferative tissues such as stem cells (Steinkraus et al., 2008; Longo et al., 2012; Wasko & Kaeblerlein, 2014). This approach was established in 1959 by Mortimer and Johnston who took the advantage that *S. cerevisiae* divides asymmetrically by budding (Mortimer & Johnston, 1959). Smaller new-born daughter cells are budding away from their mother which can thus be identified and tracked. Interestingly, daughters are rejuvenated and recover a full replicative potential, while mothers retain various aging factors (Denoth Lippuner et al., 2014). The RLS assay is still mostly performed with the age-old technique of microdissection: every 90 minutes the bud must be manually removed using a microscope equipped with a thin needle in order to keep track of the mother. Thus, the full lifespan of each mother must be individually followed from beginning to end.



Yeast aging models: RLS and CLS. RLS (left) is based on the number of divisions a single cell can perform before dying, while CLS (right) is based on the duration yeasts can survive in non-proliferating conditions. t_x : time intervals (Steinkraus, 2008).

This very laborious technique is the main limitation of RLS and usually prevents high-scale experiments. Fortunately, alternative methods are emerging and opening new perspectives. Old cells can be sorted and enriched using their size via elutriation (Laun et al., 2001), or using their cell wall via a specific biotin-streptavidin tagging system (Smeal et al., 1996). In addition, two other strategies now allow monitoring the whole RLS at medium throughput. On the one hand, microfluidic consists in trapping mother cells in small chambers and to follow their lifespan by continuous picture acquisition while new-born daughters are washed away by a steady media flow (Lee et al., 2012; Xie et al., 2012). On the other

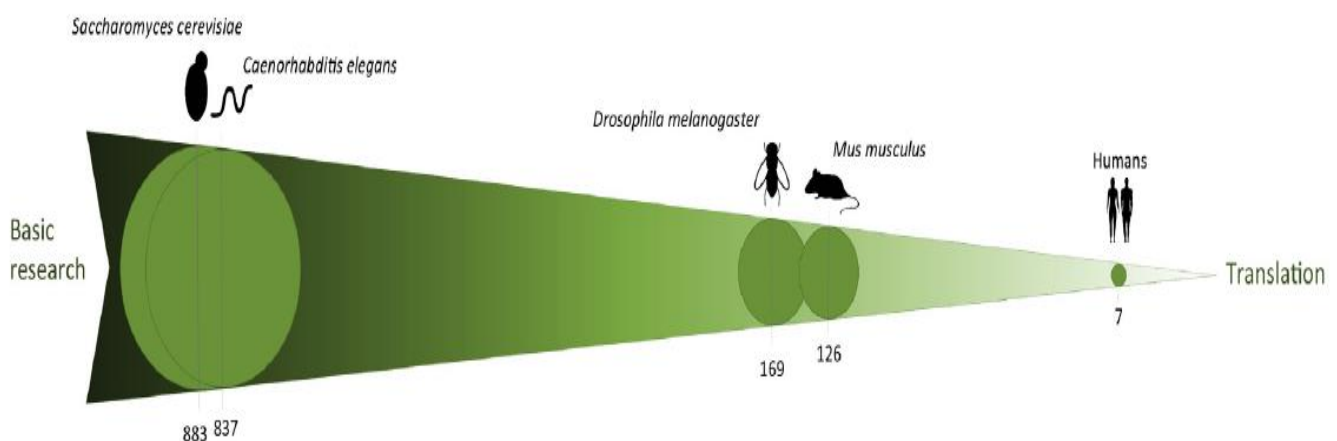
hand, the mother enrichment program (MEP) relies on an activatable genetic engineering that specifically kills the daughters (Lindstrom & Gottschling, 2009).

Second, the chronological lifespan (CLS) is defined as the duration yeasts can survive in non-proliferating conditions. It was developed as a paradigm to study the aging of post-mitotic tissues, conversely to RLS (Longo et al., 1996; Longo & Fabrizio, 2012; Longo et al., 2012). Typically, yeasts are grown and kept in liquid cultures. In less than 24 hours, glucose becomes exhausted and yeasts enter the post-diauxic phase. During this stage, they switch to a respiration-based metabolism and consume

the by-products of fermentation (especially ethanol), maintaining a high metabolic rate but slowly dividing (Werner-Washburne et al., 1996). Yeasts spend between 2 and 7 days (depending on the medium used) in post-diauxic phase before entering in a stationary phase. From this point, aliquots of the culture are regularly sampled and cellular viability is tested by checking yeast capacity to resume growth and form colonies on agar plate, a technique called colony-forming unit (CFU) (Fabrizio & Longo, 2007). While CFU is practically simple and cheap, it is labour-intensive and suffers a certain imprecision, although it remains the gold standard. Alternatively, viability can be monitored by cytometry, using viability fluorescent stainings (Ocampo & Barrientos, 2011). A strategy that is faster, more accurate and that can be scaled to high-throughput experiments (see results). High-throughput assays can also be performed using a technique based on the regrowth of viable cells in liquid cultures: a bit similarly to the principle of qPCR, the more yeasts are viable, the faster they will reach a

certain optical density (Powers et al., 2006; Murakami et al., 2008; Garay et al., 2014). A last high-throughput alternative takes advantage of the barcoded deletion collection: the deletion collection strains are grown altogether as a pool and chronologically aged before the whole DNA is extracted and the frequency of each mutant determined by microarray or sequencing (Fabrizio et al., 2010; Matecic et al., 2010; Gresham et al., 2011).

Extensive work done on yeast aging the past two decades has led to the current identification of 491 genes regulating RLS and 1261 genes regulating CLS (SGD website), making *S. cerevisiae* the best characterized model of aging. This success mainly lies on the wealth of data brought by screening the entire deletion collection. Indeed, large-scale surveys contributed to identify 20% and 77% of genes regulating RLS and CLS, respectively. This difference between the two models stems from the low scalability of RLS. The first full screen of the deletion collection was only released



***S. cerevisiae* is the best characterized aging model.** Number of genes annotated to regulate aging in different model organisms and in humans, according to GenAge database (<http://genomics.senescence.info/genes/>). The area of each circle is proportional to the number of genes. (De Magalhaes et al., 2017).

recently, at the cost of tremendous efforts (more than 2,2 million daughters were microdissected) (McCormick et al., 2015). On the other hand, the deletion collection CLS has already been processed multiple times in different conditions (Powers et al., 2006; Fabrizio et al., 2010; Matecic et al., 2010; Gresham et al., 2011; Garay et al., 2014; Campos et al., 2018). Probably because of the success of reverse genetics, forward approaches remain almost unexplored in the field of yeast aging. Only 4 studies report QTL mapping for both models: two dedicated to RLS (Stumpferl et al., 2012; Kwan et al., 2013), and the two others for CLS (Kwan et al., 2011; Jung et al., 2018).

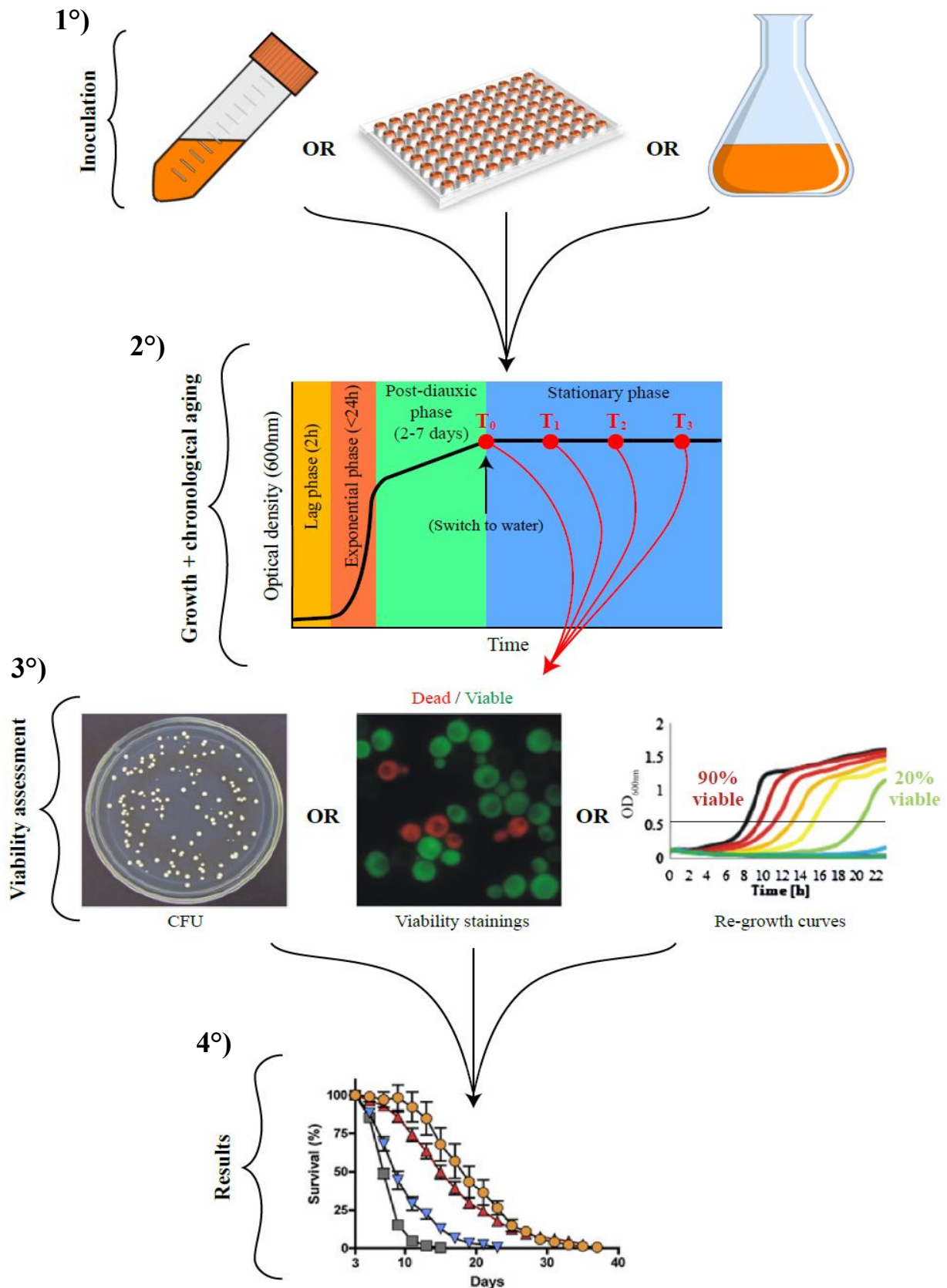
B) The yeast aging hallmarks

With aging, both models show signs of physiological decline. Replicatively aged yeasts experience sterility and slower growth rate (Lee et al., 2012; Muller, 1985; Boselli et al., 2009), whereas chronologically aged yeasts lose their replicative potential (Ashrafi et al., 1999). Similarly to higher eukaryotes, these aging characteristics stem from molecular dysfunctions. In the next paragraphs I will review the relevance and findings of *S. cerevisiae* regarding the hallmarks of aging described in the previous chapter.

B1. Genetic instability: Genetic instability is increasing with age in both aging models. In replicatively old cells, the amount of loss of heterozygosity is escalating exponentially as a result of elevated DNA damages (McMurray & Gottschling, 2003). Interestingly, increased frequency of loss of

heterozygosity has been linked with replication stress and mitochondrial dysfunction due to defective iron-sulfur cluster biogenesis (Veatch et al., 2009; Lindstrom & Gottschling, 2011). Genetic interventions impairing DNA repair, such as deletion of *SGS1* (ortholog of the human *WRN* protein involved in Werner syndrome) or *DNA2* cause premature aging and shorten RLS (Sinclair et al., 1997; Hoopes et al., 2002). The DNA damages induced during replicative aging are not transmitted to daughters which are fully rejuvenated. Therefore, it suggests that lifespan shortening by DNA instability is the result of accumulated exogenous damages on the molecule, rather than the result of irreversible mutations on essential genes.

In chronologically aged yeasts, DNA damage evidences are manifested under the form of base substitutions, indels, gross chromosome rearrangements (Longo et al., 1999; Maclean et al., 2003; Fabrizio et al., 2004; Madia et al., 2009), and also loss of heterozygosity (Qin et al., 2008). In addition, inducing replicative stress by using the dNTPs biosynthesis inhibitor hydroxyurea, or by deleting of DNA repair genes (*MEC1*, *RAD53* or *RAD27*) prevents cells to enter quiescence during stationary phase and shortens CLS (Weinberger et al., 2007; Weinberger et al., 2013; Laschober et al., 2010). Non-quiescent cells are particularly short living and prone to necrosis or apoptosis. Transcriptomic studies of non-quiescent cells reveal mRNA enrichment for proteins involved with DNA repair, recombination and rearrangement, suggesting a link between high genetic instability and cell death (Davidson et al., 2011).



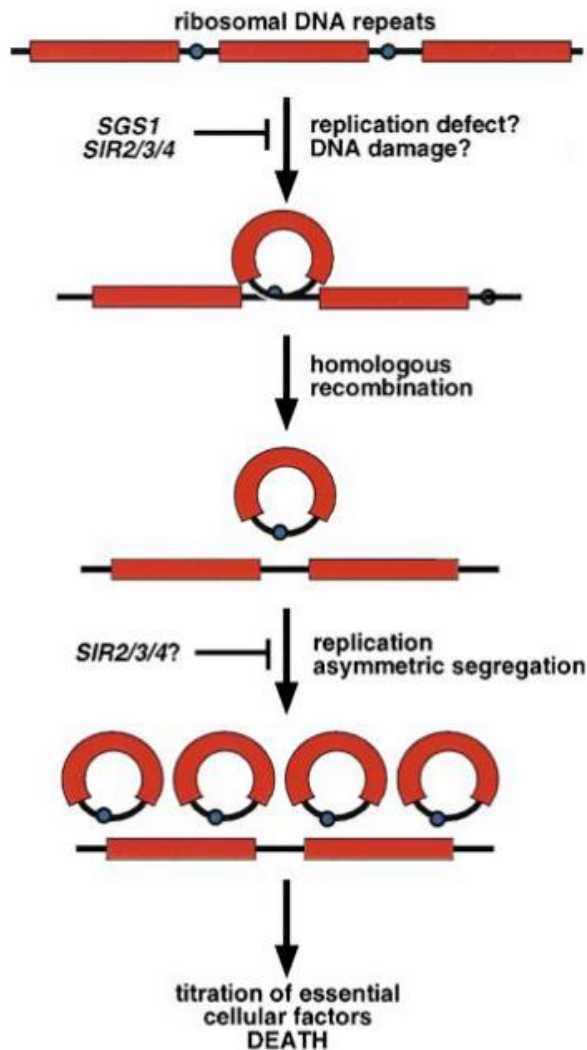
The CLS assay. Experimental procedure of the CLS assay. T_x : timepoints. (adapted from Longo et al., 2012; Kwolek & Zdrag, 2014).

B2. Telomere attrition: Yeasts constitutively express telomerase unlike mammalian somatic cells. Thus, somewhat similarly to immortal cell lines, yeasts do not experience clonal senescence (otherwise they would get extinct) and telomere shortening was ruled out from the causes of replicative aging (D’Mello & Jazwinski, 1991).

Regarding CLS, only one study reports a link between aging and telomeres. Kwan and colleagues identified a loss of function polymorphism in the protein *BUL2*, component of an ubiquitine ligase complex regulating amino acid permease trafficking (Gatbonton et al., 2006; Kwan et al., 2011). Ineffective *BUL2* leads to increased amino acid levels which eventually shorten lifespan and slightly extend telomeres through inhibition of the nitrogen responsive transcription factor *GLN3*. However, despite this common regulatory mechanism, telomere length is not affected during chronological aging, and is not considered having a role in lifespan regulation either.

B3. Epigenetic alterations: In 1997, Sinclair and Guarente discovered that episomal DNA originating from the rDNA locus, namely extrachromosomal rDNA circles (ERCs), are key drivers of yeast replicative aging (Sinclair & Guarente, 1997). The rDNA locus is repeated 80-150 times in tandem and is particularly prone to suffer double strand breaks due to replication fork stalling in between repeats (Takeuchi et al., 2003). Repair of double strand breaks generates an ERC in 50% case, consequently to the Holliday junction resolution (Laun et

al., 2007). Once an ERC is formed, it gets duplicated at every cell division because of the presence of an autonomous replication sequence, and it accumulates exponentially in mother cells that are retaining them away from daughters (Steinkraus et al., 2008; Denoth Lippuner et al., 2014). In old mothers, ERC mass gets bigger than genomic DNA itself, and disrupts cellular homeostasis. Decreasing the rate of homologous recombination by deleting *FOB1*, a protein involved in replication fork of stalling, lowers ERCs formation and improves RLS (Defossez et al., 1999). Conversely, deletion of the histone deacetylase *SIR2* promotes intrachromosomal recombination within the rDNA locus and considerably shortens RLS (Kaeberlein et al., 1999). Since this finding, histone deacetylases from the sirtuin family received a lot of attention in the aging field. The sirtuin *SIR2* has now been associated with ERC independent processes such as segregation of damaged proteins (Erjavec et al., 2007). In addition, it seems that rDNA instability in general impacts RLS more than ERCs accumulation (Lindstrom & Gottschling, 2011). Altogether, the effect of *SIR2* on lifespan could stem from a global drop in sirtuin levels with age associated with nucleosome loss, histone hyperacetylation, and decreased transcriptional silencing (Dang et al., 2009; Feser et al., 2010). However, *SIR2* activity has the opposite effect on CLS, suggesting that despite similarities, RLS and CLS mechanisms are somewhat different (Fabrizio et al., 2005).

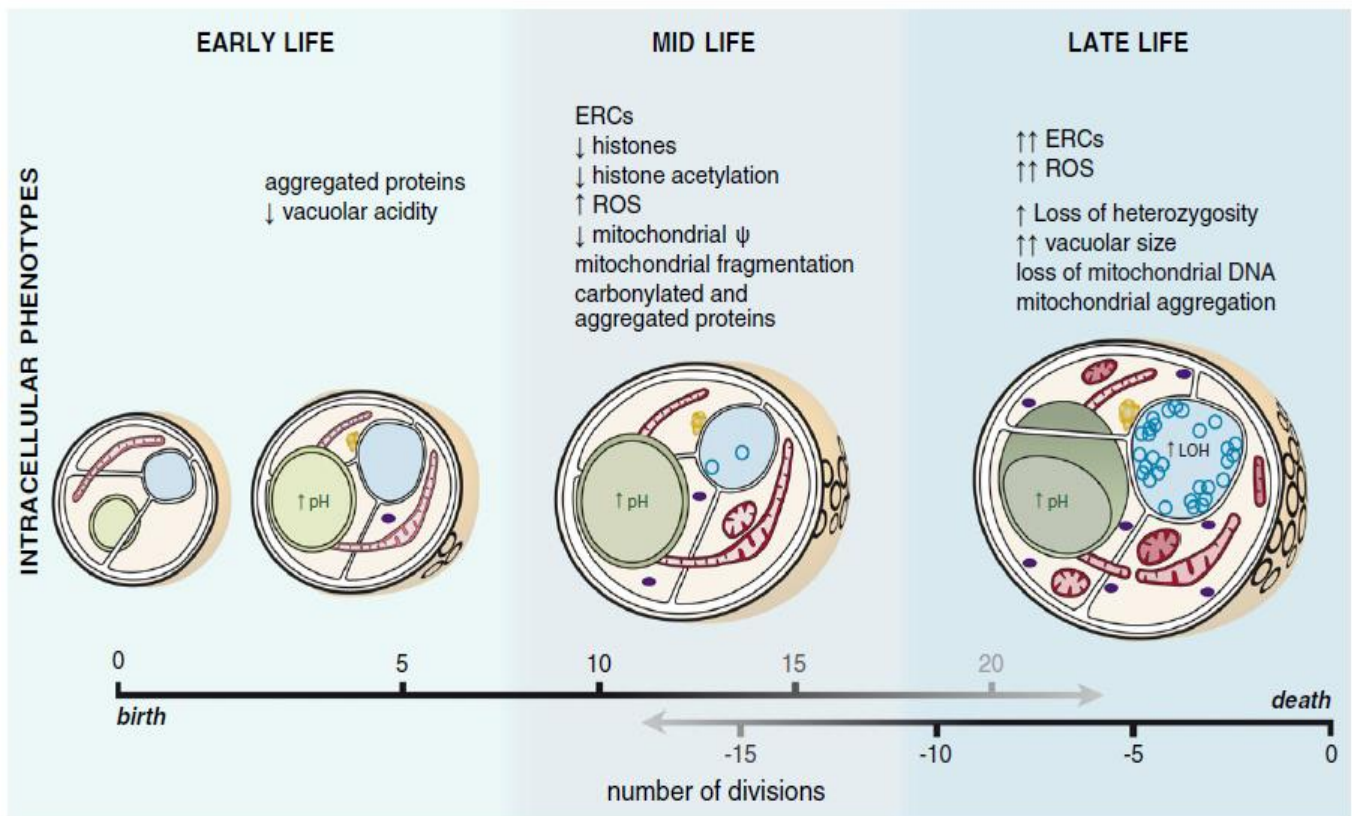


ERCs formation. ERCs are formed upon repair of double strand breaks within the rDNA locus. They accumulate specifically in mother cells and are a cause of aging. The blue dot represents a DNA replication origin (Johnson et al., 1999).

Epigenetic effects have not been much studied in the field of yeast CLS. The role of sirtuins has mainly been observed under the prism of chromatin spatial organization. Indeed, upon starvation, *SIR3* is required to gather telomeres in a large cluster at the middle of the nucleus. This telomere clustering requires ROS signalling generated by respiration during post-diauxic phase and is needed to promote survival of quiescent

cells (Guidi et al., 2015; Schafer et al., 2008).

B4. Loss of proteostasis: In 2003, Aguilaniu and colleagues found that oxidized and carbonylated proteins accumulate during yeast replicative aging and are specifically retained by mother cells (Aguilaniu et al., 2003). This partitioning of damaged proteins is orchestrated by actin cytoskeleton and is essential for daughter's rejuvenation since disruption of actin filaments with genetic intervention or latrunculin-A shortens daughter's replicative lifespan (Erjavec et al., 2007; Shcheprova et al., 2008). In addition, retention of damaged proteins requires the chaperone *HSP104*, involved in protein aggregates clearance, and the sirtuin *SIR2* as stated above, although its role remains elusive (Erjavec et al., 2007). Interestingly, recent studies found that recruitment of *HSP104* to aggregated proteins requires oxidation of the peroxiredoxin *TSA1*, highlighting a new role of ROS signalling and redox proteins in proteostasis (Hanzen et al., 2016). Proteostasis is also maintained by autophagy which is taking place in yeast vacuoles. Abrogation of vacuolar fusion by deletion of the genes *ERG6* or *OSH6* shortens RLS (Tang et al., 2008; Gebre et al., 2012). Additionally, maintenance of vacuolar acidity during aging by over-expressing the proton pump *VMA1* extends RLS. Surprisingly, this intervention also delays mitochondrial dysfunction through an unknown mechanism (Hughes & Gottschling, 2012). Along autophagy, elevated proteasome activity extends RLS as well (Kruegel et al., 2011).



Aging hallmarks during RLS. Nucleus is colored in blue, vacuole is in green, mitochondria are in red, carbonylated proteins are in purple, aggregated proteins are in yellow, ERCs are displayed as blue circles (adapted from Denoth Lippuner et al., 2014).

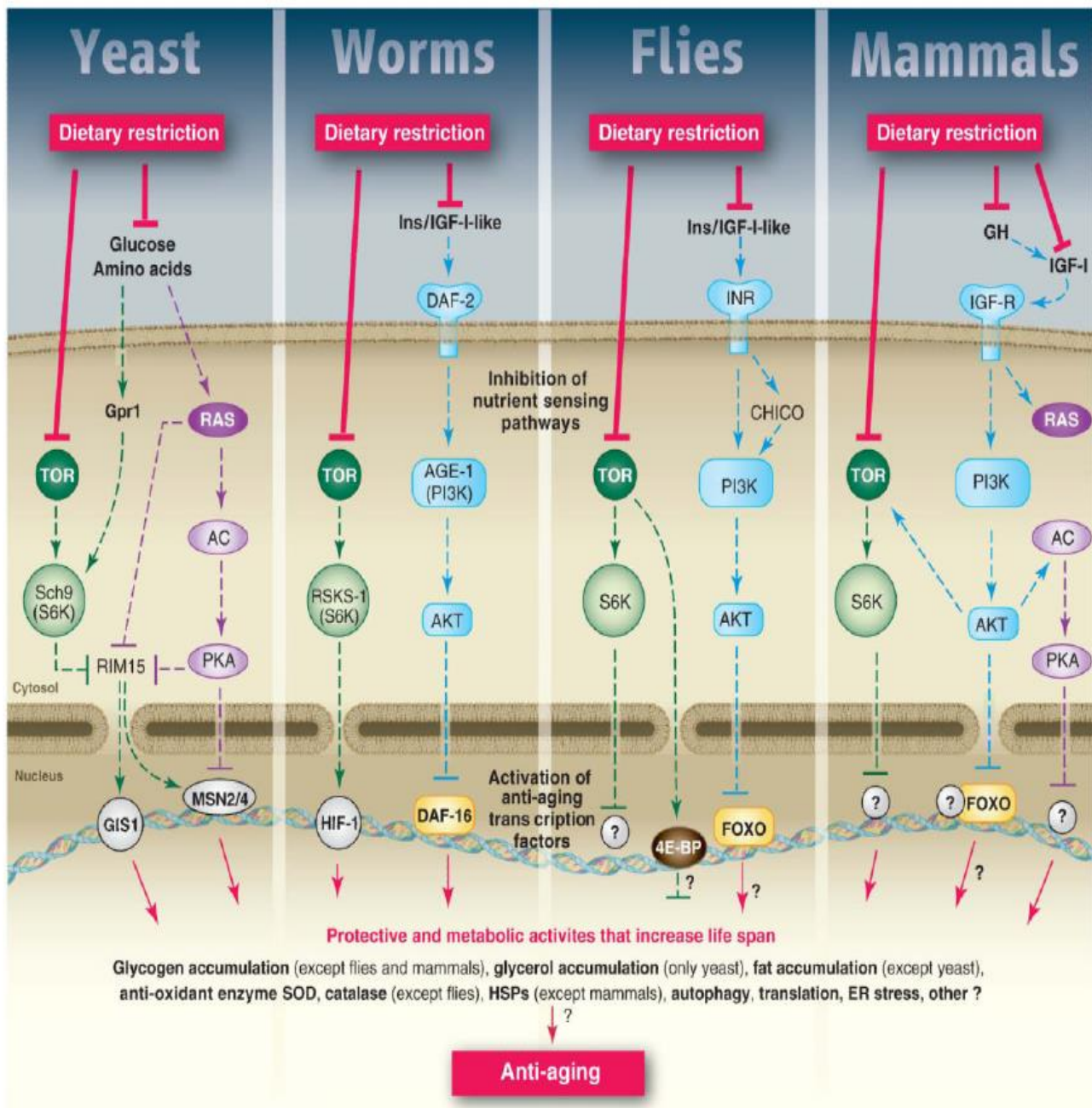
Proteostasis is also of high importance during chronological aging. While damaged protein accumulating during RLS are cleaned by mother filtration, chronologically aging yeasts cannot get rid of damaged material and thereby require sophisticated maintenance mechanisms (Stroikin et al., 2005). Moreover, CLS is performed in restricted environments, preventing growth and de novo protein synthesis. Cellular components must therefore be cautiously recycled to maintain viability over time. Presumably for this reason, autophagy is activated in response to starvation, and especially in the case of nitrogen restriction (Xie et al., 2008; Alvers et al., 2009). Consequently, abolishment of autophagy by deleting the mandatory genes *ATG1* and *ATG7* substantially shortens CLS (Alvers et al., 2009). Likewise, two genome-wide

screens have identified loss of genes encoding autophagy proteins among the most represented category shortening CLS (Fabrizio et al., 2010; Matecic et al., 2010). In addition to autophagy, proteasome activity is also increasing upon starvation and required for survival. Subsequent activation by *UMP1* over-expression, involved in proteasome maturation, extends CLS (Chen et al., 2004; Chen et al., 2006). Lastly, chaperones are also strongly induced and needed for survival in stationary phase (Miller-Fleming et al., 2014).

B5. Nutrient sensing: The first genes substantially enhancing yeast CLS were isolated by screening for heat and oxidative stress resistant mutants (Fabrizio et al., 2001), in virtue of the observation that stress resistance and longevity were

associated (Larsen, 1993; Lithgow et al., 1995). By doing so, low expression mutants of the genes *SCH9* and *CYR1* were found to consequently increase CLS in addition of conferring high stress resistance (Fabrizio et al., 2001). The genes *SCH9* and *CYR1* are core components of the target of rapamycin (TOR)/*SCH9* and *RAS*/Adenylate cyclase/PKA axis which are the two main

nutrient sensing pathways in yeast (Broach, 2012). A bit later, (partial) genome-wide screens for RLS and CLS both identified mediators of the TOR pathway as strong regulators of yeast aging (Kaeberlein et al., 2005; Powers et al., 2006). Interestingly, the kinase protein *SCH9* is a direct ortholog of mammalian *AKT* and *S6* kinases which are acting downstream of TOR and



insulin/IGF-1 pathways. On the other hand, *CYR1* encodes for the adenylate cyclase, equivalent of mammalian *AC5* which also mediates the *RAS*/Adenylate cyclase/PKA pathway in mammals. Remarkably, down-regulation of these nutrient sensing pathways were also found to increase lifespan and stress resistance in higher eukaryotes (Kenyon, 2010). From here, it became evident that a conserved regulatory mechanism of aging evolved from yeasts to humans. This common mechanism is orchestrated around the response to nutrient availability, presumably as an adaptive strategy to promote growth and reproduction during prosperous times, and survival during harsh periods such as winter.

In yeasts, the TOR pathway is the main nitrogen sensor, while the PKA pathway is in charge of integrating glucose availability (Dechant & Peter, 2008; Loewith, 2011). TOR and PKA function upstream of multiple regulatory proteins and transcription factors that will eventually mediate growth or survival (Longo et al., 2012). Activation of the pro-longevity kinase *RII15* and transcription factors *MSN2/4* and *GLS1* by reduced TOR and PKA signalling is required to extend lifespan (Pedruzzi et al., 2000; Pedruzzi et al., 2003; Fabrizio et al., 2001; Wei et al., 2008). The kinase *SCH9* is acting downstream of TOR and is also negatively impacting lifespan (Wei et al., 2009). It was found that *SCH9* can also work independently of TOR in response to low ATP levels, sensed by the yeast AMP kinase *SNF1* (Lu et al., 2011). This mode of longevity regulation is common for both RLS and CLS, although some discrepancies exist. For instance, deletion of *MSN2/4* and *RII15* extends

RLS, while they are required for CLS lifespan extension by reduced nutrient signalling (Fabrizio et al., 2004). Elucidation of the final downstream effectors of TOR and PKA pathways is still at its beginning but first results suggest that altered regulation of mRNA translation, autophagy, oxidative stress, mitochondrial respiration, DNA stability, and rDNA recombination play a role in calorie restriction induced lifespan improvement (Steffen et al., 2008; Alvers et al., 2009; Fabrizio et al., 2003; Bonawitz et al., 2007; Madia et al., 2009; Medvedik et al., 2007) (more detailed in chapter 4).

B6. Mitochondrial dysfunction: It is clear for both RLS and CLS that mitochondrial damage increases with age. In aged cells, mitochondria become aggregated instead of tubular, their membrane potential drops, they accumulate mtDNA damages, and redox sensitive proteins such as aconitase become heavily oxidised (Hughes & Gottschling, 2012; Klinger et al., 2010; Fabrizio et al., 2003; Doudican et al., 2005), eventually leading to the loss of respiratory capacity (Longo et al., 1999; Veatch et al., 2009). Interestingly, similarly to oxidised proteins, mitochondria are asymmetrically inherited between mothers and daughters (Lai et al., 2002). Mitochondria partitioning mainly relies on membrane potential; only mitochondria with high potential are transmitted to daughters. Disruption of mitochondrial potential by deletion of *ATP2*, a subunit of the ATP synthase, generates inappropriate mitochondrial segregation and causes premature aging of daughter cells (Lai et al., 2002).

As mitochondria are the main source of ROS and their dysfunction is associated

with impaired ATP production and mammalian senescence, this led multiple scientists to investigate the role of respiration in yeast aging (Kujoth et al., 2007). When glucose is present, yeasts exclusively perform alcoholic fermentation and repress mitochondrial respiration even under aerobic conditions, a phenomenon called Crabtree effect (De Deken, 1966). Therefore, even though mitochondria are vital for the biogenesis of multiple metabolic intermediates, the oxidative phosphorylation chain is facultative.

Interestingly, loss of respiration has various background dependent effects on yeast RLS, ranging from shortened to increased lifespan (Kirchman et al., 1999). In fact, the underlying effect appears mainly mediated by the retrograde response, a signalling pathway from mitochondria to nucleus which is activated to cope with mitochondrial dysfunction (Hill & Van Remmen, 2014). Deletion of the retrograde response mediator *RTG2* prevents any lifespan benefit from mitochondrial dysfunction (Kirchman et al., 1999; Borghouts et al., 2004; Miceli et al., 2012). Some studies also report the existence of a retrograde response independent pathway that would increase RLS through a SIR2 based mechanism (Caballero et al., 2011; Woo & Poyton, 2009). These mechanisms are acting regardless of ROS production. The effect of ROS on RLS is actually unclear: overexpression of the mitochondrial superoxide dismutase SOD2 and performing RLS under total anaerobic conditions shortens lifespan (Fabrizio et al., 2004; Koc et al., 2004).

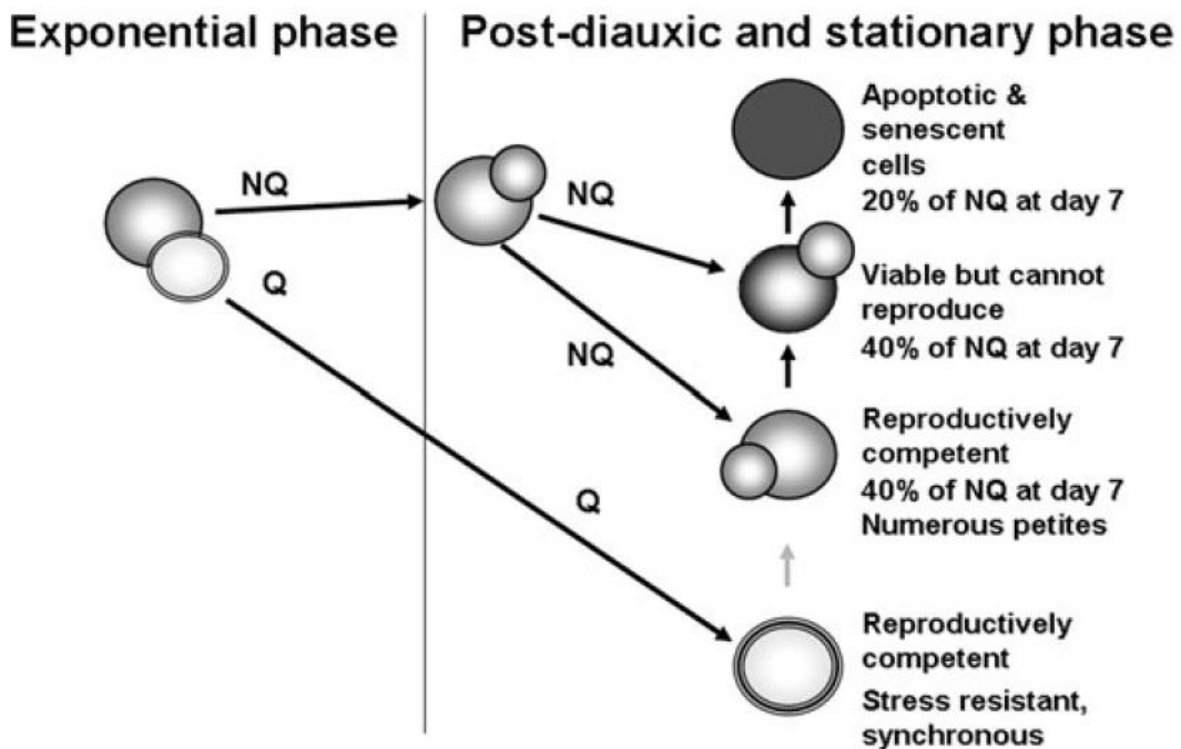
The relationship between CLS and respiration is much more straightforward.

All respiratory mutants have a relatively short CLS (Bonawitz et al., 2006; Aerts et al., 2009; Ocampo et al., 2012). As stated previously, during post-diauxic phase, yeasts switch from alcoholic fermentation to mitochondrial respiration metabolism. During this phase, yeasts substantially increase their stress resistance and start accumulating storage carbohydrates such as glycogen and trehalose which will be needed to withstand starvation (Gray et al., 2004; Cao et al., 2016). Accumulation of storage carbohydrates requires proficient respiration as the main source of energy in a glucose depleted environment (Cao et al., 2016). Conversely, activation of respiration during growth by incubating yeasts in a media with non-fermentable sugars, or by over-expressing the transcription factor *HAP4*, significantly increases CLS (Piper et al., 2006). This result seems driven by a pro-longevity ROS adaptive signal which can be reproduced by inducing mild oxidative stress during exponential phase (Mesquita et al., 2010; Pan et al., 2011). It is thought that mild oxidative stress during growth acts as an adaptive signal (hormesis) further enhancing protective mechanisms during stationary phase (Ristow & Schmeisser, 2011; Fabrizio et al., 2003).

B7. Stem cells and cellular senescence: At first glance, writing a paragraph on stem cells and cellular senescence in yeasts does not sound obvious. However, since seminal work from Werner-Washburne's lab, we know that stationary phase cultures are highly complex and heterogenic (Allen et al., 2006; Aragon et al., 2008; Davidson et al., 2011). During the transition from post-diauxic to stationary phase yeasts differentiate, similarly to stem cells, in at


least two groups: the quiescent and non-quiescent cells (Allen et al., 2006). Remarkably, it appears that cell fate is decided at the diauxic shift: unbudded virgin cells are committed to become quiescent and produce new quiescent cells during post-diauxic phase, whereas replicatively older cells will remain non-quiescent (Allen et al., 2006; Davidson et al., 2011). Moreover, quiescent cells may be of different kind according to the nature of limiting nutrients (Klosinska et al., 2011; De Virgilio, 2012). Interestingly, quiescent cells exhibit low ROS levels, contain high amount of glycogen, are genetically stable and retain high viability. Inversely, non-quiescent cells exhibit high ROS levels, are genetically instable, have dysfunctional mitochondria and are short-living (Allen et al., 2006). While quiescent cells retain the ability to resume mitotic growth in case of nutrient

repletion, non-quiescent cells rapidly lose their reproductive capacity and by definition are considered senescent. Frequently in stationary cultures, when more than 90% cells are dead, it is possible to observe a sudden increase in viability due to cellular regrowth which is fostered by increased oxidative stress (Fabrizio et al., 2004). This process is likely attributable to non-quiescent cells which might be able to exit senescence and resume growth in adverse conditions after accumulating a certain amount of mutations and epigenetic alterations, similarly to cancer cells. In fact, cell differentiation in yeasts appears to be an adaptive mechanism in which quiescent cells secure lineage survival whereas non-quiescent cells try their last chance to get beneficial mutations or merely die to feed the quiescent ones.



Yeasts encounter different cell fates upon nutrient exhaustion. Stationary phase cultures are composed of a mix of resilient quiescent cells (Q) and more unstable mitotically active non-quiescent cells (NQ). Quiescent cells are mainly made of newborn daughters and can possibly become non-quiescent with chronological age (represented by the light arrow). (Davidson, et al., 2011).

B8. Intercellular communication: Except the two mating pheromones *a* and *alpha*, yeasts do not secrete other kind of messenger molecules. Nevertheless, as unicellular organisms, they are in direct contact with their surroundings and rigorously respond to environmental cues. During growth, yeasts secrete a multitude of by-products from fermentation. In fact, it has been found that certain of these compounds can act as messengers and significantly affect cell behaviour. It is now well established that ethanol, acetic acid, and pH acidity resulting from the alcoholic fermentation process strongly impair CLS (Fabrizio et al., 2004; Fabrizio et al., 2005; Burtner et al., 2009). Indeed, these compounds were found to increase ROS levels in stationary phase and to promote apoptosis (Pan et al., 2011; Ludovico et al., 2001). Although their impact on CLS is undiscussable, whether they cause premature aging or are merely toxic is still highly debated (Longo et al., 2012). Originally, ethanol production and low pH constitute adaptive strategies that yeasts are using to outcompete other microorganisms (Liti, 2015). During stationary phase it could also promote adaptive regrowth (described in the previous paragraph) as a trade-off between reproduction and territorial control with survival.



*“World death rate holding steady
at 100 Percent”*

- The Onion, Headline
January 22, 1997

Chapter 4

Postponing aging

“For whom have my hands laboured? For whom have I suffered? I have gained absolutely nothing for myself, I have only profited the snake, the ground lion, I give up!”

While these words could be those of a PhD student after an unfortunate experiment, they were actually pronounced by Gilgamesh at the end of his unsuccessful quest for immortality. Gilgamesh was a tyrannical demigod reigning over the Sumerian capital Uruk and whose only fear was death. After a long and dangerous journey, he found in the depths of the ocean a magic plant that had the power of rejuvenation. However, the plant eventually got stolen by a snake (once again) while he was bathing. The famous Epic of Gilgamesh, written around 1200 BC, is considered as the oldest known literary work and, by extension, is also the first text relating the quest of immortality ([Jeune, 2002](#)).

The quest of immortality has always fascinated mankind and constituted a fantastic source of imagination, despite predominant scientific fatalism. What was nothing more than mystic novels and fictive quests at the beginning, started to become more concrete 25 years ago when the first genes modulating lifespan were uncovered. This discovery had a strong echo in a world of relative prosperity in which, for the first time of human history, most people are growing old and are suffering the signs of time. Following this breakthrough, a multitude of anti-aging companies emerged with different strategies, ranging from traditional pharmacological approaches to regenerative medicine ([Thomas Scott & De Francesco, 2015](#); [De Magalhaes et al., 2017](#)). Despite total lack of success until now, a plethora of new promising anti-aging compounds have been identified from research in model organisms and some of them are being clinically tested.

In this last chapter, I will review the mechanisms of calorie restriction (CR) and some of its most promising pharmacological mimetics.



Holy Grail. *Indiana Jones* was more successful than Gilgamesh in the quest for immortality.

A) Calorie restriction, when less is more

A1. Environmental modulations of aging

Complex traits are regulated by tight gene-gene interactions, but also highly depend on gene-environment interactions. Besides genetics, it is well established that aging can be modulated by a multitude of environmental cues. For instance, lowering external temperature significantly extends lifespan of poikilothermic organisms such as worms and fruit flies (Lamb, 1968; Klass, 1977). Interestingly, this result can be reproduced in mutant mice with an elevated hypothalamic temperature, leading to a 0.5°C drop of body temperature and increasing median lifespan by up to 20% (Conti et al., 2006). In addition, oxygen concentration is also a strong regulator of aging: lowering oxygen tension was shown to increase drosophila and worm lifespans through activation of the hypoxia sensor

HIF-1 (Rascon et al., 2010; Vigne & Frelin 2007; Leiser et al., 2011; Leiser et al., 2013). This result can be applied to mammalian cell lines whose senescence is delayed at low oxygen concentrations of 2-5% (which is actually closer to physiological conditions) (Campisi, 2001).

The strongest environmental impact on lifespan is seen for the parasite nematode *Strongyloides ratti* whose free living form only survives 5 days, whereas the parasitic form follows a different developmental path eventually conferring a lifespan that can exceed a year (Gardner et al., 2006). In the same fashion, queens of social insect species live much longer than workers as the result of an alternative developmental program triggered by a special diet during growth (Finch, 1990).

Interestingly, lifespan can also be affected by sexual pheromones. This is particularly well exemplified by the Australian redback spider. When no female spider is present in









Environmentally determined longevity. *Strongyloides ratti* (left) has an extremely plastic longevity ranging from 5 days (free-living) to more than a year (within host), while male Australian redback spiders (right) age prematurely when females are in their surroundings (www.WormBook.org; David Kleinert Photography).

their surroundings, male spiders develop slowly and maintain a high viability (De Magalhaes et al., 2012). More generally, in accordance with the disposable soma theory, a trade-off exists between survival and reproduction. A convincing argument comes from selection experiments with drosophila. By successively selecting drosophila with late fecundity, Rose could obtain flies with longer life durations and slower development times (Rose, 1984). This observation is confirmed in worms where deletion of germ cells extends lifespan (Yamawaki et al., 2008). Similar results can be achieved with humans as reported by studies on eunuch longevity (Min et al., 2012; Gems, 2014). Alternatively to castration, wedding was found to have a positive incidence on life expectancy since single men appear to have shorter lifespans (Kaplan & Kronick, 2006). Although both approaches might not be compatible, they are worthy of thought!

While several other environmental triggers can impact lifespan, most of them are hardly applicable to humans for obvious reasons. The most recognised and encouraging natural intervention that may contribute to improve human healthspan, in a near future, is calorie restriction (CR). The ideology of CR is based on reduction of food intake without causing malnutrition (Fontana, 2009). The first records of CR longevity benefits can be tracked back from experiments in rats performed in 1935 by McCay and colleagues (McCay, 1935). Since, lifespan increase mediated by CR has been confirmed in multiple species including dogs (up to 20% lifespan improvement) (Kealy et al., 2002), mice (up to 50% improvement) (Weindruch et al., 1986), fruit flies (up to 100% improvement) (Pletcher et al., 2002),

round worms (up to 3 fold improvement) (Klass, 1977), and also in our beloved yeasts (up to 100% improvement for RLS and 3 fold for CLS) (Jiang et al., 2000; Smith et al., 2007).

The effect of CR on hominid longevity is more debated. A first study following a cohort of rhesus monkeys subjected or not to CR, over a period of 25 years, observed a clear lifespan improvement for the animals under low food intake (20% lifespan improvement) (Bodkin et al., 2003). Two more recent and still ongoing studies have more mitigated results though. The first study still reports lower mortality rates for CR monkeys, whereas the second one claims that there is no lifespan improvement (Colman et al., 2009; Mattison et al., 2012). Regarding humans, for practical reasons, we clearly lack conclusive studies at the moment. The multiple events of famine, which inadvertently happened throughout human history, were mostly accompanied with extreme malnutrition and high mortality rates (Most et al., 2017). The only concrete beneficial effect of CR observed on human longevity comes from the inhabitants of Okinawa island in Japan. As described in the first chapter, Okinawans have the highest life expectancy worldwide, presumably thanks to their culinary habits. Their diet is close to the Mediterranean one with low protein intake and rich in fresh vegetables, fruits, sweet potatoes, soy and fish (Willcox et al., 2006). In addition they consume approximately 17% less calorie than mainland Japanese and 40% less than Americans (Most et al., 2017). Conversely, Japanese sumos have a life expectancy of 55 years old.

		Lifespan increase		Beneficial health effects	
		Dietary restriction	Mutations/drugs	Dietary restriction	Mutations/drugs
	Yeast	3 fold	10 fold	Extended reproductive period	Extended reproductive period, decreased DNA damage/mutations
	Worms	2-3 fold	10 fold	Resistance to misexpressed toxic proteins	Extended motility Resistance to mis-expressed toxic proteins and germ-line cancer
	Flies	2 fold	60-70%	None reported	Resistance to bacterial infection, extended ability to fly
	Mice	30-50%	30-50% (~100% in combination with DR)	Protection against cancer, diabetes, atherosclerosis, cardiomyopathy, autoimmune, kidney and respiratory diseases, reduced neurogeneration	Reduced tumor incidence, protection against age-dependent cognitive decline, cardiomyopathy, fatty liver and renal lesions. Extended insulin sensitivity
	Monkeys	Trend noted	Not tested	Prevention of obesity, protection against diabetes, cancer and cardiovascular disease	Not tested
	Humans	Not determined	Not determined (GHR deficient subjects reach old age)	Prevention of obesity, diabetes, hypertension Reduced risk factors for cancer and cardiovascular disease	Possible reduction in cancer and diabetes

Effect of calorie restriction and mimetic interventions from yeasts to humans. (Fontana et al., 2010).

Importantly, CR does not simply extend lifespan, it also counters age-related diseases. For instance, mice under CR are less prone to develop type 2 diabetes and cancer (Weindruch et al., 1986). Regarding rhesus monkeys, despite discrepancies about longevity benefits, all studies agree that CR promotes healthspan. Indeed, CR monkeys have lower incidence of diabetes, cancer, cardiovascular disease, brain atrophy and sarcopenia compared to control animals (Colman et al., 2009; Mattison et al., 2012).

In humans, first results also suggest that CR may protect against diabetes, hypertension, cardiovascular diseases, or cancer, although these effects could be biased by the benefits of decreased adiposity in overweight persons (Fontana et al., 2004; Meyer et al., 2006; Ravussin et al., 2015).

A new school of thought is now drifting its interest from calorie restriction to dietary restriction (DR) (Simpson et al., 2017; Brown-Borg & Buffenstein, 2017). Dietary

restriction is based on the reduction of specific macronutrients as opposed to the reduction of calorie intake. There is now multiple evidences pointing to the fact that lower protein intake can partly overlap, or even completely substitute CR (Mair et al., 2005; Grandison et al., 2009). More particularly, the amino acids methionine and tryptophan seem to be key drivers of this process (discussed in chapter 6) (Ooka et al., 1988; Troen et al., 2007).

A2. Mechanisms of calorie restriction

At first glance, the founding principles of CR are counterintuitive. When nutrients are plentiful, organisms cannot maintain themselves and age faster, whereas when energy is limited, their repair and survival capacities are improved. Some theories suggest that lower metabolic rates reduce the pace of damage accumulation and slow aging (De Magalhaes & Church, 2005). This hypothesis cannot be ruled out, since alternative developmental stages such as fungal and bacterial spores, the dauer larvae, or even mammalian hibernation, extend lifespan through periods of relative stasis in response to starvation (Klass & Hirsh, 1976; Wu & Storey, 2016). However, it is now clear that CR is not mediated by a simple drop in metabolic rates, but rather by a full reallocation of cellular energy from growth and reproduction toward maintenance and stress resistance (Walford & Spindler, 1997; Masoro, 2005). Although our understanding of the mechanisms involved remains elusive, it seems safe to claim that the genetic reprogramming induced by CR is mediated by nutrient sensing pathways, and more particularly by

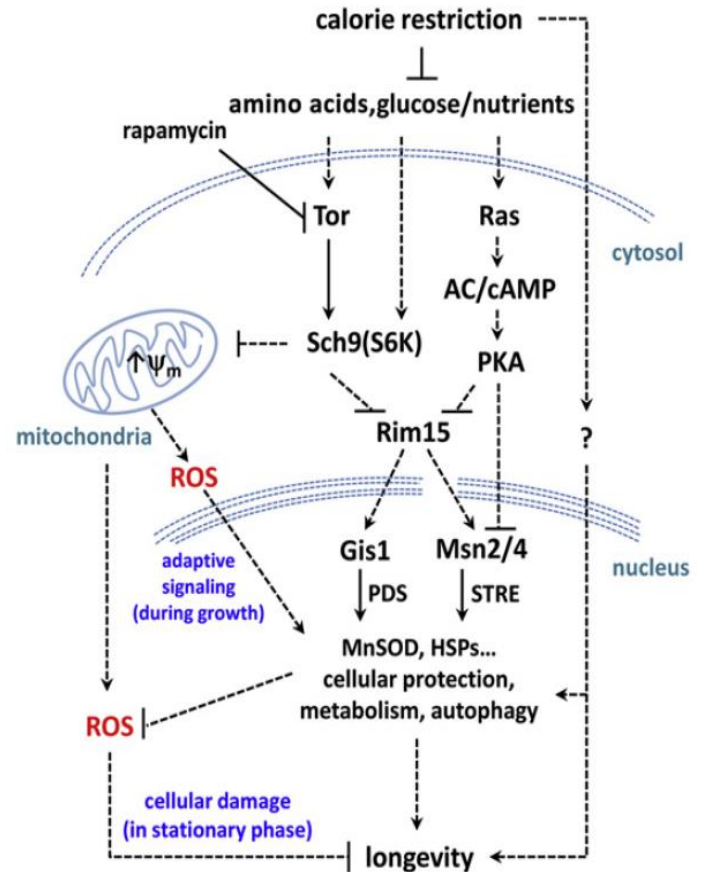
the insulin and IGF-1 hormones (De Magalhaes et al., 2012). Down-regulation of insulin/IGF-1 and TOR signalling generates a full cascade of events eventually increasing lifespan. The budding yeast *S. cerevisiae* has been pivotal to start unravelling the underlying mechanisms (Kapahi et al., 2017).

Both yeast RLS and CLS substantially benefit from CR that is commonly performed by lowering glucose concentration from 2% to 0,5% or 0,05% (Jiang et al., 2000; Smith et al., 2007). In yeasts, CR is mediated through down-regulation of the nutrient sensing pathways TOR, Ras/adenylate cyclase/PKA. Deletion of key components of these pathways is sufficient to entirely mimic CR in RLS (Kaeberlein et al., 2005). Likewise, deletion of *RAS2* and *TOR1* strongly enhances CLS, although application of CR further improves longevity, implying the existence of additional mediators (Wei et al., 2008; Wei et al., 2009). The protein kinase *SCH9*, ortholog of mammalian *S6K* and *AKT*, works downstream of TOR and Ras/adenylate/PKA and is required for nutrient signalling. Deletion of *SCH9* alone is enough to fully recapitulate *TOR1* deletion. Reversely, *TOR1* removal alone is not as efficient as *SCH9* deletion, suggesting that *SCH9* is a converging point from different pathways (Wei et al., 2008). Downstream effectors of the nutrient sensing pathway include other kinase proteins such as *RIM15*, and several transcription factors, namely *MSN2/4*, *GIS1*, *GCN4* (Wei et al., 2008; Steffen et al., 2008).

The final physiological outcomes of CR contributing to lifespan enhancement are still under investigation. A clear

phenomenon involved is the activation of respiration by CR resulting from TOR inhibition (Lin et al., 2002; Bonawitz et al., 2006; Pan et al., 2009). In RLS, respiration was originally thought to promote lifespan by activating *SIR2* through increased rates of its coenzyme NAD⁺ (Lin et al., 2000; Lin et al., 2004; Anderson et al., 2003), and further supported by the existence of crosslinks between *SIR2* and mitochondria (Caballero et al., 2011). However, this hypothesis got partly dismissed by results showing that neither respiration nor *SIR2* are required to extend RLS by CR (Kaeberlein et al., 2004; Kaeberlein et al., 2005; Smith et al., 2009). At the moment, the implication of *SIR2* in mediating CR is still debated. On the other hand, the relation between CR-induced respiration and CLS is more straightforward. As stated in the previous chapter, CLS requires an efficient shift from fermentation to respiration metabolism prior to enter stationary phase. Similarly, the use of non-fermentable carbon sources, activation of respiration by CR during growth phase acts as a pre-adaptive signalling involving ROS production, nutrient storage and sustained ATP levels, lead to extended CLS (Cao et al., 2016; Mesquita et al., 2010; Pan et al., 2011; Choi & Lee, 2013; Arlia-Ciommo et al., 2018). Alternatively, it has been proposed that the shift from fermentation to respiration metabolism limits pH acidity and prevents the accumulation of acetic acid and ethanol, which are otherwise pro-aging factors (Fabrizio et al., 2005; Burtner et al., 2009).

Besides respiration, CR has a pivotal role in promoting proteostasis. It is now well established that TOR is a potent inhibitor



Genetic mediators of CR in yeast.
(Longo et al., 2012).

of autophagy. Indeed, induction of autophagy by decreased TOR signalling is required to promote lifespan extension via CR (Alvers et al., 2009; Aris et al., 2013). Likewise, maintenance of vacuolar acidity through *SCH9* inhibition is required for autophagy and lifespan enhancement by CR (Ruckenstuhl et al., 2014; Wilms et al., 2017). Furthermore, reduced mRNA translation via decreased TOR signalling is a key player in for RLS extension by CR (Steffen et al., 2008; McCormick et al., 2015). However, it is not known at the moment whether this effect is linked to proteostasis.

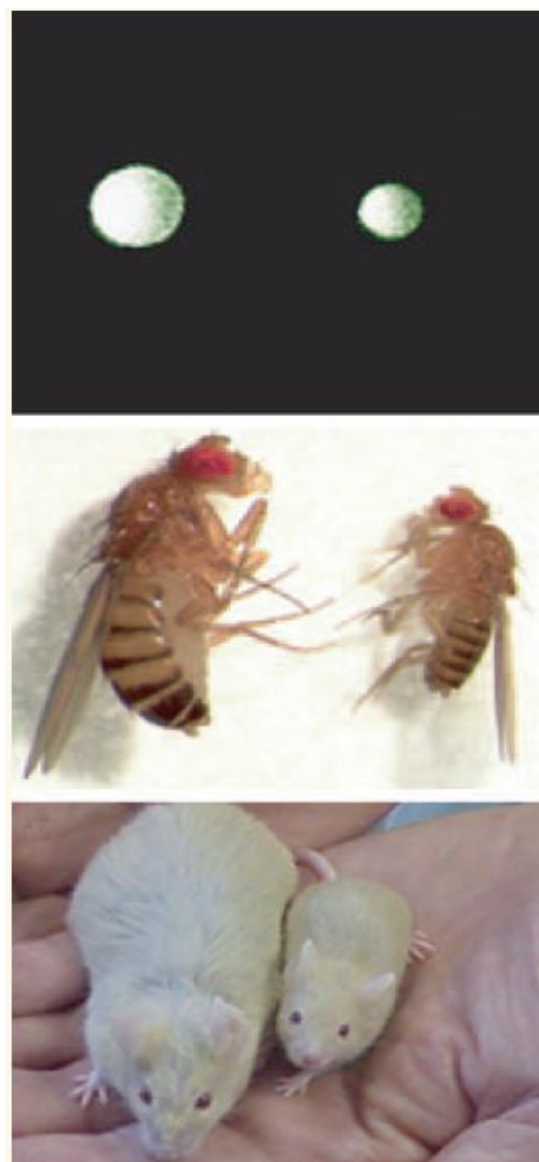
In addition, CR has been found to improve genome maintenance. Application of CR, or

inhibition of *SCH9* is enough to counter the detrimental effect of DNA replication stress induced by hydroxyurea, or by inactivation of DNA repair proteins *SGS1* and *RAD27* (Weinberger et al., 2013). Similarly, CR increases rDNA stability and protects against genotoxic stress through chromatin remodelling (Riesen & Morgan 2009; Dang et al., 2014). Interestingly, CR is also linked with genome stability. When nutrients are plentiful, *SCH9* promotes DNA translesion synthesis via activation of the zeta polymerase, leading to higher rates of point mutations (Madia et al., 2008; Madia et al., 2009).

Lastly, CR is well known to increase stress resistance, a process that is mainly mediated by the transcription factors *MSN2/4* and *GIS1* which are required to increase CLS when *SCH9* is deleted (Fabrizio et al., 2004). More particularly, enhanced oxidative stress resistance is needed since deletion of the superoxide dismutase *SOD2* prevents CLS extension (Fabrizio et al., 2003). Moreover, the anti-oxidant peptide glutathione is also mandatory to profit of CR (Mannarino et al., 2008). The role of redox balance in RLS is more complicated though. Activation of the peroxiredoxin *TSA1* in response to CR is essential to promote RLS extension (Molin et al., 2011). However, deletion of *MSN2/4* extends RLS, while *SOD2* over-expression shortens it (Fabrizio et al., 2004). It is thought that this discrepancy between RLS and CLS stems from activation of protective enzymes and stress resistance pathways which can prematurely stop cell division (Fabrizio et al., 2004). Nevertheless, ROS signalling was recently associated with chaperone regulation and proteostasis (Hanzen et al.,

2016). Thus, it appears oxidative stress is not merely a source of damage, but rather a sophisticated signalling system which can have both positive and negative outcomes on lifespan when dysregulated.

The overall architecture of CR starts to take shape thanks to the wealth of data brought from the different model systems. However, many black holes remain and will



Trade-off. Calorie restriction and genetic mimetics reduce and delay growth, but increase lifespan. Yeast *SCH9* deleted (top) and deficient IGF1 fly (middle) and mouse (bottom) (Longo et al., 2005).

need clarification in order to make CR efficient for humans. Strikingly, CR can sometimes have detrimental effects on lifespan when combined with certain single deletions in both RLS and CLS (Schleit et al., 2013; Campos et al., 2018). In higher eukaryotes, the effect of CR is also closely tied with the genetic background: it fails to extend lifespan in certain wild mice (Forster et al., 2003) and have very variable outcome on recombinant inbred mice, ranging from 98% lifespan extension to 68% lifespan reduction (Liao et al., 2010). These results are presumably applicable to humans. It is actually estimated that most persons who already have a healthy lifestyle would actually not get any benefit from CR (Phelan & Rose, 2005).

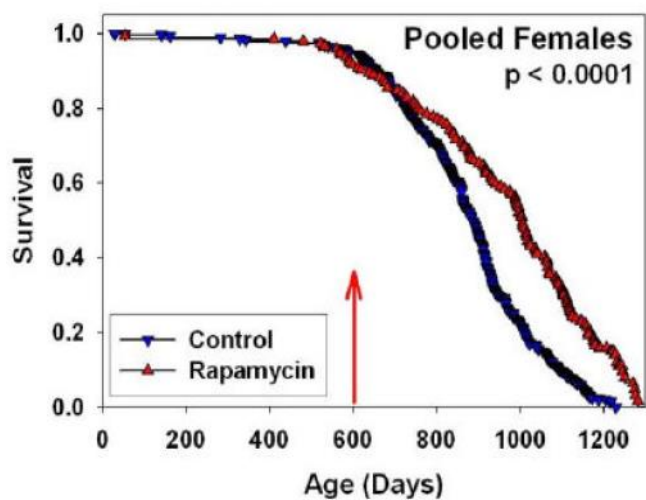
B) Mimetics of calorie restriction

Although the effect of CR on human longevity is still debatable, it may have some benefits on health, including weight loss (obviously), increased insulin sensitivity and reduced risk of cardiovascular diseases (Mattson et al., 2017). However, applying CR in modern societies is somewhat challenging. Current approaches mainly rely on either intermittent or periodic fasting programs. Intermittent fasting is based on recurrent starvation for relatively short durations (16-48 hours), while periodic fasting is based on reduced calorie intake during longer time frames (2 to 21 days or more). Nevertheless, since it is in the human nature to try to get the best with minimal efforts, and because CR can also be accompanied by a certain number of undesirable effects such as permanent hunger, anxiety, depression, tiredness and

decreased libido, several companies are striving to develop consequence-free drugs mimicking CR (Dirks & Leeuwenburgh, 2006).

B1. Rapamycin, from chemotherapy to lifespan extension

Rapamycin is a lipophilic molecule produced by the bacteria *Streptomyces hygroscopicus* located on Easter Island, also known as Rapa-Nui. When discovered and purified in the 1970s, rapamycin was first used as an anti-fungal agent. Subsequently, rapamycin was found to be a direct inhibitor of TOR and became popular for its anti-proliferative properties on mammalian cells. Thanks to this property, rapamycin received clinical approval in 1999 and has been used as an immunosuppressant to prevent graft rejection and in chemotherapy to prevent tumour progression (Loewith, 2011; Li et al., 2014). In the meantime, inhibition of TOR was discovered to promote life extension in model organisms (Kapahi et al., 2004;



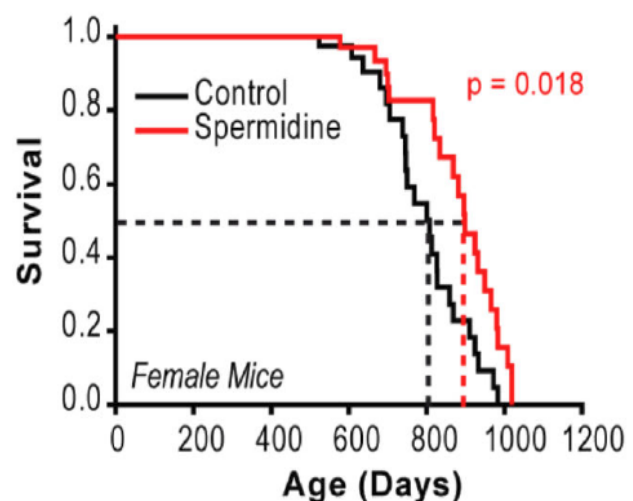
Rapamycin improves mice lifespan. Red arrow corresponds to the addition of rapamycin to the diet (Harrison et al., 2009).

(Kaeberlein et al., 2005; Hansen et al., 2007). More recently, rapamycin was successfully used to increase mice lifespan, confirming a potent pro-longevity role of this drug even in mammals. Indeed, rapamycin can enhance mice lifespan by 15% for females and 10% for males, even when administrated at late age (Harrison et al., 2009). Similarly to CR, rapamycin also delays cardiovascular and neurodegenerative diseases (Malagelada et al., 2010; Flynn et al., 2013). Although the effects are not earth-shattering, it is important to mention that simply delaying aging by 2,2 years in the US would contribute to save more than \$7 trillion over a period of 50 years (Goldman et al., 2013). Rapamycin appears as an intriguing drug at the crossroads between growth and survival that can prevent cancer progression on one hand, and delays aging on the other hand. However this duality has a price: since rapamycin is not cytotoxic, it does not eradicate cancer cells which eventually leads to a relapse (Li et al., 2014), and since rapamycin inhibits proliferation it comes with undesirable effects and prevents long term usage (Kaeberlein, 2014). To turn around these issues, a proper drug dosage will be required to optimise its effect either in cancer treatment, or in anti-aging therapy. Additionally, pharmaceutical companies are trying to develop more specific analogues, namely rapalogs.

B2. Resveratrol

Since the seminal discovery of their pro-longevity effect on yeast aging, the histone deacetylase sirtuins have gained a lot of interest in aging research (Kaeberlein et al., 1999). While the original role of

sirtuins found in yeasts was to limit ERCs formation, it has later been linked with multiple biological processes due to age-associated transcriptional dysregulation mediated by increased histone acetylation (Dang et al., 2009). Following results obtained with yeasts, over-expression of sirtuins was also found to improve lifespan in round worms and fruit flies (Tissenbaum & Guarente, 2001; Rogina & Helfand, 2004). Since sirtuin targeting appeared as a promising candidate for anti-aging interventions, drug screening was performed to look for agonists of *SIRT1*, the mammalian orthologue of *SIR2* (Howitz et al., 2003). Quite interestingly, the screening identified the polyphenol molecule resveratrol, which can be found at high doses in grapes, and by extension in wine (and also in grape juice, but it is obviously much less exciting). The longevity secret of French people was finally pierced! However, the hype around resveratrol did not last long: its effect on invertebrate models was



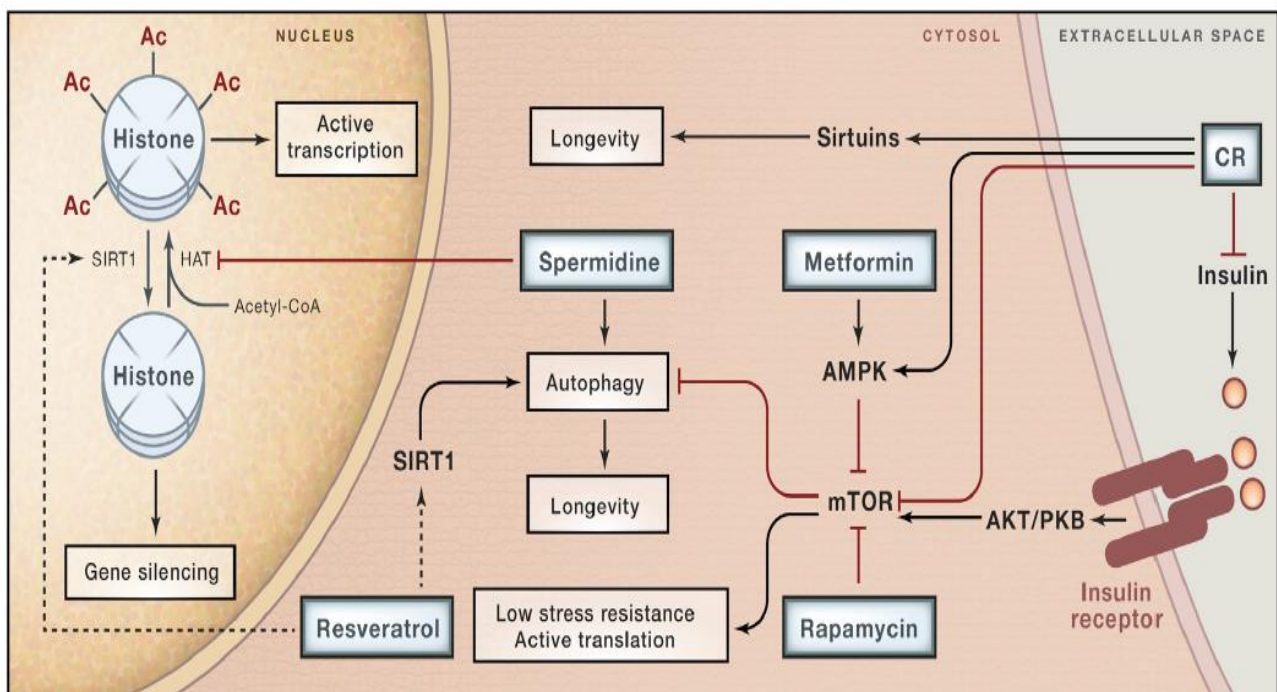
Spermidine improves mice lifespan. Spermidine administration was started 4 months after birth (Eisenberg et al., 2016).

relatively contrasted, and it failed to extend mice lifespan (Hector et al., 2012; Miller et al., 2011; Strong et al., 2013). This limited success was proposed to result from inefficient activation of sirtuins by resveratrol (Kaeberlein et al., 2005), and from contradictory data about the role of sirtuins in the aging process of higher eukaryotes (Burnett et al., 2011). Nevertheless, localised brain over-expression of *SIRT1* was reported to increase mice lifespan (Satoh et al., 2013). Hence, it is possible that other sirtuin agonists will encounter a better success in the future.

B3. Spermidine

Biochemical studies performed during human aging reported a significant decrease in polyamine levels in multiple

organs (Scalabrino & Ferioli, 1984). This observation led researchers to investigate the role of polyamines in aging, and more particularly spermidine, whose name is derived from the place it was originally found, the semen. In 2009, Eisenberg and colleagues found that administration of exogenous spermidine could substantially increase lifespan of yeasts, round worms, fruit flies, and human cells (Eisenberg et al., 2009). More recently, spermidine was found to improve mice cardiovascular function and lifespan by 10% (Eisenberg et al., 2016). The mode of action of spermidine is still unclear, but first evidences suggest that it inhibits histone acetyltransferase and thus prevents histone H3 deacetylation. Somehow, this chromatin alteration increases autophagy which is required for lifespan extension similarly to CR (Eisenberg et al., 2009). However, besides



Simplistic mode of action of CR mimetics. (De Cabo et al., 2014).

autophagy, epigenetic modifications elicited by spermidine probably have a much wider spectrum of effects, including the prevention of age-induced histone hyper-acetylation. Spermidine appears to be one of the most promising anti-aging drugs, with no undesirable effect noted so far (Soda et al., 2009). Nevertheless, additional studies are needed to better understand underlying mechanisms.

and is currently undergoing clinical trials to evaluate its effect on human health (Barzilai et al., 2016).

B4. Metformin

Metformin is a licensed drug involved in glucose metabolism which is commonly used as a treatment against type 2 diabetes (Barzilai et al., 2016). Interestingly, metformin was also found to promote lifespan and healthspan in round worms and mice (Anisimov et al., 2008; Anisimov et al., 2011; Cabreiro et al., 2013; De Haes et al., 2014). In addition to its role in decreasing insulin-resistance, metformin was reported to inhibit mitochondrial electron transport chain and to activate the energy sensor AMPK (Liu et al., 2011; Bridges et al., 2014; Duca et al., 2015). Metformin was also proposed to increase lifespan in round worms by altering the microbiome and inducing methionine restriction (Cabreiro et al., 2013). However, metformin failed to increase fruit fly lifespan, although it is presumably due to an imbalanced dosage of the drug (Slack et al., 2012). At the moment, it is unclear whether metformin pleiotropic effects are independent from each other or constitute a cascade of events. While a lot of work is still needed to unravel underlying mechanisms, metformin has been used with an excellent safety record for more than 20 years in diabetes treatment

RESULTS

Objectives

Our team, *Population genomics and complex traits*, mainly focuses on understanding genome evolution and dissecting the genetic architecture of quantitative traits in *S. cerevisiae*.

On the one hand, the team has been historically working with natural strains from different *S. cerevisiae* lineages and deeply characterised their genome architecture (Liti2009, Bergstrom2014, Yue2017). More recently, the team has co-led *The 1002 Yeast Genomes Project* which provided the most comprehensive view of *S. cerevisiae* genome evolution by collecting and sequencing more than a thousand strains (Peter2018).

On the other hand, the team has developed state-of-the-art forward genetic strategies aimed at dissecting the genetic architecture of complex traits. These approaches mainly rely on highly recombinant outbred crosses which are subsequently used for linkage analysis. It has already been successful for studying selective pressure from various nutrient sources, thermal sensitivity or resistance to chemotherapeutic agents (Parts2011, Salinas2012, Cubillos2013, Vazquez-Garcia2017).


When I joined the Liti team my main interest was to apply this know-how to aging biology. Yeast aging has quasi exclusively been studied in the classic lab strain S288C and its derivatives, with only few exceptions (Kaya2015, Qin2008). While extensive reverse genetic studies have undoubtedly established the foundations of yeast genetics, the next challenge, in the upcoming era of personalised medicine, is to understand the nature of phenotypic variations between individuals. To fulfil this objective, I have taken advantage of the resources developed by the lab to study

how genetic and environmental factors shape yeast chronological aging variation.

The results of this work will be presented in the form of two independent scientific articles that are currently in preparation and will be shortly submitted.

The first article is not exclusively focused on aging, but rather in understanding the life history of *S. cerevisiae*. In this collaborative project co-led by myself and Mateo De Chiara (bioinformatics), we screened the CLS and sporulation capacity of all the strains from the 1002 Yeast Genomes Project. Sporulation and chronological survival are two key features developed by yeasts to cope with the adverse environments in which they spend most of their lifetime. The idea behind this project is to understand how CLS and sporulation were shaped throughout evolution and to identify associated genetic drivers species-wide. This study constitutes the most comprehensive phenotypic description of these two traits.


The second article focuses on dissecting the genetic and environmental basis of CLS variation. I performed linkage analysis using a pool of 1056 diploid segregants derived from an advanced intercross between two natural isolates. CLS was determined in synthetic media as well as calorie restriction, and in presence of the pro-longevity drug rapamycin, which are two promising anti-aging interventions. This project helps to unravel the complex nodes of gene-gene and gene-environment interactions which will be eventually required to deepen our understanding of aging, or any other complex trait, and to apply personal medicine strategies.



*“The most incomprehensible thing
about the world is that it is
comprehensible”*

- Albert Einstein

Domestication shaped the *Saccharomyces cerevisiae* natural life cycle



Matteo De Chiara^{1}, Benjamin Barré^{1*}, Agurtzane Irizar¹
and Gianni Liti¹*

Abstract

Humans shaped the evolution of plant and animal agricultural crops via domestication. This process transformed species traits as a consequence of selection acting upon segregating genetic variation. The industrial microbe *Saccharomyces cerevisiae* evolved alongside humankind for several millennia and multiple selected breeds are characteristics of fermented beverages. Recent discoveries of a reservoir of natural wild *S. cerevisiae* population isolated worldwide provide an opportunity to understand the impact of domestication. Here, we provide a species wide view into life history traits that constitute essential aspects of the yeast life cycle. We measured and analysed growth, sporulation and chronological life span across multiple environments in a vast collection of approximately one thousand sequenced isolates. We observed dramatic quantitative variation in all the life cycle traits, with some examples that drastically differ from what we learned by studying laboratory strains. Indeed, we unveiled a clear dichotomy between wild and domesticated strains. Specifically, wild strains appear to have maintained a fully functional life cycle in all his components and efficiently trigger sporulation process when entering stationary phase regardless the environmental condition. In contrast, domesticated strains appear more specialised in resisting specific stresses and have repeatedly lost or weaken their sexual phase. Candidate gene and genome wide association approaches indicate multiple genetic accidents that underlie such quantitative variation with many examples that underlie population histories. These traits likely represent key species fitness components and the natural variation revealed here likely reflects differences in selection regimes experienced during their evolutionary histories.

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Introduction

Domestication of wild species is initiated by controlled breeding and followed by artificial selection. Humans domesticated many plant and animal species for at least 12000 years with the aim of improving desirable agricultural traits. While domestication has greatly contributed to the expansion and development of humankind, it has also profoundly altered species subjected to this process. Many striking domestication examples can be observed in popular plant and animal crops that drastically differ from their respective ancestors (Driscoll et al., 2009). These phenotypic improvements go along with selection acting upon the genetic diversity segregating in the species. Modern crop improvement approaches rely on sophisticated genotyping technologies that enable rationalised controlled breeding.

Beyond plants and animals, microbes have also been extensively exploited for agricultural purpose, although the breeding and selection schemes were not directly controlled. The budding yeast *Saccharomyces cerevisiae* is a key industrial microbe that evolved alongside with humans for several millennia. Earliest evidences of *S. cerevisiae* domestication dated back at least 9000 years ago in Asia (McGovern et al., 2004). Subsequently, proofs of early winemaking (8000-7000 years ago) were discovered in the Caucasus mountains (McGovern et al., 2017) and first traces of *S. cerevisiae* were identified in Egyptian wine jars (5000 years ago) (Cavalieri et al., 2003). The yeast biotechnology industry shifted from the spontaneous or artisanal fermentations to pure-stock breeds that were

isolated, stored and inoculated, following the advent of applied microbiology. The advent of early yeast genetics coincided with the attempt to develop designed breeding in industrial beer strains (Barnett, 2007).

Over time, the application of *S. cerevisiae* expanded to a broad range of fermented foods and beverages including wine, beer, dairy, rice, cocoa, coffee, bread and agave fermentation. This abundance of products provided a large array of specialised artificial niches, which have likely contributed to the emergence of several specialized domesticated breeds (Liti & Carter et al., 2009; Gallone et al., 2016; Strobe et al., 2015; Cromie et al., 2013; Goncalves et al., 2016). Furthermore, this process was associated with extensive movements of fermented products and technologies, contributing to the human-driven dispersion of the species. Altogether, the artificial selection and the human-driven dispersion moulded the yeast genomes and in parallel shaped the ensemble of the species phenotypes.

However, while yeasts from domesticated environments were repeatedly and abundantly isolated over the past century, the presence of *S. cerevisiae* in wild environments outside the human activity was scarce and long debated (Boynton & Greig, 2014; Liti, 2015). This has limited our understanding of yeast natural history and prevented to precisely infer the effect of human driven domestication. Recently, field surveys revealed the ubiquitous presence of *S. cerevisiae* across multiple natural environments and shifted our view of budding yeast ecology (Liti, 2015). The availability of large cohorts of natural strains enabled to compare wild and

domesticated breeds. Both wild and domesticated strains can be grouped in genetically diverged lineages based on an ecological and geographical niche or on a specific fermented product. However, we reported that distances among lineages, measured as single nucleotide polymorphisms, are much more pronounced among wild strains (Peter & De Chiara et al., 2018). In contrast, domesticated strains appear to differ more in term of genomic complexity in the form of aneuploidies, higher ploidies, variable gene content and copy number (Bergstrom et al., 2014; Peter & De Chiara et al., 2018; Dunn et al., 2012; Goddard et al., 2005). We previously suggested that the genetic variation observed in domesticated strains fuel phenotypic diversity, while the accumulation of SNPs seems more neutral (Liti & Carter et al., 2009; Warringer et al., 2011; Bergstrom et al., 2014). Indeed, domesticated strains are more fitted to specific conditions that mimic industrial settings such as stresses and carbon sources, while their survival in natural environments and sexual reproduction capacity has decayed (Warringer et al., 2011; Gallone et al., 2016; Driscoll et al., 2009). This dichotomy between domestic and wild strains, mirror the differences previously reported between the *S. cerevisiae* and the wild relative *S. paradoxus* (Bergstrom et al., 2014).

Fields surveys and population genomics are naturally followed by laboratory experiments that enable to link ecology and genomic variation to organismal phenotypes. The phenotype ensemble can be used as a proxy of global organismal fitness, providing crucial information on how different

individuals will perform in wild and domestic environments. Yeast has an incredible history as a model system and virtually any aspect of biology has been deeply investigated in this single cell eukaryote. Decades of yeast genetic experiments provided protocols to precisely control the life cycle by simply shifting yeast cells through different defined media.

In nutrient rich conditions, budding yeasts rapidly proliferate and expand their population size by mitotic growth. This process can be quantitatively decomposed into lag, rate and yield, with each of these phases that can be relevant in competitive growth conditions. Sugar rich media promote alcoholic fermentation and inhibit respiration. During this step, yeasts favour the utilization of simple fermentable sugars, such as glucose, through glycolysis. In this phase, yeasts are in their exponential growth phase and their doubling time is at its fastest rate. When rich fermentable sugars become limiting, yeasts switch their metabolism from fermentation to respiration and consume the ethanol previously produced by glycolysis. The shift between fermentation and respiration is referred as diauxic shift. Two to seven days later, when nutrients are limited, yeasts enter the stationary phase, which is characterized by an overall low metabolic activity. This phase of the lifecycle is of primary importance given that most existing single cell organisms are thought to spend most of their lifetime in a non-dividing state with limited windows of mitotic reproduction due to global nutrient scarcity in the wild. To survive these starvation periods, yeasts developed multiple sophisticated physiological responses such as sporulation

(Freese et al., 1982), and quiescence (Gray et al., 2004). Sporulation leads to the formation of gametes wrapped into a special spore wall, while quiescence promotes the entry in a low metabolically active G0 state. In both cases, spore and quiescent cells have substantially enhanced stress resistance and abundant nutrient storage, designed to overcome harsh periods. The duration cells can survive in non-proliferative conditions is referred as chronological lifespan (CLS) (Longo & Fabrizio, 2012). These life cycle features are complex traits tightly regulated by both the genetic background and the environments. Growth, sporulation and CLS are all extremely variable across natural populations and linkage analysis revealed many quantitative trait loci (QTLs) (Wilkening et al., 2014, Gerke et al., 2009, Jung et al., 2018).

In this work, we analysed the lifecycle phenotypic landscape across a large collection of 987 sequenced *S. cerevisiae* isolates. This collection samples the full breadth of *S. cerevisiae* geographic, ecological and historical origins and reveals surprising and pronounced differences in the natural yeast life cycle. Wild clades appear to have the ability to use a broader array of growing substrates, while domesticated strains have developed strong resistance to stresses encountered in industrial settings. This dichotomy becomes more evident in nutrient limiting conditions in which wild strains mainly respond by initiating sporulation, while domesticated strains have mostly lost this capacity and mainly rely on quiescence. We used multiple genotype-to-phenotype approaches to reveal the genetic determinants underlying such striking variation. We observed multiple causative

genomic variants, with many of them specific to individual subpopulations and reflecting genetic accidents in their evolutionary histories. Overall, our work indicates that human driven domestication has shaped the budding yeast life cycle and we suggest that wild lineages have a closer resemblance to the ancestral state.

Results

Wild strains have higher growth fitness in adverse environments

While microorganisms spend most of their lifetime in non-proliferative conditions, mitotic proliferation capacity remains a primary fitness component. This trait might be even more relevant for industrial strains, which are repeatedly inoculated in rich fermenting environments and have shorter non-proliferative periods. We re-analysed in depth the end-point growth capacity (growth yield) of 971 isolates in 36 different environmental conditions (Peter & De Chiara et al., 2018). Overall, wild clades were performing better than domesticated in 16 conditions (Fig. 1A and S1). Wild strains had better growth capacity in all conditions related to stresses, which they might encounter in the wild, such as high temperature (42°C), caffeine, oxidative stress and KCl, which is consistent with previous findings (Gallone et al., 2009). Wild strains also showed a better ability to use alternative carbon sources (ribose, xylose, sorbitol, galactose, glycerol, ethanol, but not acetate), and a broader resistance to chemical stresses (benomyl, cycloheximide, methylviologen, anisomycin, nystatin and hydroxyurea). Interestingly, wild isolates

within the domesticated Wine/European clade (feral strains) were also performing better in some alternative carbon sources (galactose, acetate and xylose) compared to their closely related anthropic counterparts (Fig. S2).

In contrast, domesticated strains have growth advantage only in 5 conditions: copper sulphate, fluconazole, 6-azauracil, sodium arsenite and NaCl (Fig. 1B and S3). Their fitness advantage in these conditions does not appear to be a coincidence since copper can be found in brewing fermenters, in addition of its usage as a pesticide in oenology, similarly to arsenite. Also, sodium and chloride ions have been extensively used as fertilizers in the form of sodium nitrate or

ammonium chloride.

Regarding the other conditions it is complicated to determine whether they are strictly representative of wild or industrial habitats, especially since some nutrients or stresses can be overlapping. Nevertheless, it globally appears that wild strains have kept a broader capacity to grow in adverse environments and resist to various kind of stresses. This observation might be more attributable to defect accumulation in domesticated strains that specialized to defined industrial settings and lost their ability to cope with unconventional nutrients or stresses (Goncalves et al., 2016; Driscoll et al., 2009; Gallone et al., 2016). However, feral strains seem to quickly re-

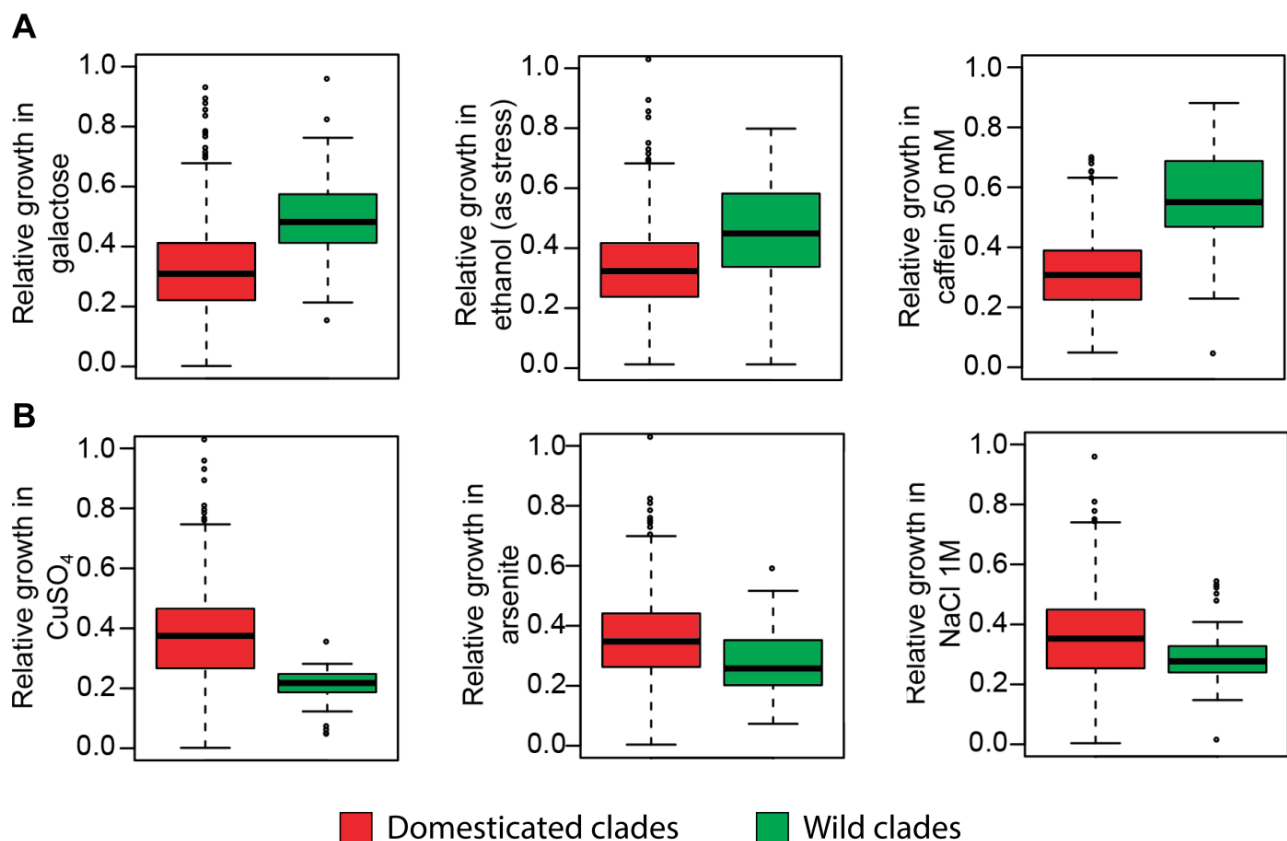


Figure 1. Growth fitness is shaped by domestication. Boxplots representing the growth yield of domesticated (red) and wild (green) strains in different environments. (A) Wild strains are performing better in adverse environments and with alternative carbon sources. (B) Domesticated strains are performing better in man-made environments.

acquire the growth capacity lost in domestic environments.

Domestication impairs sporulation efficiency

Yeast sporulation can be induced in mating type heterozygous diploid cells in response to fermentable sugar and nitrogen deprivation (Honigberg & Purnapatre, 2003; Zaman et al., 2008). Sporulation is a complex trait regulated by multiple

quantitative trait loci and its efficiency is known to be highly variable among yeast strains (Gerke et al., 2006; Deutschbauer & Davis, 2005). Here, we quantified sporulation efficiency across the whole sequenced strain collection in conditions optimised to trigger efficient and rapid sporulation response in laboratory strain backgrounds (see methods). The total amount of asci and the number of spores per asci were monitored at an early (24 hours) and a late (72 hours) time points post KAc

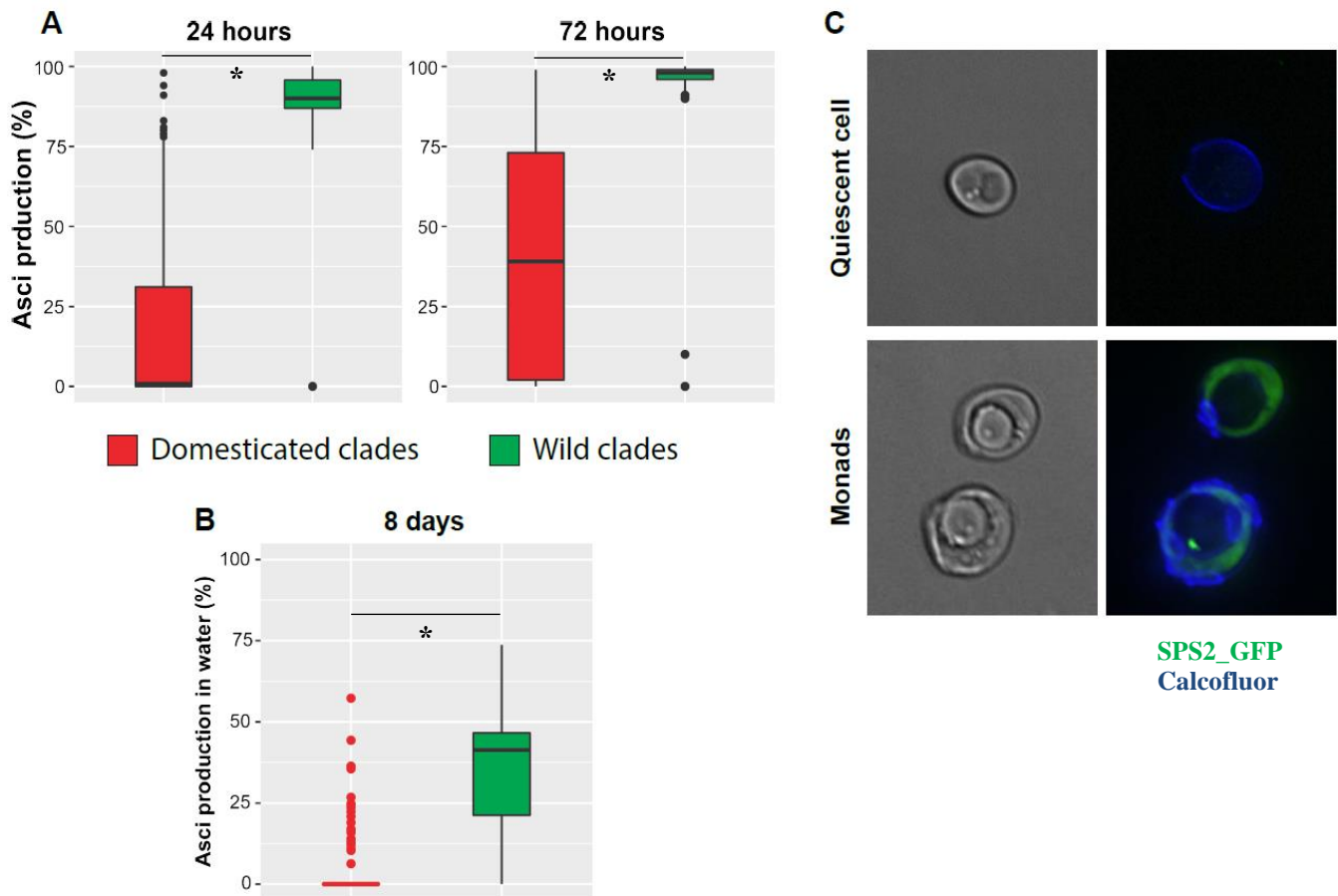


Figure 2. Wild strains are extremely prone to sporulate. Boxplots showing sporulation efficiency of domesticated (red) and wild (green) strains. Asci are the sum of monads, dyads, tryads, and tetrads. (A) Sporulation efficiency in optimal conditions after either 24 or 72 hours of induction (KAc 2% - see methods). (B) Sporulation efficiency in water after 8 days, determined by FACS (see methods). (C) Microscopy pictures of the North American isolate BC233 with *SPS2* GFP tagged, and stained with calcofluor. *SPS2* is a specific protein of spore walls. Picture showing a quiescent cell (top) and monads (bottom). * $p < 0.01$ (1-sided Wilcoxon)

induction. These time points were chosen to capture a broader picture of the sporulation efficiency landscape since some strains could produce almost 100% asci in less than 24 hours, while some others sporulated at much slower pace.

We found that all wild isolates (N=54) were able to quickly initiate sporulation, unlike the domesticated ones (N=503), which were significantly slower. Nearly all wild strains (except 3) reached at least 74% of asci formation within 24 hours (median = 90%), while 50% of the domesticated strains did not even start to sporulate. Wild strains also had better sporulation efficiency after 72 hours of induction, compared to domesticated strains with a median of 98% asci formation versus 36% (Fig. 2A and S4).

Isolates belonging to clades assigned neither to wild nor to domesticated environments (N=293) isolated from a mixture of sources and included human isolates (both clinical and carrier) have a sporulation efficiency distribution similar to domesticated clades (Fig. S4). Domesticated clades are much more heterogeneous than wild clades and globally less efficient. French Dairy, French Guiana, African beer and Sake lineages have extremely low sporulation efficiency and all isolates in the Wine/European *S. boulardii* subclade do not sporulate (Fig. S5).

We next investigated the capacity of our isolates to sporulate in plain water, a simple condition that mimics periodic starvations experienced in natural habitats. This capacity was determined by cytometry, based on cellular morphology (see methods and Fig. S6). Surprisingly, 88% of the wild isolates were able to form asci in water,

while only 0.6% of the domesticated strains could sporulate (Fig. 2B). Sporulation efficiency in water was much lower compared to KAc induction though. In this extreme condition, only monads and dyads were produced due to lack of sufficient resources for the maturation of the four gametes (Fig. 2C) (Taxis et al., 2005).

We further investigated the capacity of a small subset of isolates to sporulate in exhausted media. A total of 8 strains able to sporulate in water (4 wild and 4 domesticated) and with a high sporulation efficiency in KAc (>75%), were grown in YPD media for 3 days. We did not detect sporulation in any of the domesticated strain whereas 3 out of 4 wild strains were able to form asci (from 15 to 45% sporulation efficiency) although no complete tetrads were observed.

Overall, wild strains are extremely prone to sporulate across diverse environmental conditions.

Chronological lifespan varies across lineages and conditions

When environmental conditions are not sufficient to support proliferation, yeasts can exit the cell cycle and enter in an alternative G0 development stage termed quiescence. The duration cells can survive in non-proliferating conditions is referred as chronological lifespan (CLS) and is an essential fitness component to overcome starvation periods. CLS is also commonly used as a paradigm to study aging of post-mitotic tissues such as neurons (Longo & Fabrizio, 2012).

We investigated CLS variation in different environments. Initially, we measured CLS in phosphate buffered water (PBS) for 734 sequenced isolates. However, CLS could not be monitored in wild isolates (except for six strains) since these strains were sporulating in this condition. The percentage of viable cells ranged from 0.5% to 96.8% after 20 days of chronological ageing. We observed a drastic species wide variation in CLS with French Guiana and African Cocoa isolates

being the longest living clades, while French dairy and African beer were the shortest ones. Human isolates were long-living compared to domesticated strains (Fig. 3A).

In addition, since CLS is a complex trait highly environment dependent, we monitored the longevity of a large subset of 572 euploid diploid isolates in different conditions known to slow down aging: calorie restriction (CR), and rapamycin

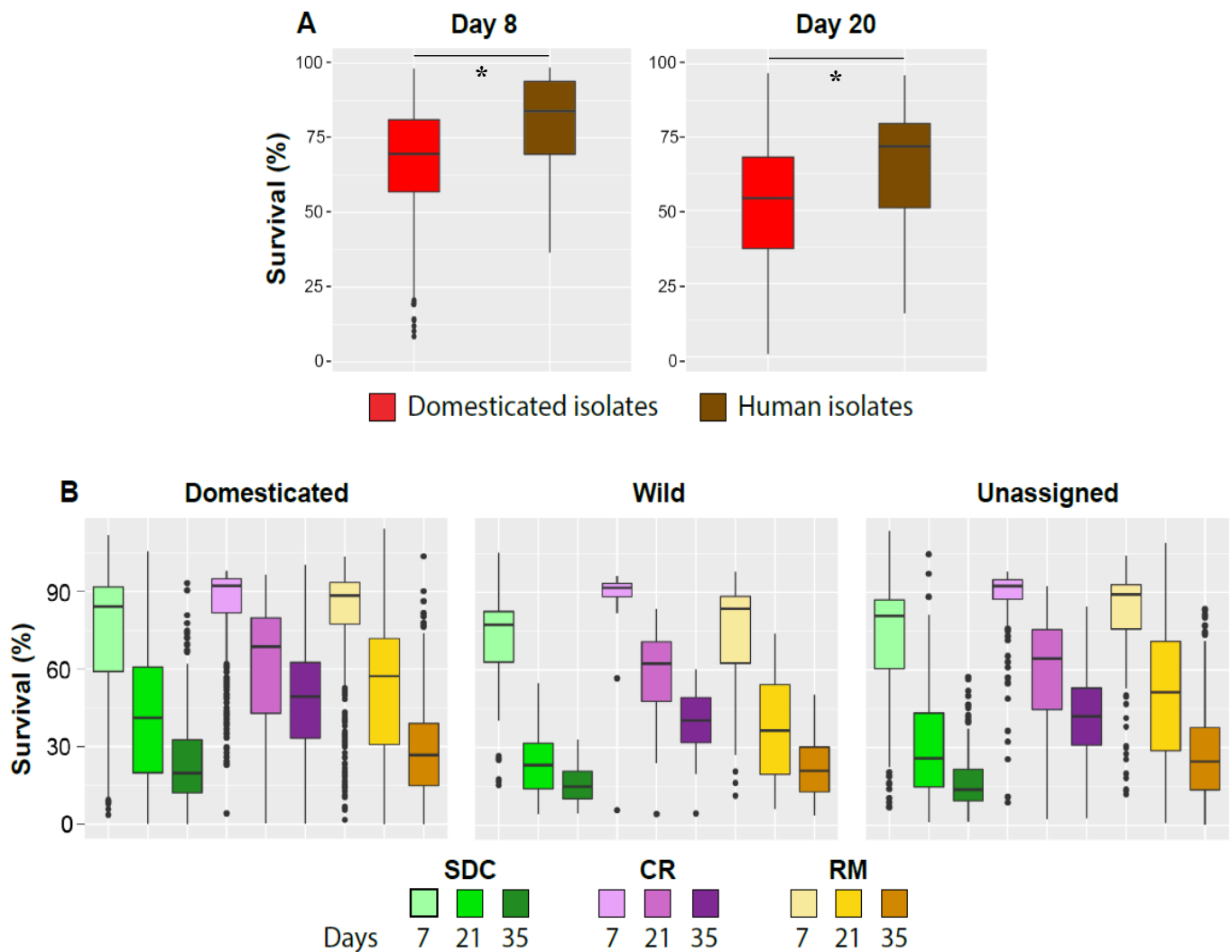


Figure 3. CLS is highly variable species wide. Boxplots representing CLS values in different environments. (A) Viability of domesticated and human isolates after 8 or 20 days of aging in PBS (B) Viability of domesticated, wild, and unassigned strains in either SDC, or CR, or RM at different time points. * $p < 0.01$ (1-sided Wilcoxon).

administration (RM). Rapamycin is known as a drug mimicking CR through direct inhibition of the TOR pathway (Loewith, 2011). Synthetic media (SDC) was used as a control, and viability was estimated at three different time points (7, 21, and 35 days post-stationary phase).

Since wild strains do not sporulate in SDC based media, we could this time obtain a CLS estimation for them. Overall, CLS was equivalent in wild and domesticated strains, excepted in 3 conditions in which domesticated strains were in average better: SDC days 21 and 35, and CR day 21 ($p < 0.01$; 2-sided Wilcoxon) (Fig. 3B). However, most of the variation was explained by specific lineages. The Taiwanese and the French Dairy clades had the shortest CLS with less than 30% of cells alive after 7 days in SDC. In contrast, French Guiana, Mexican agave and West African Cocoa clades confirmed their long lifespan with more than 60% of survival rate after 21 days in SDC (Fig. S7). The West African Cocoa is the only clade that did not improve lifespan in CR (Fig. S7). Since they are already long living in SDC, it is possible that they naturally harbour genetic polymorphisms mimicking calorie restriction. Apart from this exception, both CR and RM globally increased lifespan across all genetic backgrounds, although the efficiency of each intervention was clade dependent. However, the effect of CR appears in average stronger in all clades compared to RM (Fig. S7).

Finally, since sporulation appears as a quiescence alternative favoured by wild strains to survive starvation, we monitored spore lifespan by microdissection. We measured spore viability after KAc induction, using a wild (North American -

AKN) and a domesticated (Wine/European - BTN) isolates that were subsequently swapped to water. After 8 weeks of chronological aging in water, spore viability did not decline at all (more than 90% viability). Interestingly, this was the case for both strains, indicating that although domesticated strains are less prone to initiate sporulation, their spores are as resistant as the ones of wild strains. These data suggest that sporulation is a better route than quiescence to survive starvation. However, because of the plurality of stationary cultures and of quiescence states, a direct comparison between quiescence and sporulation remains difficult (Allen et al., 2006; Klosinska et al., 2011).

Overall, there are no striking CLS differences between the domesticated and wild groups as a whole, instead there are clear separations at the clade level. This variation could also reflect differences in other kind of stress resistance with pleiotropic effects which could translate into lifespan variation.

Sporulation deeply modulates *S. cerevisiae* genetic landscape

In *Saccharomyces* species, sporulation and meiosis are tightly linked with each other, implying that the capacity of strains to sporulate will impact gamete production and breeding rate. The existence of a general negative correlation between sporulation and heterozygosity has been previously observed (Magwene et al., 2011). Indeed, we found that sporulation efficiency is higher in homozygous isolates (Fig. 4A). This fact is particularly evident for wild strains that are all extremely proficient at sporulating and

nearly completely homozygotes. This result might look unexpected since the outcome of sporulation is sexual reproduction, contributing to outbreeding. However, *Saccharomyces* sporulation in the vast majority of cases is followed by automixis (or intratrad mating) causing long run of homozygosity (Tsai et al., 2008). More rarely, haploid cells switch mating type and perform haplo-selfing leading to complete homozygous diploids. Only a tiny fraction of the spores is predicted to mate with gametes from other cells resulting in outbreeding. Gametes produced during sporulation, hence, mainly escape outbreeding and favor haplo-selfing, which could be a strategy to purge deleterious mutations and uncover recessive beneficial heterozygosities through a process defined as “genome renewal”

(Mortimer, 2000). Nevertheless, there is no global correlation between sporulation and level of heterozygosity (Fig. 4B). This result is well illustrated by a group of clades (Sake, French Guiana, Wine/European *S. boulardii* subclade), which exhibit a low amount of heterozygosity (up to 1 heterozygous SNP for kb in heterozygous regions) while having a poor sporulation capacity. In these clades, their extended runs of homozygosity seem correlated with extensive events of loss of heterozygosity (LOH), which occur at higher rates in domesticated isolates (Peter & De Chiara et al., 2018). The mitotic LOH events likely explain the lack of global correlation between sporulation and heterozygosity. Therefore, while wild strains maintain a high sporulation rate allowing them to rapidly increase and fix genetic

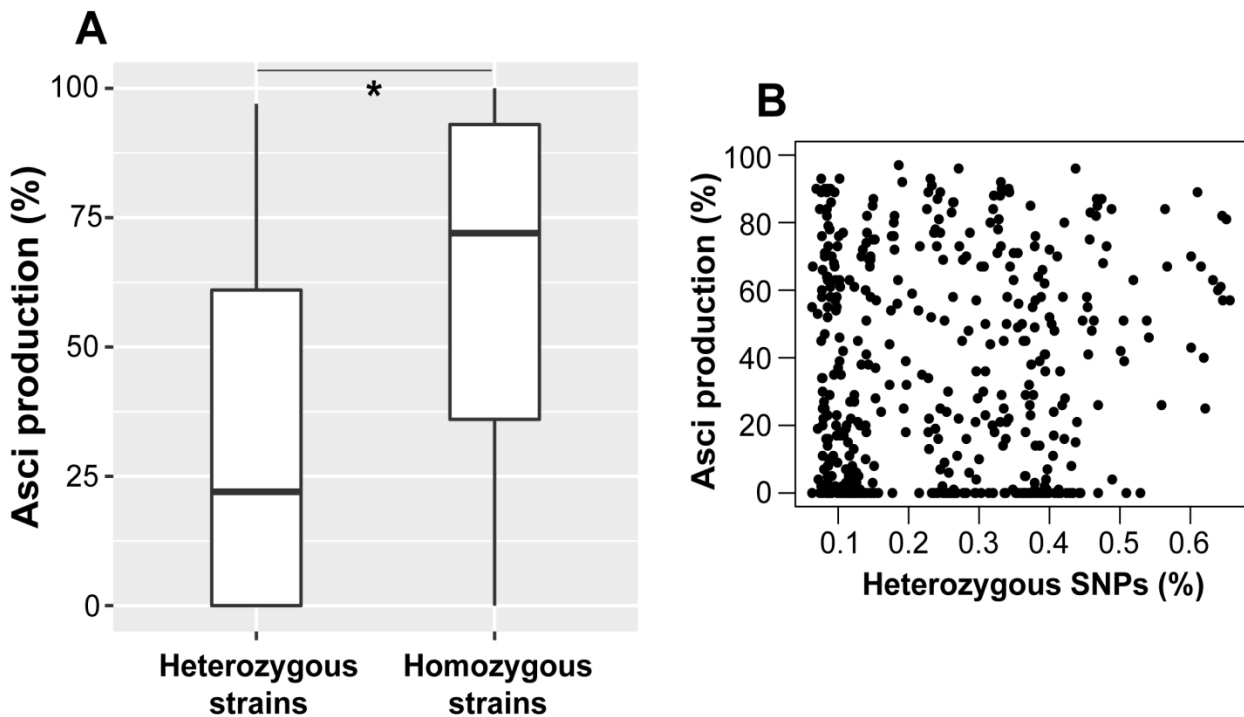


Figure 4. Sporulation and genome wide heterozygosity. (A) Sporulation efficiency distributions for homozygous and heterozygous strains after 72 hours of induction in KAc. (B) Correlation between asci production and heterozygosity frequency in delimited heterozygous regions, 72 hours post-KAc induction. * $p < 0.01$ (Wilcoxon).

polymorphisms through inbreeding and haplo-selfing, domesticated strains appear to have favoured asexual reproduction and rely more on the fixation of recessive mutations through mitotic recombination (Gerstein et al., 2014, Vazquez-Garcia et al., 2017).

Aneuploidy but not polyploidy negatively impacts sporulation

Genome analysis of the 1011 sequenced strains revealed a significant enrichment for aneuploidies and higher ploidies among domesticated clades (Peter & De Chiara, 2018). We compared sporulation efficiency across the strain collection according to their genome and chromosomal contents. We observed that sporulation efficiency is strongly inhibited in the presence of aneuploidy at both early and late time points (Fig. 5A). Interestingly, we observed that sporulation efficiency is further impaired in strains with multiple chromosomal aneuploidies (Fig. 5A). A weak general negative correlation between increased number of aneuploidies and sporulation efficiency was detected ($R = -0.24$, pearson $p\text{-value} = 4.854e-13$). Interestingly, there was no difference between strains with gain or loss of aneuploid chromosomes. This result is surprising since aneuploidies from chromosome loss ($2n-1$) will inevitably lead to inviable gametes. Overall, aneuploidy appears as major determinant of the poor sporulation ability with effect size calculated as omega-squared from ANOVA of 0.045 at

24 hours and 0.073 at 72 hours (restricting the analysis to diploid strains). Chromosomal aneuploidies might entirely explain the poor sporulating capacities of the Ale and African beer clades, which have not evident LOF in sporulating genes (see below).

In contrast, polyploidy does not impair sporulation (Fig. 5B). There is no correlation between ploidy and ascii production, although we found a slightly lower sporulation efficiency for euploid polyploid isolates compared to euploid diploids at 24 hours but not at 72 hours. This holds true for both triploid and tetraploid strains, which are known to face different chromosomal pairing and segregation scenarios (St Charles et al., 2010). The slower sporulation of polyploids might reflect longer DNA replication time during the meiotic S phase. Alternatively, since polyploids are clustered into few given subpopulations, their slow sporulation at 24 hours could result from their genetic background rather than their ploidy.

Chronological lifespan is affected by both aneuploidy and polyploidy

Similarly to sporulation, we observed a strong detrimental effect of aneuploidy in chronological aging (Fig. 5C). However, we did not observe a cumulative effect due to additional aneuploidies. In addition, polyploids also appear to have a strong detrimental impact on CLS (Fig. 5D). Diploids have substantially higher viability

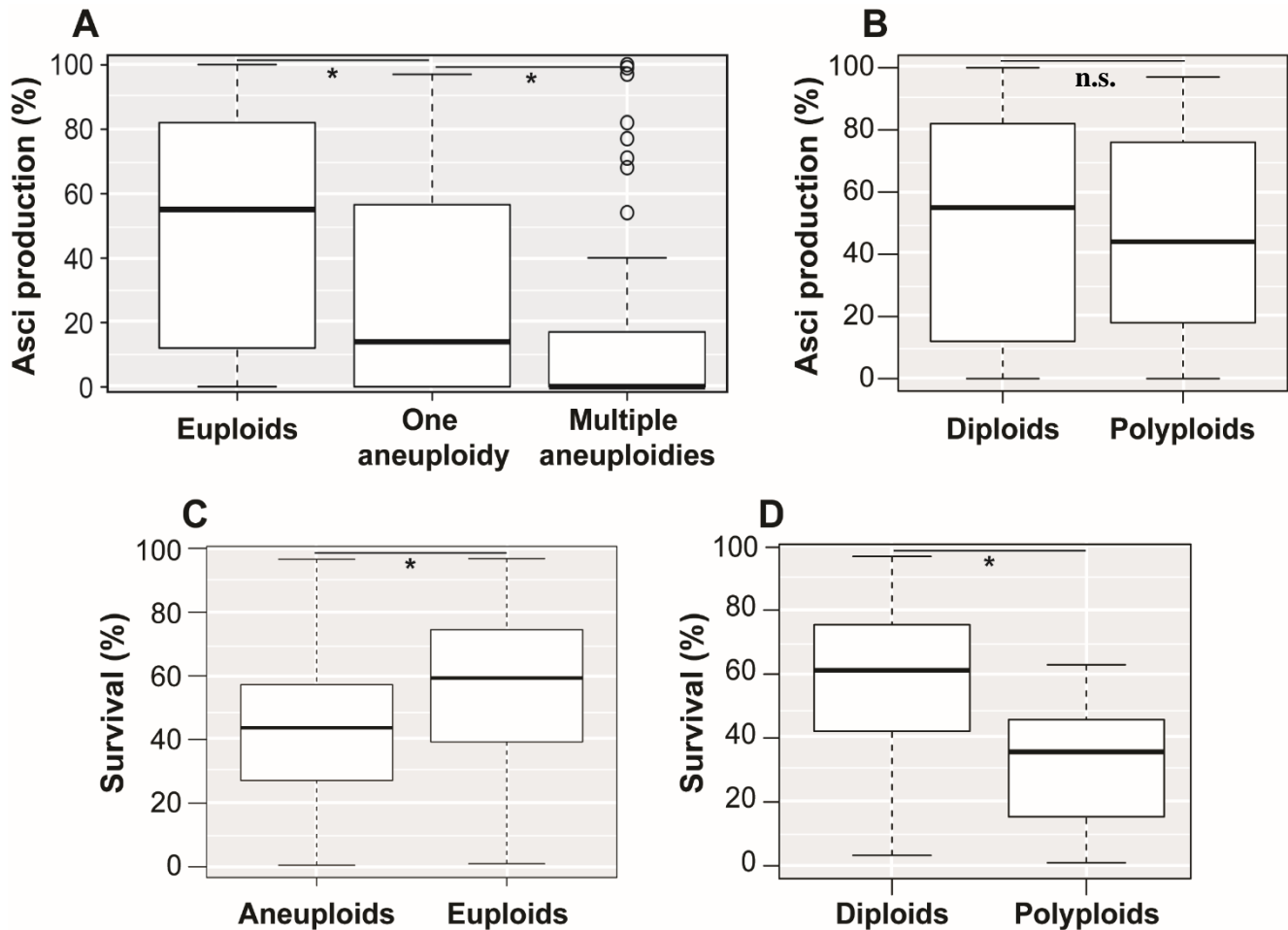


Figure 5. Aneuploidy and polyploidy strongly impact sporulation and CLS. (A). Boxplot representing sporulation efficiency of euploid and aneuploid isolates after 72 hours of KAc induction. Aneuploid isolates were split into two categories according to the amount of aneuploidies they harbour. (B) Boxplot representing sporulation efficiency of diploid and polyploid isolates after 72 hours of KAc induction. (C) Boxplot representing CLS of euploid and aneuploid isolates after 20 days in PBS. (D) Boxplot representing CLS of diploid and polyploid isolates after 20 days in PBS. * $p < 0.01$ (1-sided Wilcoxon). n.s. Non-significant.

rates at both day 8 (75% versus 58% median survival) and day 20 (61% versus 35% median survival). Since polyploids are enriched in aneuploidies, the analysis was restricted to euploid diploid and euploid polyploid isolates. The detrimental effect of aneuploidy was already reported in yeast replicative aging, but not in the chronological one (Sunshine et al., 2016). It was found that replicative aging is shortened due to unbalanced protein synthesis caused by the additional chromosome, leading to proteostatic stress. Regarding CLS, it plausible that a similar mechanism is

occurring, both for aneuploidy and polyploidy, although we have no evidence supporting this model.

Multiple mutations impair sporulation in domesticated yeasts

The lifecycle phenotypes studied here were extensively characterised at the mechanistic level in the laboratory strain genetic backgrounds. We compiled a catalogue of 251 genes related to sporulation and meiosis to apply a candidate gene approach. We

searched for deleterious and loss of function (LOF) variants that could impair sporulation. We run SIFT on the SNVs catalogue as well as calculated small indels causing frameshifts to systematically predict occurring LOF.

This approach provided compelling LOFs in key sporulation regulators in poorly sporulating clades and subclades. For example, we observed LOFs in *RIM15* and *SPO74* in the Sake clade. The *RIM15* variant has already been experimentally validated in another study (Bergstrom et al., 2014). Similarly, the *S. boulardii* subclade has private homozygous frame-shifts in *MND1* and *SHE10*, which might contribute to their complete loss of sporulation capacity. In multiple cases, we observed several deleterious variants in sporulation related genes, co-occurring with a LOF in a major sporulation regulator. Striking examples are the French dairy isolates which harbour a start loss on meiosis-specific transcription factor *NDT80* and nonsense mutations *OSW2*, *MND2*, *SPR6*, *DOM34*, *AQY1*. Similarly, French Guiana strains have a nonsense mutation in the meiotic master regulator *IME1* as well as a frame-shift in *MPC54* and LOFs in *DIT2*, *BMH2*, *DON1*, *NUD1* and *AQY1*.

We hypothesised that the accumulation of multiple deleterious variants in different domesticated clades reflects relaxed selection on sporulation ability. To test this theory, we compared genome-wide distribution of deleterious mutations predicted by the SIFT algorithm as “deleterious” or “highly disruptive” within domesticated and wild clades. In total, domesticated clades showed more deleterious mutation genome wide with an average number of 824 affected genes

against 666 in wild clades. We observed a strong enrichment of deleterious mutations in meiotic genes in domesticated clades, while they were significantly preserved in wild strains (Fig. 6A). These results underlie a strong selective pressure on maintaining efficient spore formation and sexual reproduction for wild isolates. In contrast, domesticated strains have accumulated mutations in sporulation associated genes, suggesting a lack of selection, or even a negative selection, of this trait in man-made environments.

Interestingly, we found 9 Wine/European strains that were able to efficiently sporulate (up to 60% at 72 hours) but only produced dyads due to the presence of LOF in either *SPO12* or *SPO13*, two genes required for meiosis completion and tetrad formation (Klapholz & Easton Esposito, 1980). Seven of these isolates are very closely related and contain a premature stop codon in *SPO13* (G86*) as well as a frameshift caused by insertion in *SPO12*. The other two, also closely related with each other, harbour two disrupting mutations in *SPO12*: a frameshift and a premature stop codon (K103*). Despite these mutations that block meiosis after the first division, these strains have maintained the ability to form spores, perhaps indicating that this event is recent and degeneration of other genes might follow, unless there is some selection in maintaining a partially functional meiotic programme that enables the first meiotic division and the formation of diploid spores. Among these isolates that only form dyads, one strain appears to have accumulated massive segmental duplications genome-wide. This finding might indicate that strains with partially or non-functional

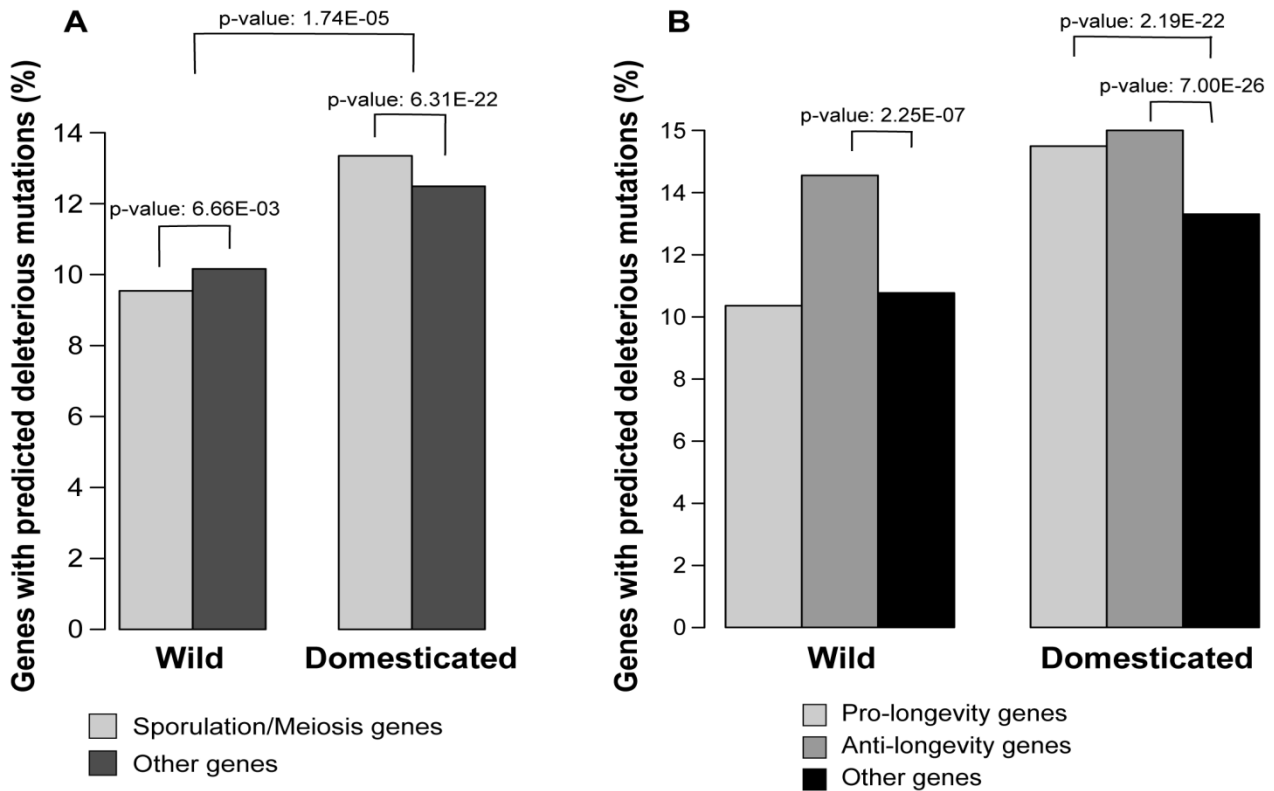


Figure 6. Sporulation and pro-longevity genes are protected in wild strains. Bar plots showing the frequency of deleterious and highly disruptive mutations found either in sporulation related genes (A), or in pro and anti-longevity genes (B), compared to the genome-wide rate. Deleterious and disruptive mutations were predicted with the SIFT algorithm. p-values were calculated with 1-sided Fisher test.

sexual cycle do not have the selective constraints of exchanging genetic material with other individuals and might evolve unique genome structures.

Losses of function do not impair chronological lifespan

We used a similar candidate gene approach for CLS, testing genes whose deletion is referenced to enhance or decrease lifespan (Matecic et al., 2010). In addition, we also included genes belonging to the AMPK and cell wall integrity pathways which are required to promote survival in stationary phase (Cao et al., 2016). In contrast with sporulation, we did not observe any strong signature of gene inactivation that would significantly impact CLS. Among interesting candidates, we observed private LOFs in the

French Dairy *SIC1* gene, which could partly explain their very short lifespan, and in French Guiana *PCL7*, which could contribute to their long lifespan. The lack of strong LOF signature may be explained by the absence, or the low frequency, of detrimental mutations within key CLS regulators. In addition, it is possible that the effects of gene deletion, which were referenced in a laboratory strains, are neutral in other genetic backgrounds, especially since lab strains appear to be phenotypic outliers (Warringer et al., 2011).

We next considered deleterious polymorphisms as predicted by SIFT and partitioned genes promoting and shortening lifespan in the laboratory strain background. In wild strains, we observed deleterious

mutations enriched in genes which extends CLS when deleted. In contrast, deleterious mutations were enriched in both pro and anti-longevity genes in domesticated strains (Fig. 6B). These findings reinforce the idea of relaxed negative selection in domesticated strains as a consequence of recent population expansions in human made artificial niches (Bergstrom et al., 2014). Alternatively, since most of genes regulating CLS have trade-off effects with growth (Delaney et al., 2011), it is possible that different domesticated clades have accumulated either pro or anti-longevity mutations according to their needs. The observation that wild strains accumulate mutations in anti-longevity genes probably highlights their need to develop efficient survival strategies in the wild. In addition, several genes positively regulating CLS and sporulation are overlapping, such as *RIM15*, or *RAS2*, or *IRA1* among others (Cao et al., 2016; Toda et al., 1985; Cannon et al., 1994). Hence these mutations could have been co-selected to improve both traits, or are simply hitchhikers of sporulation. Nevertheless, while this mutation enrichment may contribute to make wild strains more resilient in natural habitats, it does not make them fitter than domesticated isolates in synthetic media.

Referenced QTNs are rare and genetic background dependent

Sporulation has been studied extensively as a quantitative phenotype and multiple quantitative trait nucleotides (QTNs) that contribute to sporulation variation were discovered (Gerke et al., 2009; Tomar et al., 2013; Ben-Ari et al., 2006). This collection

of natural alleles might provide different insights from screens of the deletion that were restricted to laboratory strain backgrounds. We compiled a list of known QTNs and quantified their contribution to the sporulation variation in the sequenced strain cohort. The frequency of QTNs is extremely variable ranging from very rare like *TAO3* (E1493Q) only detected in two strains, or *SPO74* (C16A) not present in any strain of the sequenced collection, to *MKT1* (D30G) present in all the isolates except two mosaic haploid strains (Tomar et al., 2013, Deutschbauer & Davis, 2005). However, the *MKT1* D30 allele is known to be a rare derived variant present in the S288C background with pleiotropic effects impacting many phenotypes (Strope et al., 2015; Peter & De Chiara et al., 2018).

Two deleterious QTNs in *IME1* (L325M and A548G) segregated in a Wine/European and Oak North American cross (Gerke et al., 2009). We found in the Wine/European and Alpechin clades 19 strains homozygous for the L325M mutation, 5 strains carrying L325M and A548G but no strains carrying only the A548G. Despite the high relatedness of these strains, their sporulation efficiency varied broadly ranging from less than 10% (six strains) to more than 90% (three strains) (Fig. 7A). In addition, strains grouped by all possible *IME1* genotype combinations at positions 325 and 548 do not quantitatively differ in sporulation (Fig. 7B).

The *HOS4* (A1384G) and *SET3* (A1783T) variants were detected using association mapping in the SGRP strain panel (Liti & Carter et al., 2009, Tomar et al., 2013). We found 26 strains homozygous for *HOS4* (A1384G) mutation, 64 homozygous for

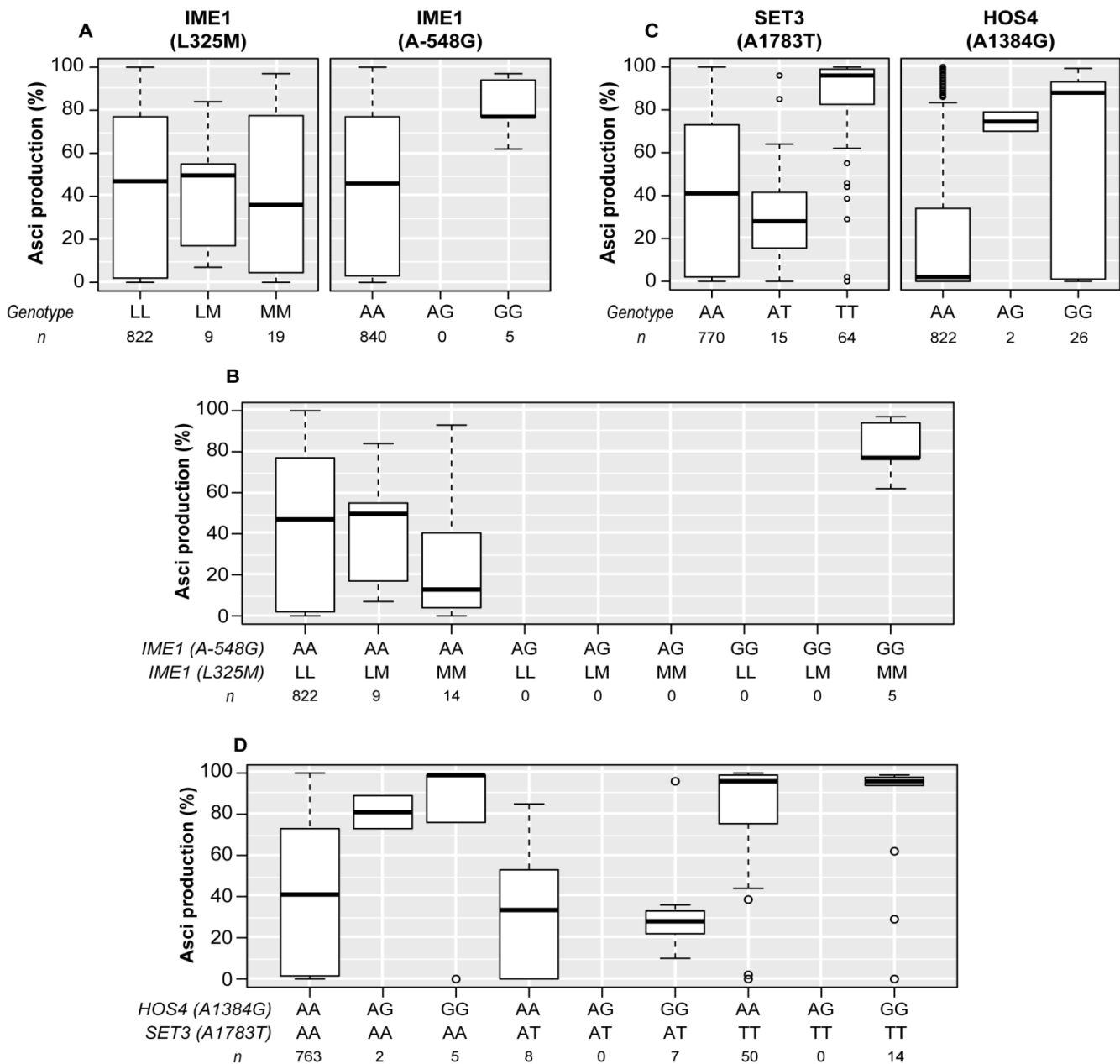


Figure 7. The effect of referenced QTNs on sporulation is highly variable within natural isolates. Bar plots showing sporulation efficiency (72 hours post-KAc induction) according to the genotypic distribution of different QTNs identified in previous studies. **(A)** Effect of *IME1* QTNs at the population level. **(B)** Effect of the different combinations of *IME1* QTNs. **(C)** Effect of *SET3* and *HOS4* QTNs at the population level. **(D)** Effect of the different combinations of *SET3* and *HOS4* QTNs.

SET3 (A1783T), and a total of 14 isolates homozygous for both mutations. Homozygous strains for *SET3* A1783T had a substantially higher sporulation rate (+130% median increase). On the other hand, isolates carrying the *HOS4* A1384G do exhibit sporulation increase only for

isolates harbouring the homozygous allele of *SET3* (Fig. 7C and 7D). These results indicate that the *HOS4* QTN has an effect dependant on the specific state of the *SET3* allele or by different background dependency. Several additional QTNs were homozygous and at high frequency within

our yeast collection. The *MCK1* (C1112A) is present in 89 isolates, *RSF1* (D181G) in 177 isolates, *PMS1* (A122G) in 161 isolates and *FKH2* (A1498C) in 422 isolates. However, none of these QTNs significantly impacted the sporulation (Fig. S8). Altogether, analysis of known QTNs was partially limited by the low frequency of several variants. For the abundant QTNs, except for *SET3*, we did not observe an effect, perhaps indicating the interaction with genetic background plays a key role.

Natural variation in *IME1* globally regulate sporulation efficiency

The availability of whole genome sequencing enables to apply a genome wide association studies (GWAS) approach. We ran the GWAS using the complete set of genomic features that include SNPs, losses of function, gene presence/absence and copy number variation filtered for a minor allele frequency (MAF) of 5%. Analysis of the narrow sense heritability h^2 indicates that a large part of the phenotypic variance, across all traits, is explained by genome-wide genetic variants segregating in the population and suggest the GWAS feasibility (Fig. 8A).

GWAS results for 24 hours of KAc induced sporulation detected 351 SNPs and 6 other types of variants. The high number of hits might reflect high number of false positive given this trait has a lower fraction of the variance explained by the variants detected (Fig. 8A). This might be driven by the low differentiation of the phenotype at this time point which has a skewed distribution, or by the presence of LOFs with large effect sizes undetectable due to their strong association

with population structure. GWAS detected 82 variants after 72 hours of sporulation, with 55 overlapping with the 24 hours time point (Fig. 8B). The vast majority of these hits is again represented by SNPs, except for *SAM4* LOF and presence/absence of a non-reference homolog of the cytochrome c subunit. Across the two time points, 384 unique genetic markers are identified and 30 of them (6 detected at both time points) are affecting 19 sporulation candidate genes or their flanking intergenic regions (upstream or downstream). The GWAS hits are significantly enriched for sporulation genes (11%, $p\text{-value} = 3.90\text{e-}05$, chi-squared) compared to their genome occurrence (5%).

The *IME1* master regulator of the meiotic program was the main hit with five variants; four of them were detected at both time points (Fig. 8B and S9A). There is no overlap between the variants identified here by GWAS and the ones previously mapped by linkage (Gerke et al., 2009), consistent with the limited contributions of the QTNs at the population level. We detected two hits in the promoter region (A-325C, C-181T) and three nonsynonymous within the coding sequence (H78R, N311Y, and two minor alleles of the same codon E316V and E316G). The N311Y allele is only detected at 24 hours and isolates carrying this variant have lower sporulation efficiency at 24 hours but no effect at 72 hours, suggesting that this variant slows the sporulation program (Fig. S9B). Instead, the H78R mutation promotes sporulation and segregates at high frequency in the population (27%). Nevertheless, this allele is not present in wild clades and is a derived allele (when taking *S. paradoxus* as outgroup). Its effect is particularly striking

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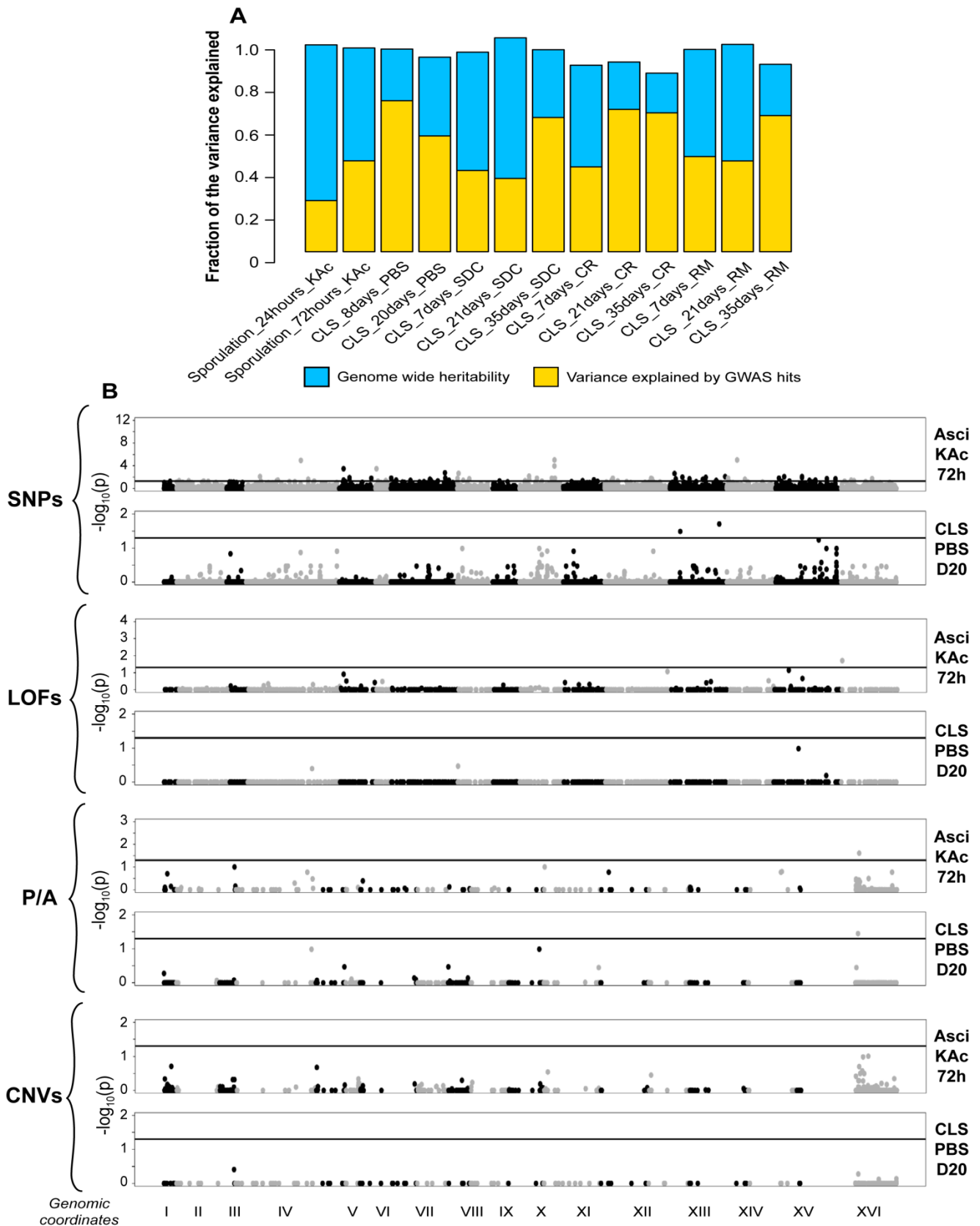


Figure 8. GWAS reveal new genetic variants regulating sporulation and CLS. (A) Narrow sense heritability and phenotypic variance explained by GWAS predicted genetic determinants. (B) Association scores for sporulation (72 hours post KAc incubation) and CLS (8 days in PBS) phenotypes of genome wide markers and non-reference ORFs (for both presence/absence and copy number variation). SNPs: Single Nucleotide Polymorphisms. LOFs: Loss of functions. P/A: Gene presence or absence. CNVs: Copy number variation. MAF > 5%. The switch between grey and black areas delimits the 16 chromosomes.

when restricting the dataset to the domesticated strains (Fig. S9C). Overall, GWAS revealed new alleles that quantitatively affect the sporulation and segregate at high frequencies in the population, while fail to identify variants mapped by candidate gene approach and linkage analysis due to their low frequencies and population stratification (Fay, 2013).

Chronological lifespan causative variants are condition dependent

We applied the GWAS approach to the chronological lifespan phenotype across all the environments and time points in different strain subsets (see methods). We detected nine significant variants in PBS, with some of them replicating across time points (Fig. 8B). We next run the GWAS on the 572 strains subset in the three SDC, CR and RM environments and detected a total of 288 unique significant hits (Fig. S10 A, B, C, D). Most of these hits (N=177) were identified only in a specific condition or time point, while only a small fraction (N=8) were found in at least 5 time points across conditions. We detected a particularly recurrent hit in chromosome X that was mapped at all SDC time points, and also at early CR and RM time points. This interval contains 13 strongly linked SNPs within the *SDH1b* gene region. *SDH1b* is a paralog of the gene *SDH1* which encodes the succinate dehydrogenase protein, which is required to perform respiration. It is plausible that *SDH1b* regulates CLS through modulation of mitochondrial respiration which is needed to promote yeast survival in stationary phase (Bonawitz et al., 2006; Piper et al., 2006). These polymorphisms are

strongly linked and widespread in the population (MAF ranges from 16% to 47%). Only one variant is non-synonymous and associated with shorter CLS (Fig. 9).

Among the other ORFs features, we observed two hits that strongly replicated across conditions and affect gene candidates previously associated with CLS. Specifically, a common LOF is found in 5 conditions/time points, affecting the *WHI2* gene, including PBS as well. The only another hit which is common to both SDC and PBS is a SNP on the *Gsf2* gene in the chromosome XIII.

Surprisingly, there is no overlap between genetic determinants identified in CR and RM. The three CR time points revealed a very strong hit among the variable ORFs segregating as presence/absence in the population. This hit points to the *HPF1* cell wall protein, found in 69 isolates, and is significantly associated with reduced lifespan at all time-points. An additional hit, also detected at all CR time points, is found in the upstream region (position -2065) of *HPF1* again. These results underlie the emerging role of the *HPF1* gene in regulating chronological aging among natural yeast populations (see chapter 6).

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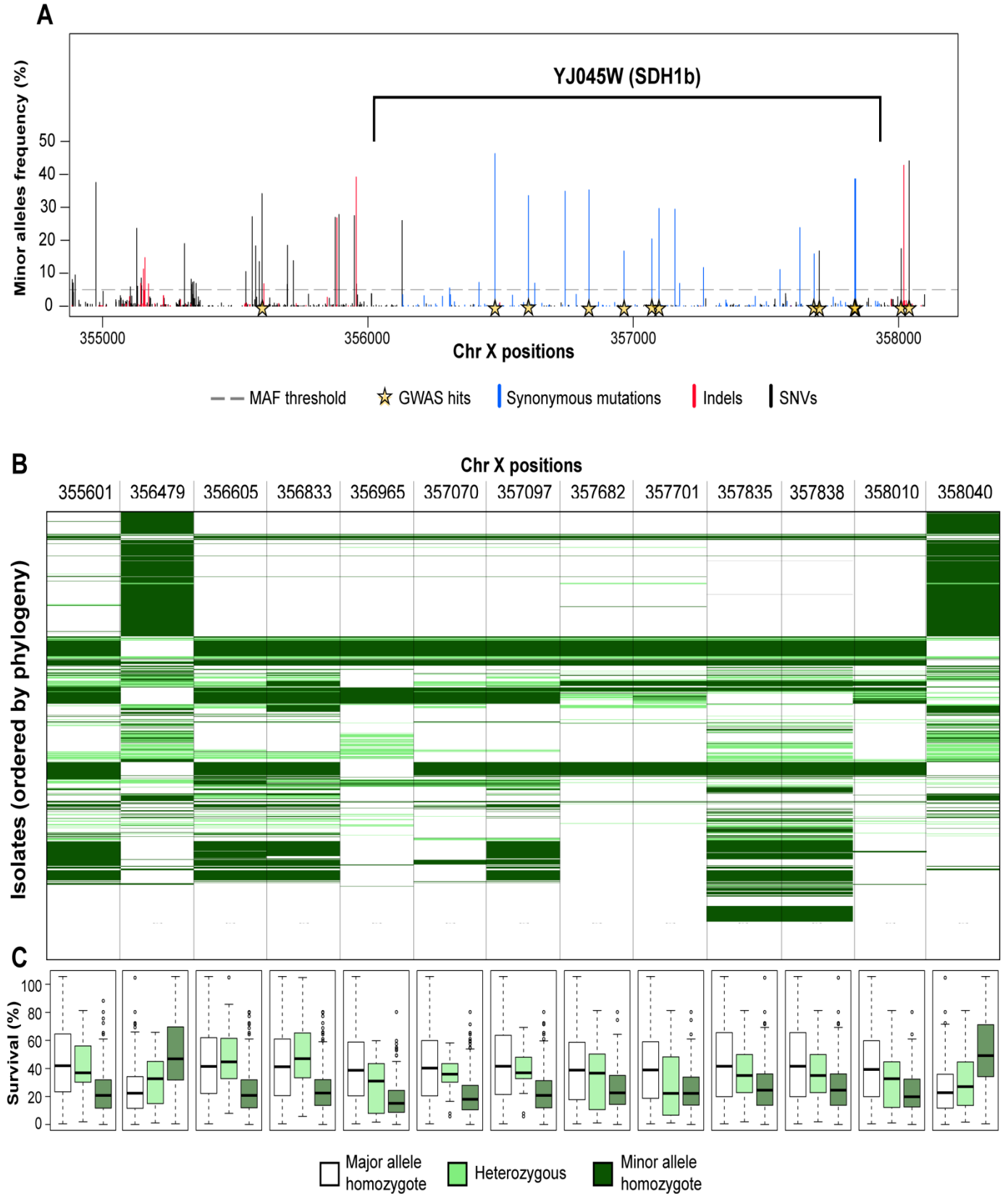


Figure 9. SDH1b variants modulate CLS. (A) Summary of the thirteen QTNs (yellow stars) identified by GWAS at SDH1b locus. (B) Distribution of *SDH1b* variants among the 572 isolates tested for CLS in exhausted media. (C) CLS of the 572 euploid diploids after 21 days of chronological aging in SDC according to the *SDH1b* genotype they harbour (indicated above).

S. cerevisiae wild strains phenotypically resemble *S. paradoxus*

One scenario that explains the phenotypic dichotomy between wild and domesticated strains is that human selection has profoundly affected traits in domesticated strains. Because *S. paradoxus* has not been extensively domesticated, we suppose that isolates of this species should be phenotypically close to wild *S. cerevisiae* strains. To test this idea, we monitored the CLS and the sporulation capacity (in water) of 12 *S. paradoxus* isolates and compared the values obtained with those of 49 domesticated and 23 wild strains that were rerun in the same batch to avoid noise from experimental stochastic variations. The 12 *S. paradoxus* chosen are representative of all the four major subpopulations of the species (Yue et al., 2017). Domesticated *S. cerevisiae* strains showed quantitative and qualitative differences compared with both

wild *S. cerevisiae* clades and *S. paradoxus*. Specifically, they had less sporulating isolates and a lower yield among the few isolates that were able to sporulate in water (Fig. 10A). In contrast, variability in the phenotypic values (even within clades) is significantly higher compared to both wild *S. cerevisiae* and *S. paradoxus*, which instead have comparable variances. F-tests also demonstrated that the variance of CLS is significantly higher for domesticated clades (>3.5 times) compared to either wild *S. cerevisiae* (3.6 times, fisher test p-value = $1.74e-3$) or *S. paradoxus* (4.7 times, fisher test p-value = $8.56e-3$; Fig. 10B). Overall, these results support the phenotypic relatedness between wild strains of the *S. cerevisiae* and *S. paradoxus*, while domesticated *S. cerevisiae* strains are highly variable and distinct.

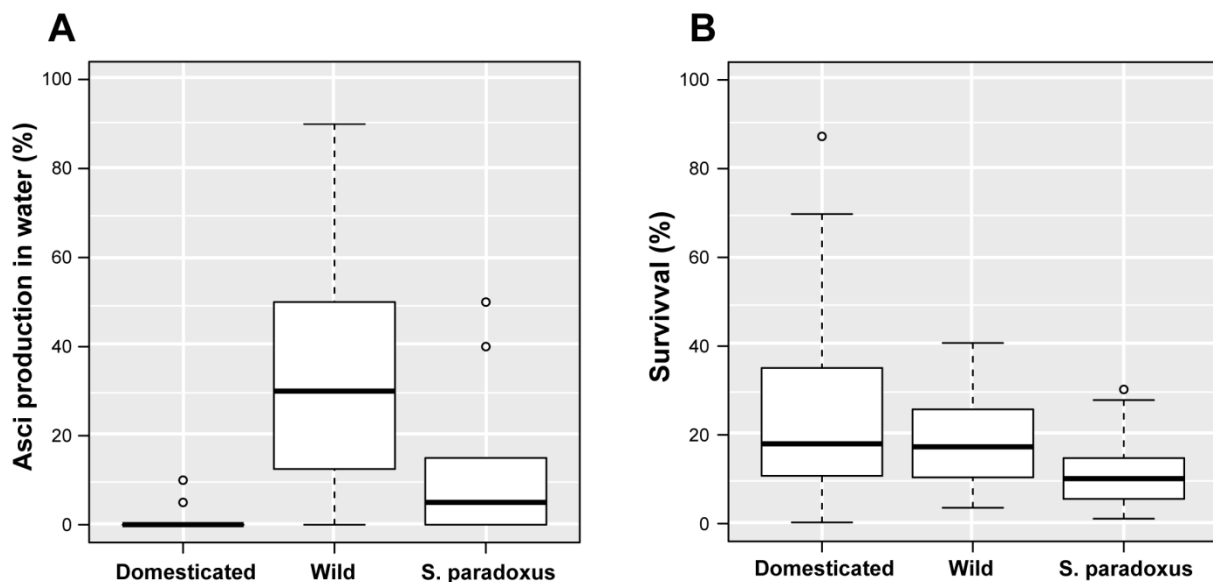


Figure 10. *S. paradoxus* is phenotypically close to wild *S. cerevisiae* isolates.

Phenotypic behavior of a subset of 49 domesticated and 23 wild strains of *S. cerevisiae*, compared to 12 representative isolates of *S. paradoxus*. (A) Capacity to perform sporulation in water. (B) CLS after 19 days in SDC.

Discussion

We presented a broad overview of the quantitative phenotypic variations in key components of the yeast life cycle in both wild and domesticated clades. Wild clades evolved to thrive in conditions where carbon sources are scarce and diversified. They are superior in their ability to use alternative carbon sources such as ribose, xylose, sorbitol, galactose, glycerol and ethanol. When nutrients become limiting, wild strains rapidly trigger the sporulation process. The formation of sexual spores appears as an efficient strategy to resist stress (Coluccio et al., 2008). In addition, our results indicate that these cell types are extremely long living and therefore spores could represent the state how isolates in natural environments bypass long periods of starvation. Surprisingly, we found that sporulation is triggered even in total absence of energy source, possibly using nutrients stored previously, although producing almost only asci with single spores (monads).

In stark contrast, we observed a strong deviation from the natural lifecycle in human related environments and we proposed that this is likely driven by the domestication. Domesticated clades grow on richer media where sugars are abundant and can support large population sizes. Growth curves across a large panel of conditions previously highlighted the ability of domesticated strains to rapidly grow (short doubling time) that will enable to outcompete coexisting microbes (Liti & Carter & al., 2009; Warringer et al., 2011). They also were exposed to several physical and chemical stresses and have evolved

strong tolerance to some of these conditions such as resistance to copper and arsenite. However, this specialization to a human made environment has likely resulted in relaxed selection for some component of the natural life cycle. This is consistent with enrichment of deleterious variants that accumulated during the evolution of domesticated clades, perhaps as rapid recent expansion in these sugar rich niches (Bergstrom et al., 2014). This quantitative variation in deleterious variants is much evident in the dataset presented here and enable to test accumulation of deleterious variants within specific cellular processes, pathways and gene sets.

The repeated loss of the sexual cycle in multiple lineages and strains represent a striking difference between domesticated and wild strains. The inability to trigger the sporulation has profound consequences on their lifestyle and evolution. First, when nutrient shortage occur, cells can only persist in post mitotic non-dividing state and survive by chronological ageing with a fraction of the cells entering quiescence. Second, meiosis is a key mechanism for the species evolution. Diploid *S. cerevisiae* that undergo meiosis can exchange genetic material with other individuals (outbreeding) or simply reshuffling their own genomic information (inbreeding) and purging deleterious mutations. The inability to purge mutations is consistent with a more severe accumulation of deleterious mutations. Part of this process can be rescued by mitotic recombination and indeed we observed that loss-of-heterozygosity from mitotic recombination is a hallmark of domesticated strains (Peter & De Chiara et al., 2018). However,

inactivation of the sexual cycle result in relax selection for mating with other individual in the population and this will evolve in the accumulation of major genomic changes such as SVs, aneuploidies and polyploids.

The phenotypes investigate are complex traits and GWAS provided some insights in their genetic determinants. However, several striking examples that were identified by gene candidate approach were undetectable using the GWAS. These large-effect inactivating variants are typically restricted to specific clades and likely driven by genetic drift. Different quantitative genetics approaches such QTL mapping in haploid segregants (Bloom et al., 2015; She & Jarosz, 2018) and diploid phased outbred lines (Hallin & Märtens et al., 2016) enable complete variance decomposition of a genetic trait. However, many of these variants are likely functional and relevant for a specific cross and will provide limited information on the phenotypic variability of the population as a whole.

The quantitative phenotypic variation appears to be higher in domesticated strains, despite their higher relatedness compared to the wild strains. This is consistent with their evolution in much diversified niches and likely driven by pangenome variation (Peter & De Chiara et al., 2018). In contrast, wild strains appear more phenotypically more homogenous, despite their genetic distance is paradoxically 3-fold higher. This can be reconciled by evidences that SNP variation within wild strains is more neutral, consistent with the variant prediction effect presented here. The more neutral evolution is also consistent with evolution in more homogenous natural niches of the wild

strains, while domesticated strains have fast diverged between each other. Surprisingly, we detected phenotypic relatedness between wild *S. cerevisiae* and the wild closed relative *S. paradoxus* despite 10-fold higher genetic distance compared to wild-domesticated *S. cerevisiae* strains. Indeed, at the genomic level we observed the *S. paradoxus* evolving by neutral sequence divergence and not by pangenome (Bergstrom et al., 2014) and being much more homogenous compared to *S. cerevisiae* despite the higher intra species genetic distance (Liti & Carter et al., 2009, Warringer et al., 2011). We suggested that the wild *S. cerevisiae* represent the ancestral pre-domesticated state of the species and future analysis will further test this scenario.

Material and methods

Datasets of yeast strains

In addition to the assignment of each strain to a different ecological class on the bases of the substrate of isolation (domesticated, wild, human or unknown), each clade has also been assigned to domesticated and wild as previously reported (Peter & De Chiara et al., 2018). In brief, clades were classified as ‘domesticated’, if over two third of the isolates within the clade were of anthropic origin, or ‘wild’, when more than two third of the isolates were isolated from pristine wild ecosystems. Clades not reaching this threshold were marked as ‘unclassified’.

We used a total of previously sequenced 987 isolates (Peter & De Chiara et al., 2018) and restricted some analysis to specific subsets. Sporulation efficiency was measured in 850 isolates. We removed haploid strains, which were mostly previously engineered for HO

deletion and 2 isolates with growth defects in pre-sporulation media. Chronological lifespan in water was tested on the whole strain collection of 987 isolates while data for chronological lifespan in SDC and associate conditions (caloric restriction growth or rapamycin presence) were collected only for euploid diploid isolates. Isolates with sporulation in water phenotype and isolates having insufficient number of cells or high level of cell aggregation were excluded for FACS analyses. Overall, we collected data for 735 isolates in water and 572 for the SDC, SDC+CR, and SDC+RM.

Induction and quantification of sporulation

All strains were grown in YPD (2% dextrose, 1% yeast extract, 2% peptone, 2% agar) before being 1:50 diluted into 10mL of the pre-sporulation media YPA (2% KAc, 1% yeast extract, 2% peptone) and grown 48 hours at 30°C (250rpm). Next, strains were transferred to sporulation media (2% KAc) into 250 ml flasks at a 5:1 volume/medium ratio and a final optical density (OD₆₀₀) of 0.6. Flasks were kept at 23°C and shaken at 250rpm.

To estimate sporulation efficiency, at least 100 cells per sample were counted at 24 and 72 hours post KAc incubation using an optical microscope (Zeiss Axio Lab.A1). Sporulation efficiency was defined as the number of asci divided by the total number of cells counted.

Spontaneous sporulation in water

Sporulation in water was determined concomitantly with the chronological lifespan experiment (see below). The number of asci was quantified based on cellular morphology (granulosity and size)

specific gating using SSC and FSC parameters of a FACS Calibur flow cytometer (Becton Dickinson). The morphology of sporulating cells looked quite peculiar and was confirmed using a pSPS2-GFP reporter strain, a late sporulation protein secreted into the spore wall (Gerke et al., 2006). The content and proportion of asci produced in this condition were visually confirmed by microscopy for a subset of samples.

Chronological lifespan in water assay

Cells grown overnight in liquid YPD were 1:100 diluted in 200 µL of fresh YPD in a 96 well plate and incubated 24 hours at 30°C. 20 µL were then transferred into a new plate containing 180 µL of phosphate saline buffer (PBS). Cells were washed twice with PBS, and finally resuspended in 200 µL of PBS (day 0).

Chronological lifespan in SDC, CR and RM assay

Cells grown overnight in liquid synthetic complete medium (SDC) (2% dextrose, 0.675% yeast nitrogen base (Formedium), 0.088% complete amino acid supplement (Formedium), pH buffered to 6.0 with 2.5M NaOH) were 1:100 diluted in 200 µL of either fresh SDC, or calorie restricted SDC (CR) (0.5% dextrose instead of 2%), or SDC supplemented with rapamycin (RM) (0,025 µg/mL rapamycin) in a 96 well plate and incubated at 30°C for the whole duration of the experiment. Ageing time points started to be counted 72 hours post inoculation at saturation of the culture.

Cell viability

Cell viability was determined by flow cytometry based on the uptake of the fluorescent molecules propidium iodide (PI) and YO-PRO-1 iodide (YP). At each time point, 5 μ L of cells were transferred in 100 μ L of staining solution (PBS + 3 μ M propidium iodide (Sigma) + 200 nM YP (Invitrogen)) in a 96 well plate and incubated 5min in the dark at 30°C. The samples were then analysed on a FACS-Calibur flow cytometer (Becton Dickinson) using a High Throughput Sampler (Becton Dickinson) device to process 96 well plates and detecting fluorescence with FL-1 (YP) and FL-3 (PI) channels.

Propidium iodide and YO-PRO-1 are membrane-impermeable nucleic acid binding molecules that can only enter into dead cells whose cellular membrane is impaired. Therefore, non-fluorescent cells were counted as viable, whereas the bright ones were considered dead. Propidium iodide was previously shown to be a valuable choice for quick and reliable CLS assay ([Ocampo & Barrientos, 2011](#)). YO-PRO-1, unlike PI, is able to penetrate apoptotic cells which are encountered, along with necrotic cells, during the course of chronological aging ([Herker et al., 2004](#); [Wlodkowic et al., 2009](#)).

Spore chronological lifespan

Sporulation was induced similarly to the protocol described above. Cells were kept 3 days in KAc 2% before being washed twice with water and resuspended in the same final volume of water. Flasks were incubated at 23°C and shaken at 220 rpm for the whole experiment. At each time point 300 μ L

of cells were collected from flasks, centrifuged, and resuspended in 100 μ L of dissection buffer (sorbitol 2 M, EDTA 10 mM, sodium phosphate 100 mM, zymolase 1mG/mL) for 1 hour at 37°C. Each time 32 tetrads were dissected on YPD plates with a micro-dissecting microscope SporePlay+ (Singer). Plates were incubated 3 days at 30°C before the number of growing colonies was counted to determine spore viability.

Loss of function predictions

Losses of function prediction was based on SIFT software and presence of indels causing frameshifts. Homozygous presence of non-sense mutations or mutation causing start or stop loss were considered as highly damaging the gene function. Presence of loss of function has been registered also for genes in which homozygous frameshift were found by mapping (frameshifts were predicted as a net value of insertion and deletion not divisible by 3).

Statistical tools

All statistical test was performed in R.3.1.1. Function `wilcox.test` (performing a two-sided Mann-Whitney test) was used to calculate p-values unless explicitly stated.

Genome wide association

The VCF file available for the sequenced strains ([Peter & De Chiara et al., 2018](#)) was converted and filtered using `plink` (v1.9) with $MAF > 0.05$ (5%). Also, non-biallelic SNPs were retained and, in this case, only data concerning the major of the minors alleles have been used. Same MAF filter has been applied for presence/absence and copy number variation (coded as single copy versus multiple copies) matrices reported in

(Peter2018) and for presence of LOF. These ORF features were manually added to the plink SNPs files, coded as alleles with simulated genomic positions.

GWAS, heritability and genome inflation factor were all run and calculated with fast-lmm. Correction for population structure while running the GWAS was performed using the SNPs with $MAF > 0.005$ (0.5%). To calculate heritability and genome inflation factor, the actual genetic variants used as markers were also retained to calculate the population structure.

To reduce confounding factors, only data from diploid euploid isolates have been used, resulting in 618 isolates for sporulation, 572 for growth in SDC/RM/CR condition (excepted for RM at 35 days, for which 571

isolates are available) while, for CLS in water, 494 and 504 were respectively used for 8 and 20 days.

Supplemental tables

(Not provided)

Table S1. Differences in resilience in stress conditions.

Table S2. Contingency matrices.

Table S3. Candidate gene list.

Table S4. All GWAS hits.

Supplemental figures

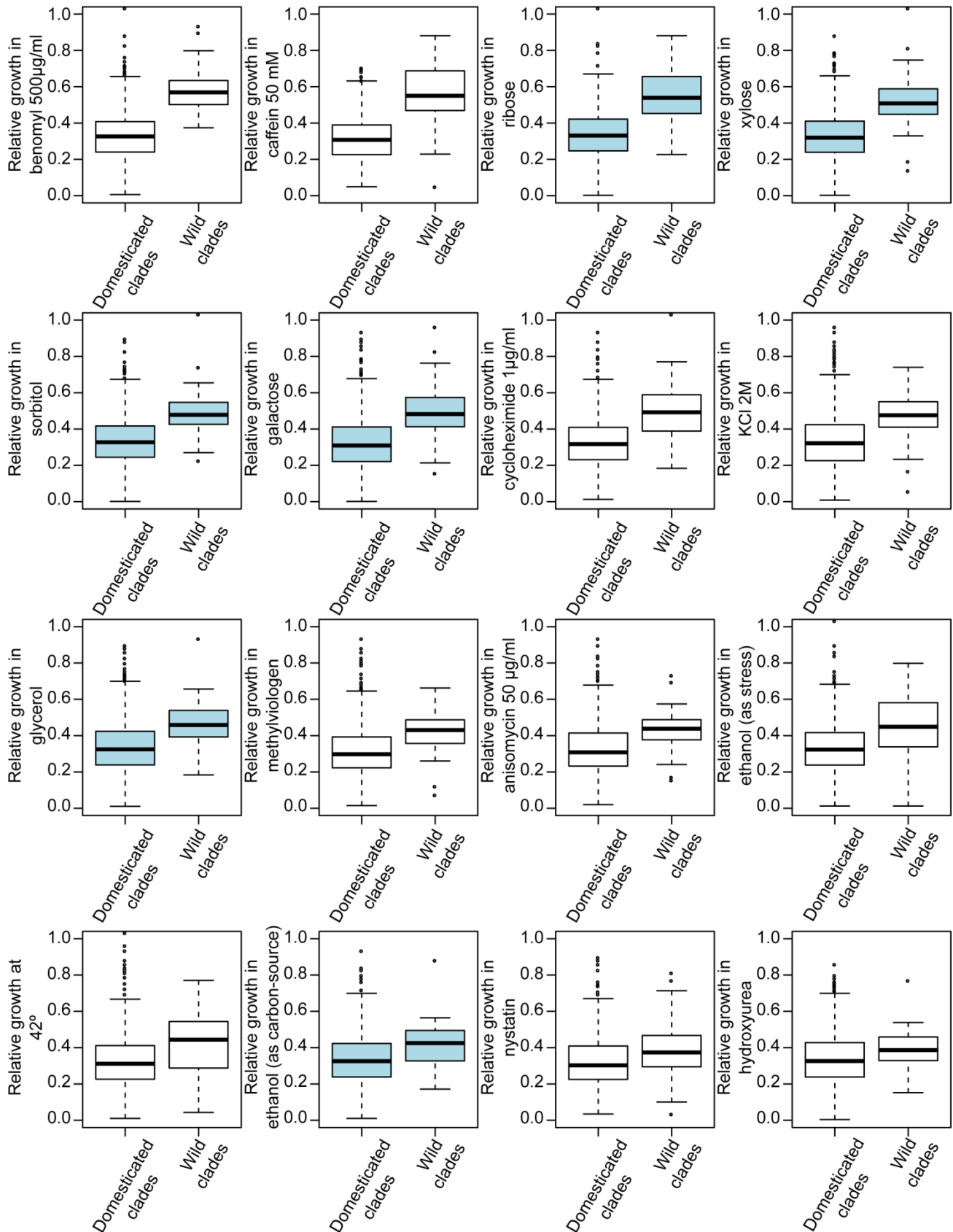


Figure S1. Growth superiority of wild strains.

Boxplot distribution of 16 phenotypes in which wild strains have better resilience than domesticated strains. Usage of alternative carbon sources, which represents a large fraction of these phenotypes, is highlighted in blue.

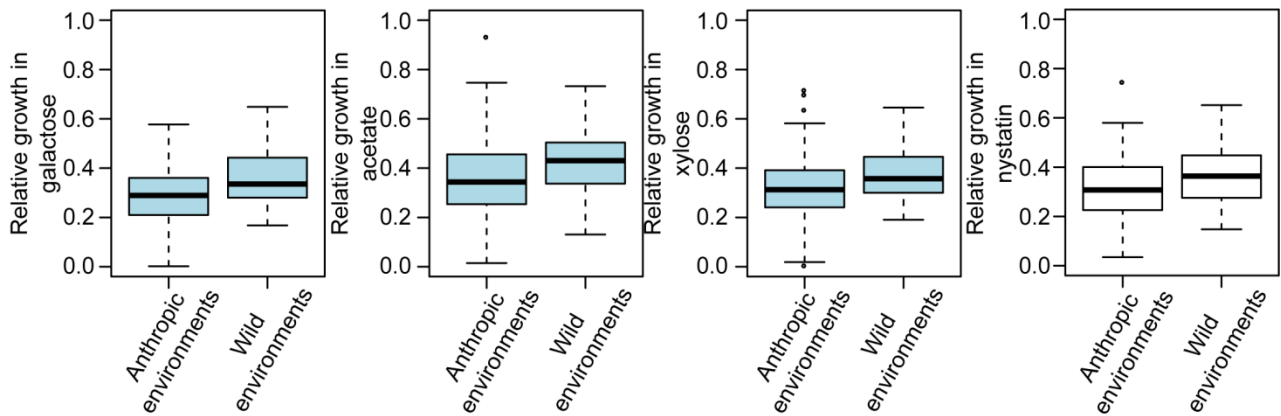


Figure S2. Growth superiority of feral strains.

Comparison of Wine/European isolates isolated from anthropic (domesticated) or natural (feral) environments. Only conditions with significant differences are shown. Feral isolates have a better resilience for growth using alternative carbon sources (highlighted in blue).

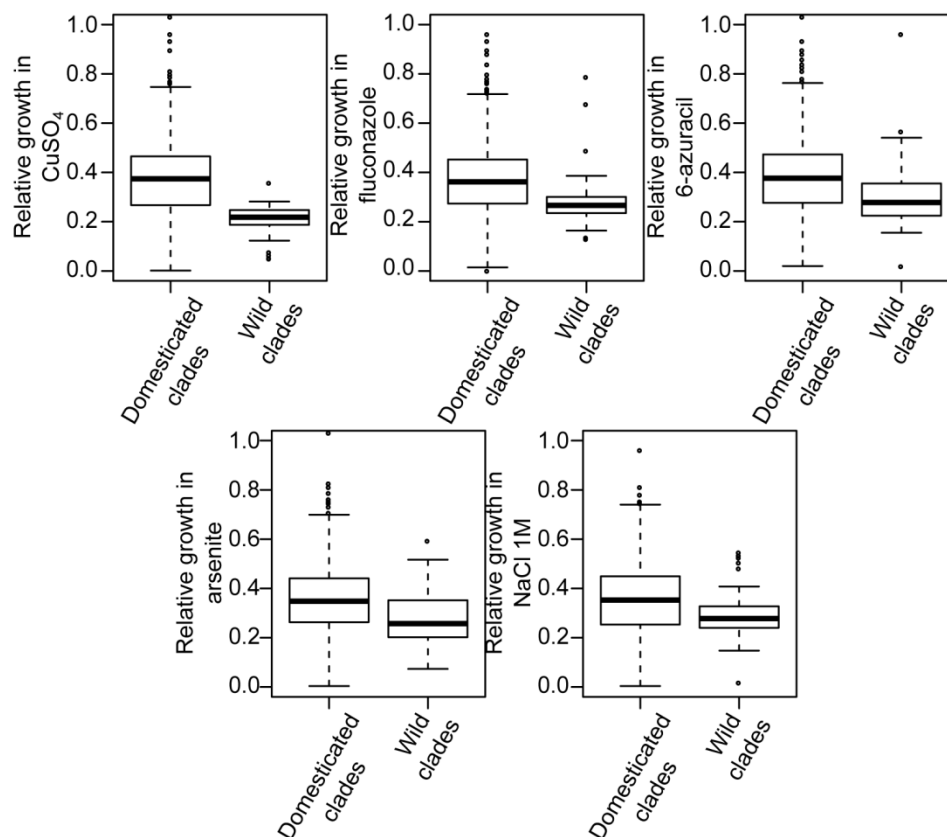


Figure S3. Growth superiority of domesticated strains.

Boxplot distribution of five phenotypes in which domesticated strains have better resilience than wild strains.

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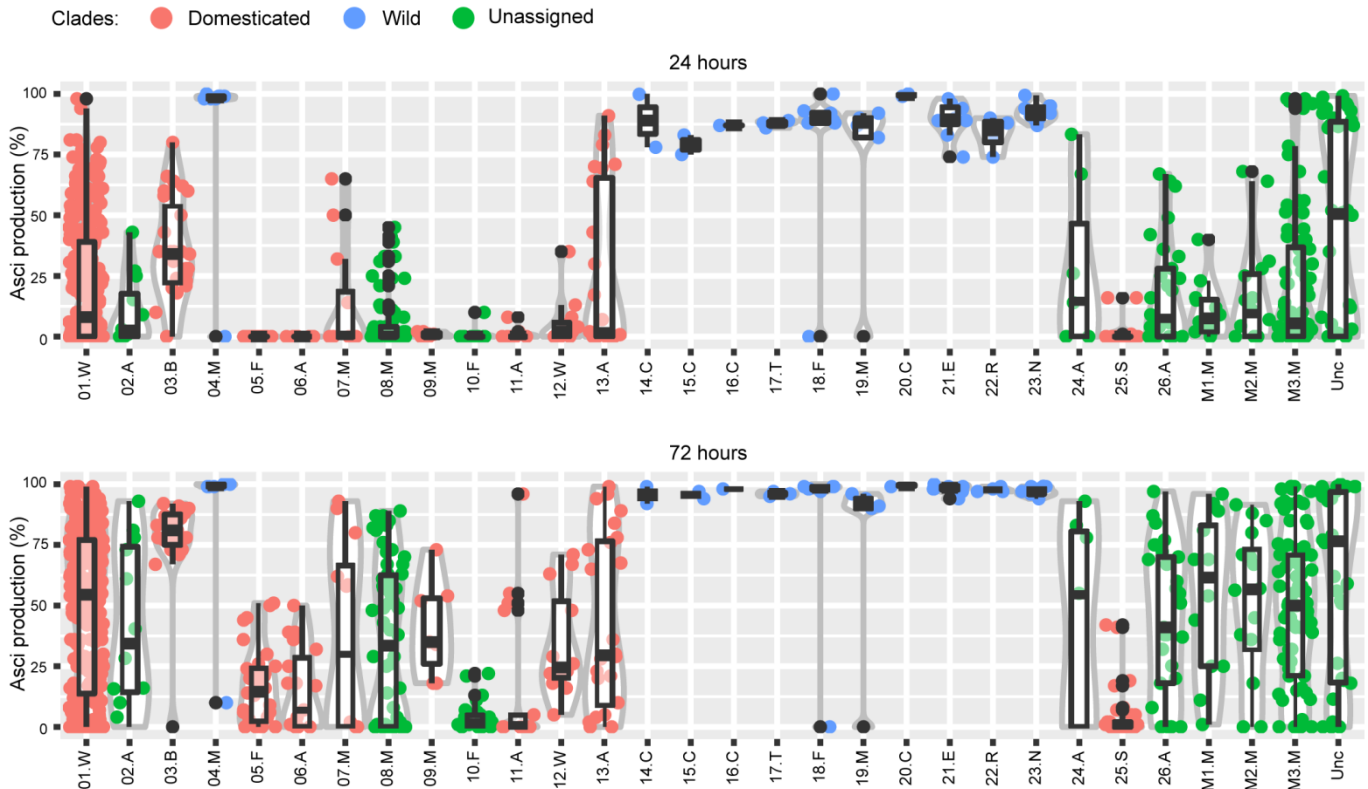


Figure S4. Sporulation efficiency across subpopulations.

Distribution of the Asci production at 24 hours and 72 hours for each phylogenetic clade. Dot colors indicate domesticated clades (red), wild clades (blue) and unassigned clades (green).

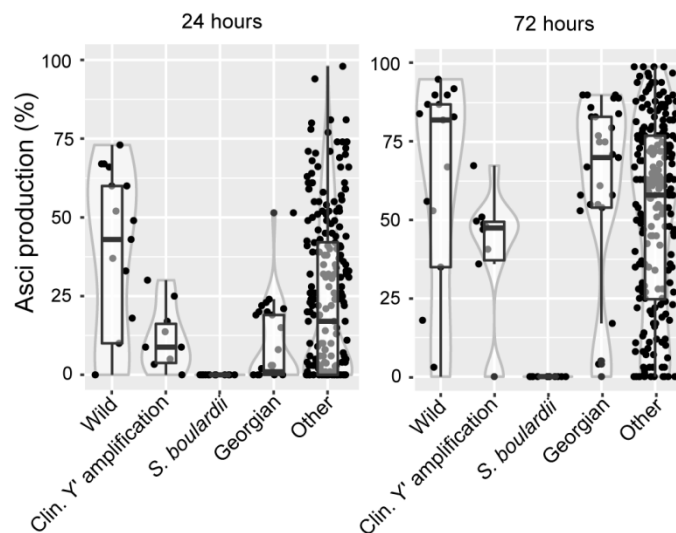


Figure S5. Wine/European strains sporulation efficiency.

Distribution of the asci production at 24 hours and 72 hours for the four subclades within the Wine/European clade. The *S. boulardii* subgroup has completely lost the ability to sporulate.

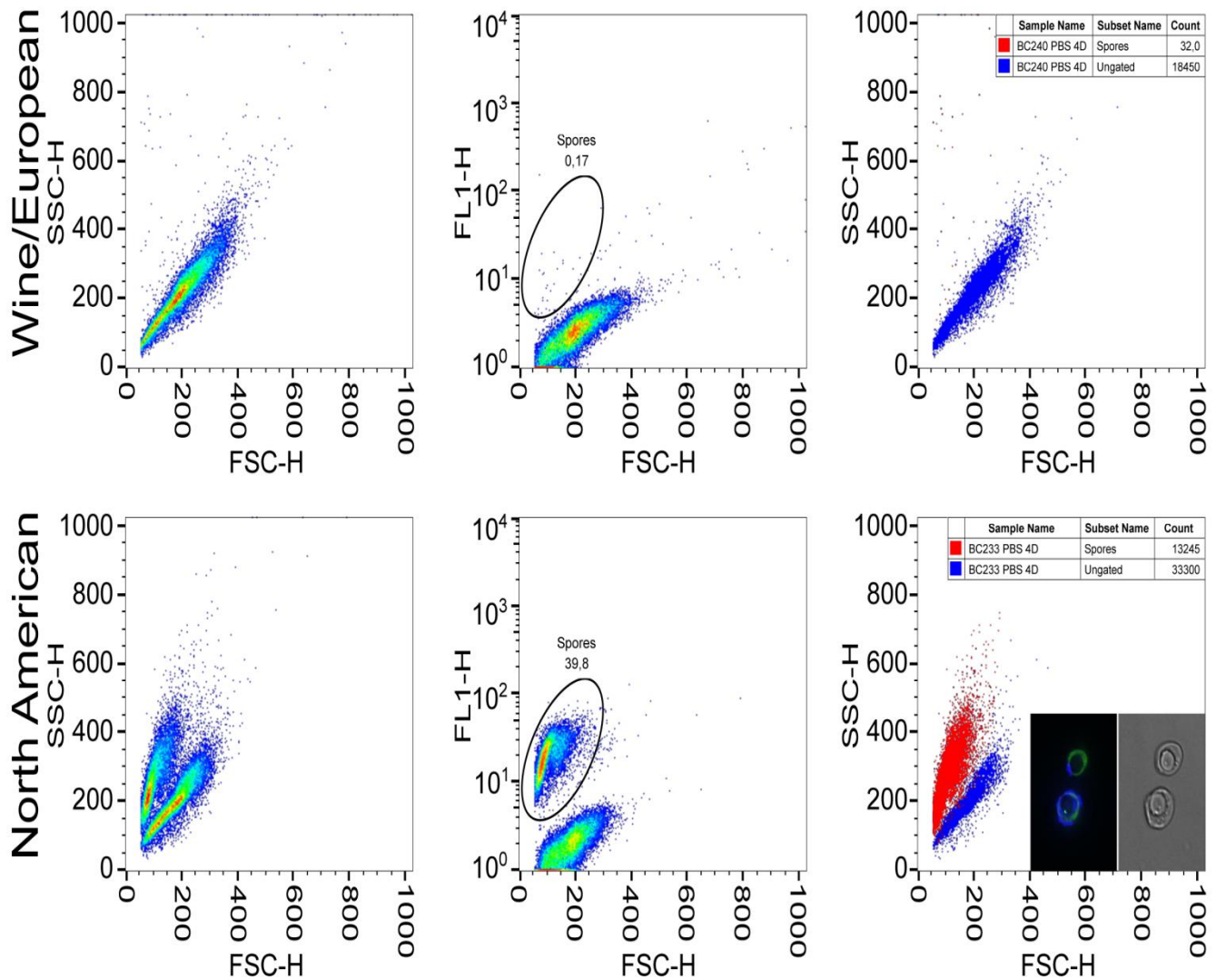


Figure S6. Sporulation in water.

FACS analyses of a wild North American and a domesticated Wine/European strains engineered with a GFP-*SPS2* fusion protein. Cells were pre-grown in YPD and incubated in water for 4 days. North American isolate shows the presence of two distinct populations, one composed by monads as confirmed by microscopy inspection (inset panels). The dots on the rightmost panels are colored accordingly to the GFP signal: fluorescent cells (red) and non-fluorescent cells (blue).

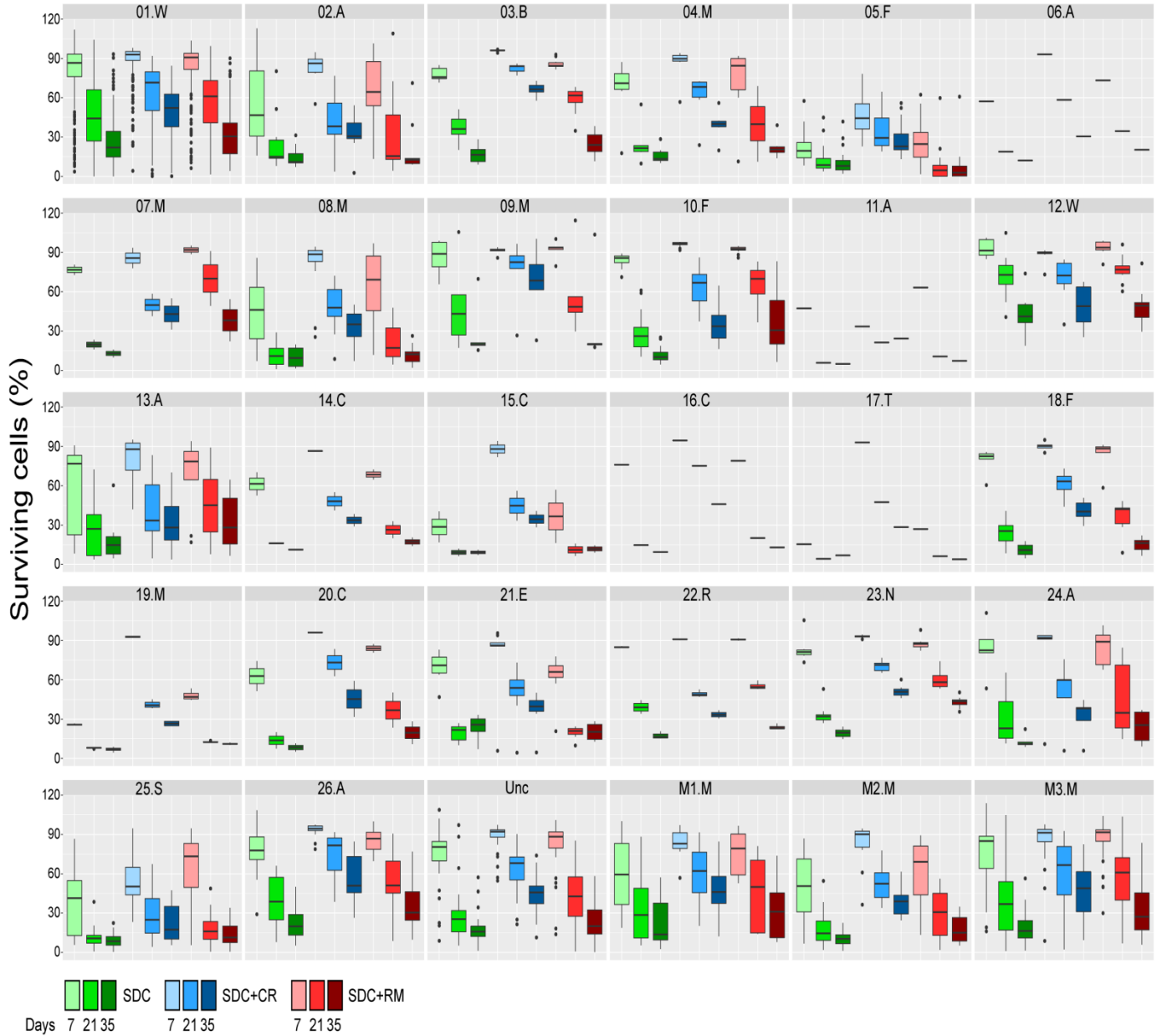


Figure S7. Chronological aging across lineages.

Boxplots show the percentage of surviving cells for each clade at each condition and time points. Shortest lifespan is found in French Dairy (5.F) while French Guiana (10.F), Mexican agave (09.M) and West African Cocoa (12.W) have the longest lifespan.

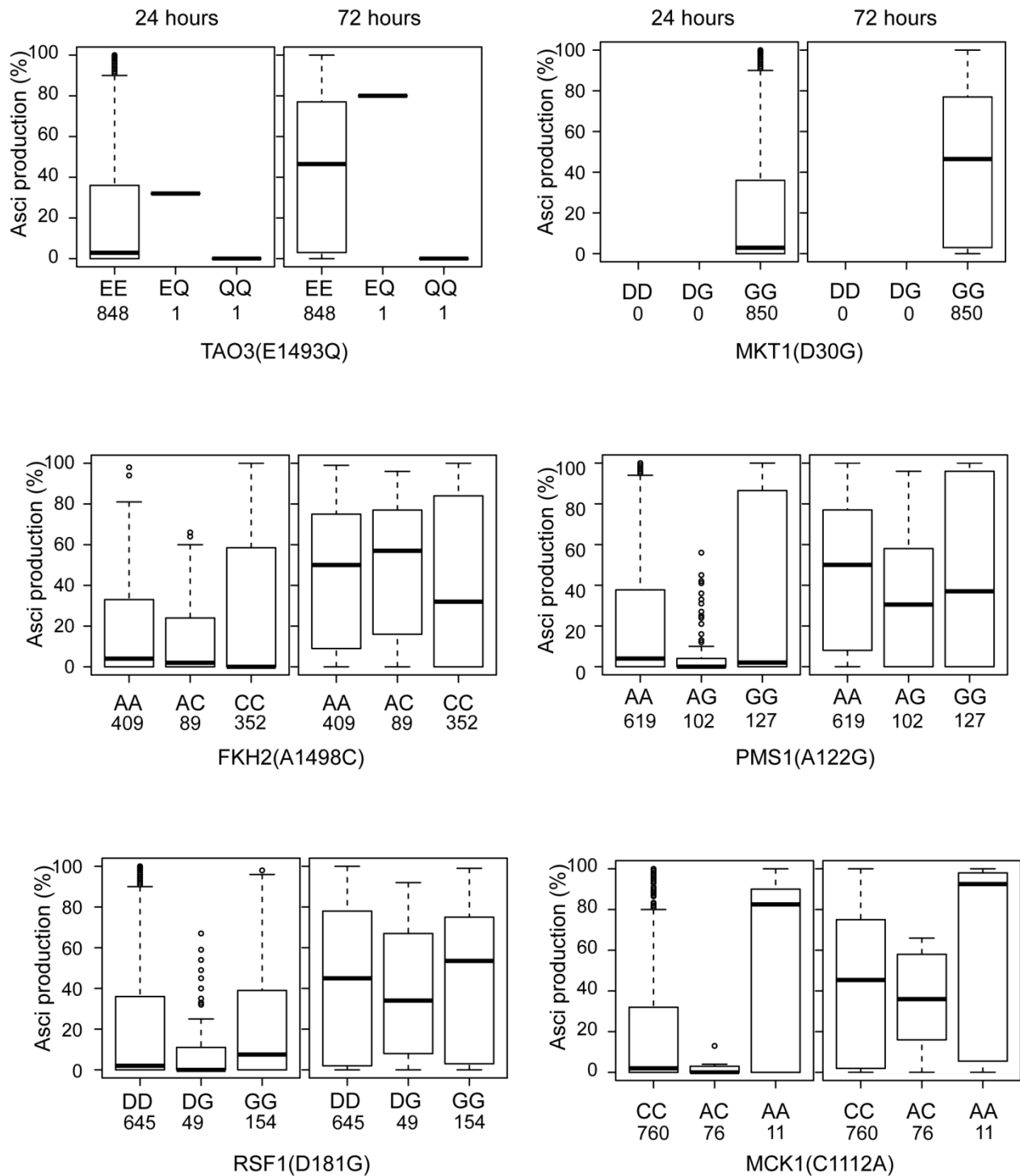


Figure S8. QTNs have limited effect on global population variation.

The boxplots report the distribution of sporulation at 24 and 72 hours of isolates harbouring different QTNs referenced in the literature.

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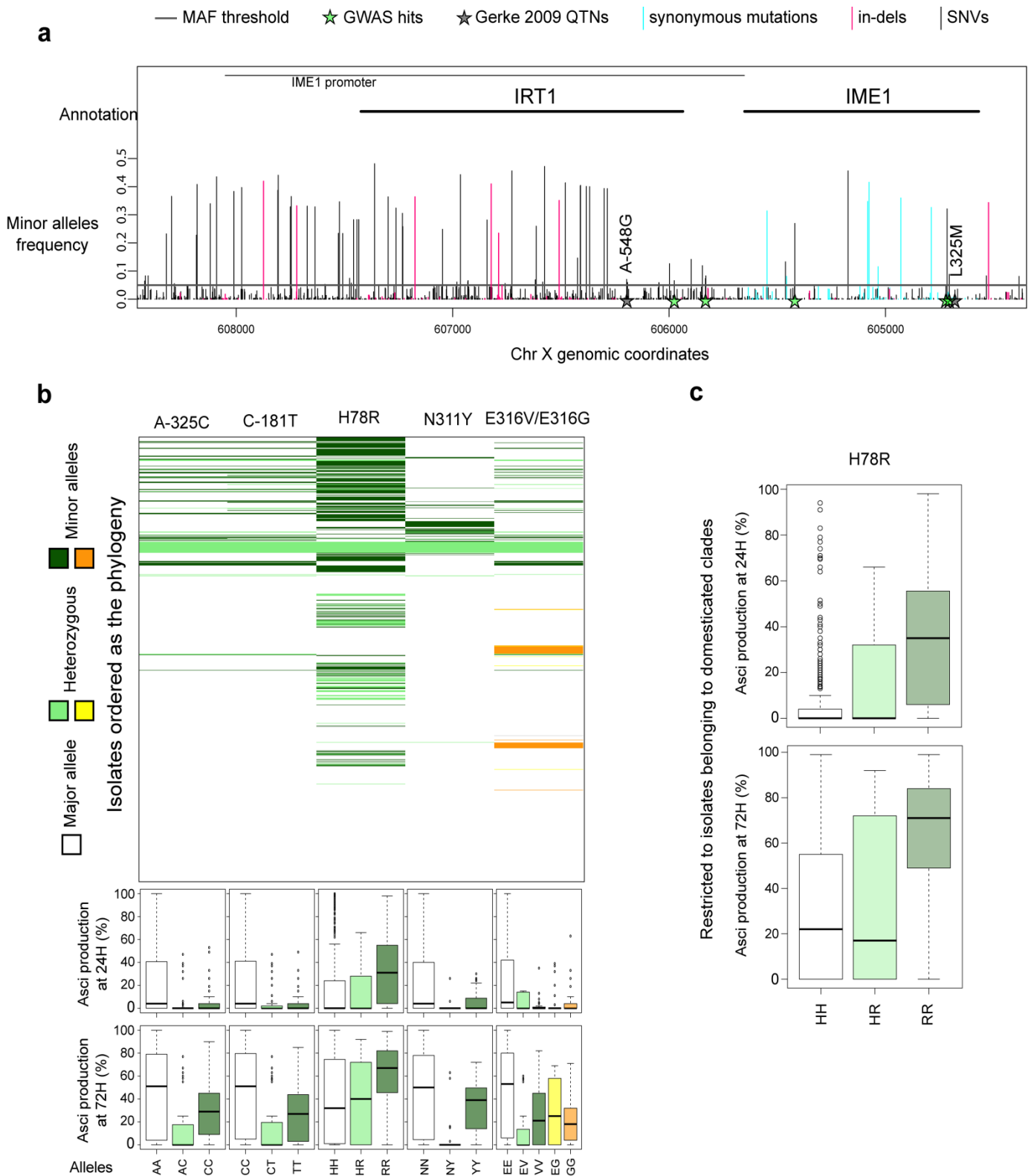


Figure S9. *IME1* variants detected by GWAS. (A) Five sporulation GWAS hits impact the *IME1* gene and its promoter. None of these correspond to the SNPs identified previously (Gerke et al., 2009), which do not pass the MAF threshold of 5%. (B) Several hits possibly in linkage and all of them associated to a detrimental effect on spore production, excepted for H78R, which is also the most abundant in the population (~27%). (C) H78R is only found in domesticated clades and when the dataset is restricted only to these clades, the positive effect of such allele is strikingly apparent.

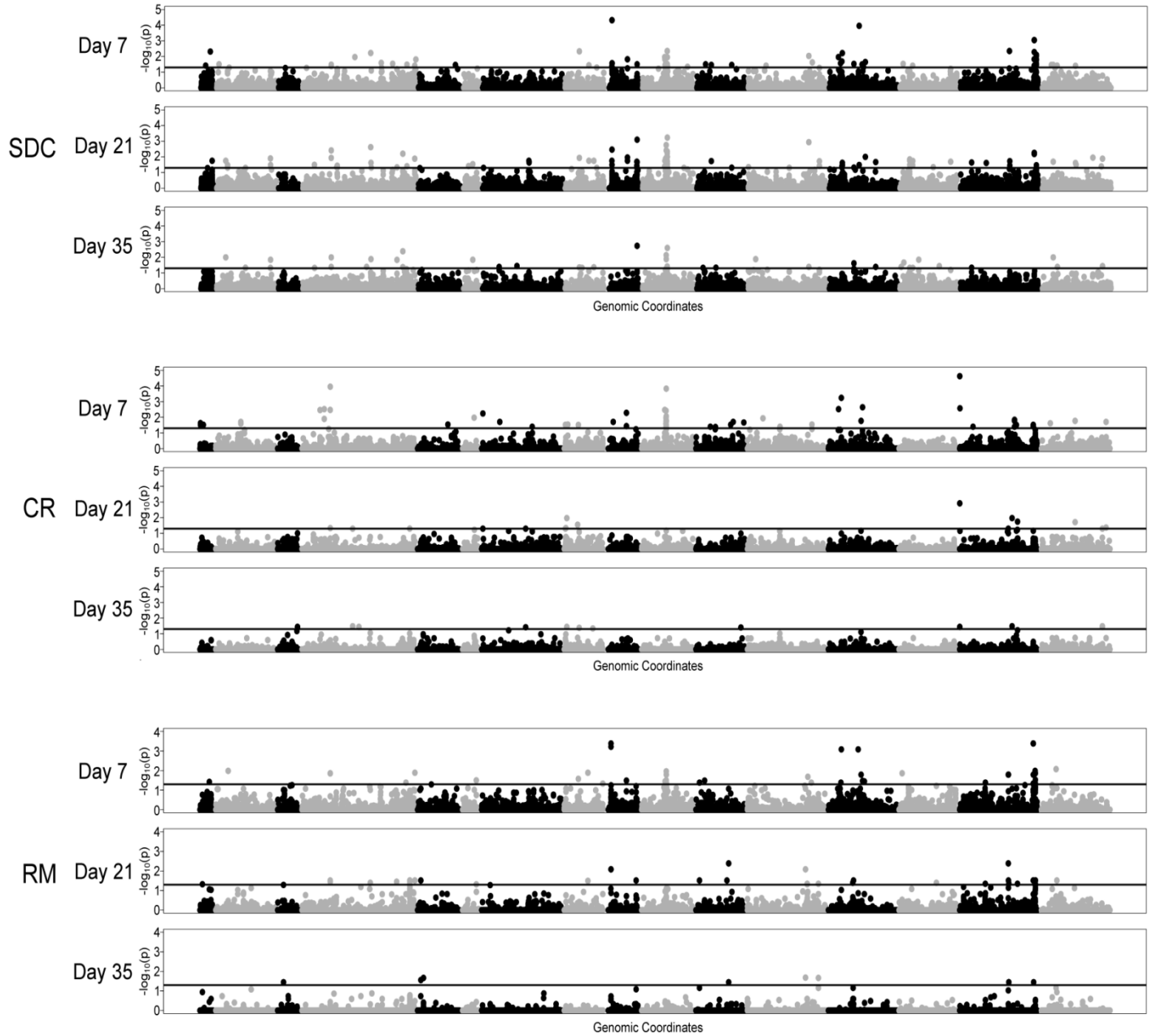


Figure S10A. CLS SNPs hits detected by GWAS.

Association scores of the SNPs with MAF >5% across the 16 chromosomes for CLS. The switch between gray and black dots delimits the 16 chromosomes.

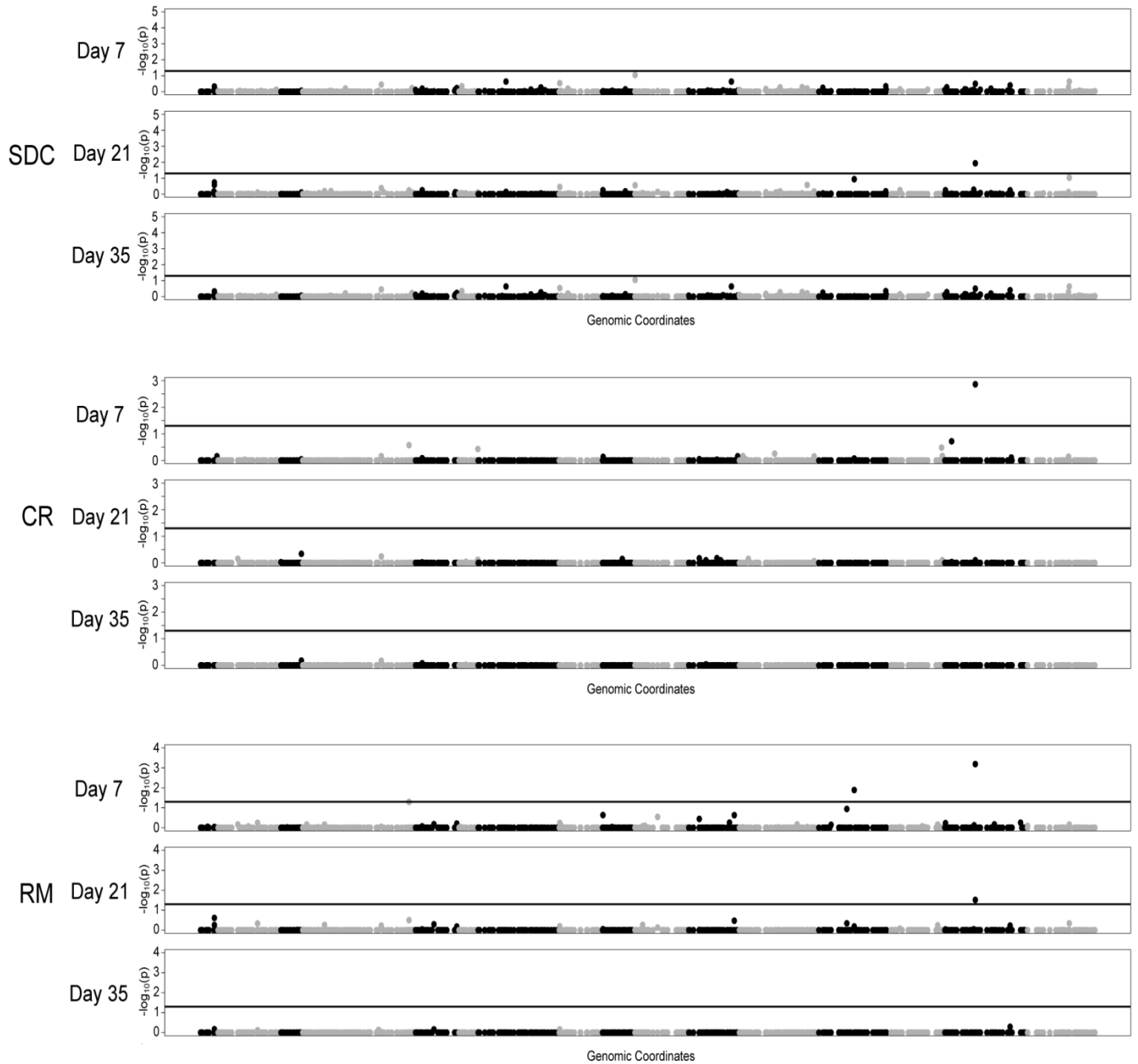


Figure S10B. CLS loss-of-function hits detected by GWAS for CLS.

Association scores of the predicted loss of functions with MAF > 5% across the 16 chromosomes for CLS. The switch between gray and black dots delimits the 16 chromosomes.

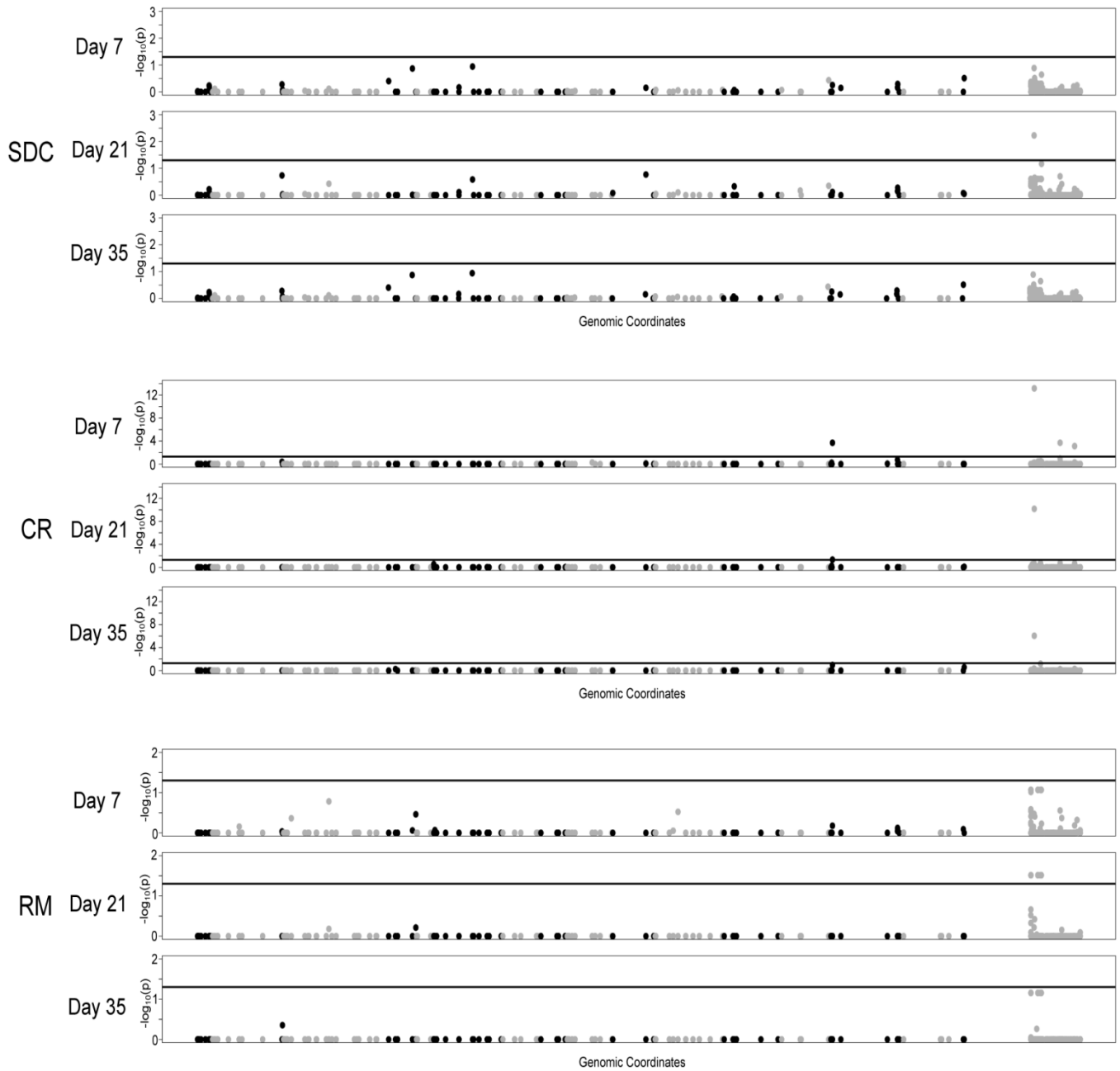


Figure S10C. CLS gene presence/absence hits detected by GWAS.

Association scores of the presence/absence markers with MAF >5% across the 16 chromosomes and the non-referenced material for CLS. The switch between gray and black dots delimits the 16 chromosomes, the mitochondria and the non-referenced material.

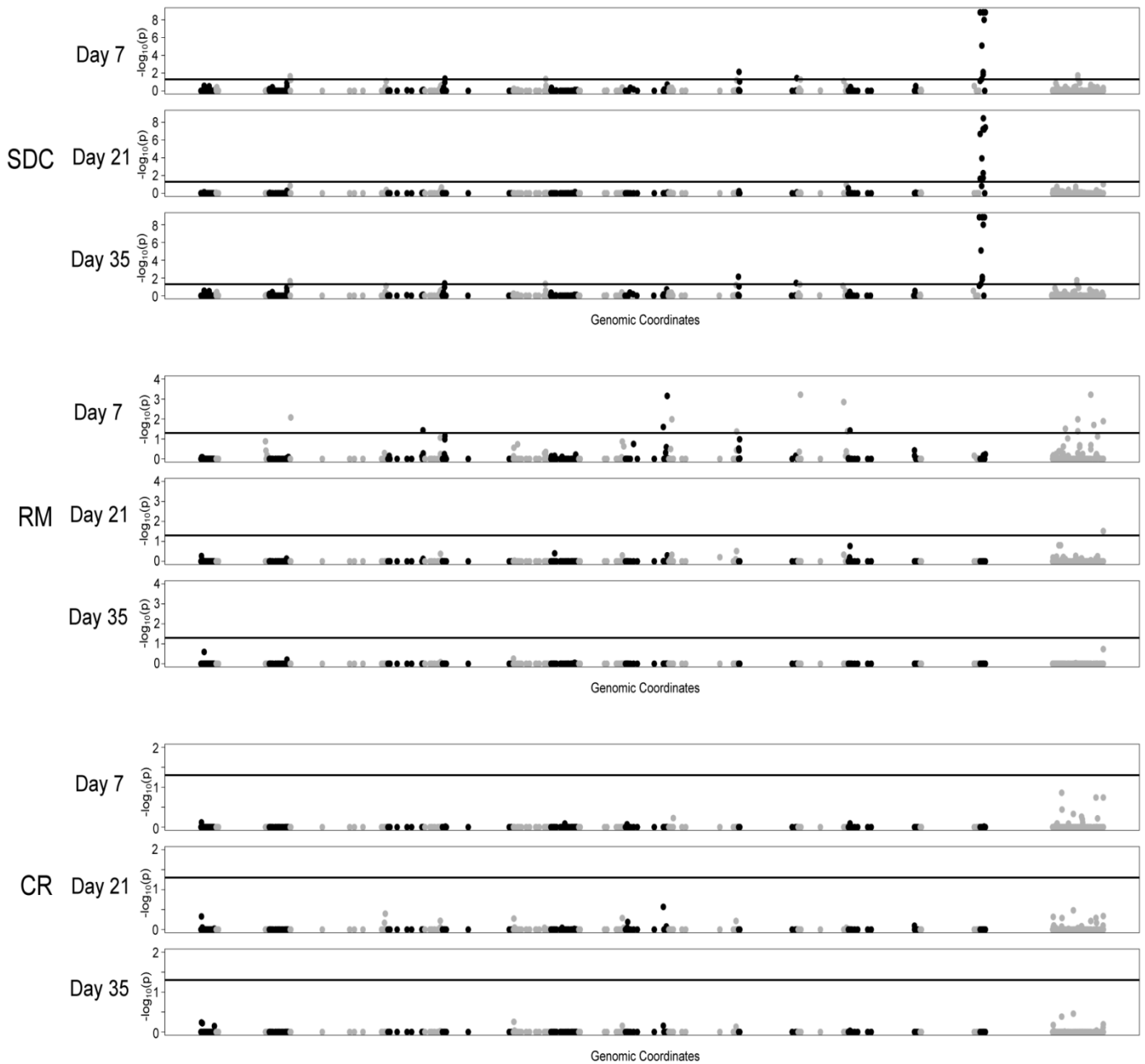


Figure S10D. CLS CNVs hits detected by GWAS.

Association scores of the CNVs with MAF >5% across the 16 chromosomes and the non-referenced material for CLS. The switch between gray and black dots delimits the 16 chromosomes, the mitochondria and the non-referenced material.



*“Life is really simple, but we insist
on making it complicated”*

- Confucius

Natural intragenic tandem repeat expansions shorten yeast chronological lifespan by altering the redox homeostasis

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Abstract

Aging is a classical complex trait varying quantitatively among individuals and is affected by both the genetic background and the environment. Identifying the causal genetic variants underlying variation in longevity and understanding their interaction with the genetic background and the environment remains a major challenge. In this work, we use the budding yeast, *Saccharomyces cerevisiae*, to identify environmental and genetic factors contributing to aging. While extensive classical genetic studies have helped to discover several genes involved in the regulation of both Replicative (RLS) and Chronological (CLS) lifespans using laboratory strains in standard conditions, there are only few studies exploiting natural variations in natural populations. Here we performed linkage analysis using 1056 diploid segregants, derived from a two diverged parents, and phenotyped CLS to map quantitative trait loci (QTLs). The CLS were determined across multiple time points in three distinct environmental conditions: complete media, calorie restriction (CR) and in the presence of rapamycin (RM). We mapped a total of 30 unique QTLs including 6 shared across different environments and 24 specific to a condition. The two strongest detected QTLs were linked with natural polymorphisms in the cell wall glycoproteins *FLO11* and *HPF1*. Interestingly, both genes had massive intragenic tandem repeat expansions in one of our founder parent. While the short allele versions of *FLO11* and *HPF1* did not impact CLS, tandem repeat expansions within those genes were sufficient to confer a dominant detrimental effect that was partially buffered by rapamycin treatment. Further investigation revealed that the extended form of *HPF1* induces flotation and higher oxygenation of the culture during exponential phase, leading to the perturbation of redox homeostasis, remodelling of methionine and lipid metabolism, alongside with activation of misfolded protein response and ribosome biosynthesis, altogether contributing to CLS shortening. Taken together our results reveal complex trait architecture of CLS in natural population and suggest that intragenic repeats variability can transform the overall organismal fitness.

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Introduction

Aging is a biological process impacting almost all living organisms (Jones et al., 2014; Finch, 2009). It consists of a progressive decline in biological functions eventually leading to death of the organism. The overall process is thought to be resulting from the accumulation of various types of damaged biomolecules contributing altogether to the loss of cellular homeostasis (Gladyshev, 2016). The pace at which these events are happening is further regulated by genetics, environment, and stochasticity, making ageing a remarkably complex and highly diverse trait within the tree of life. While aging is the highest risk factor for a large number of diseases, little is known about the underlying molecular mechanisms. It has long been considered that aging rate could not be modulated until the first genes regulating lifespan were identified in the beginning of the 90s with the onset of classical genetics (Kenyon et al., 1993; Sun et al., 1994). As of today, hundreds of genes have been annotated to impact lifespan and associated signalling pathways have been laid down (Kenyon, 2010). In addition of genetic components multiple environmental conditions such as calorie restriction (CR) (Pletcher et al., 2002; Jiang et al., 2000; Weindruch et al., 1986; Klass, 1977; Colman et al., 2009), oxygen reduction (Rascon & Harrison, 2010; Leiser et al., 2013) or lower temperature (Sestini et al., 1991; Conti et al., 2006; Leiser et al., 2011) were found to extend lifespan. The most studied one is undoubtedly CR, which is leading to healthspan and lifespan extension promoted by the reduction of nutrient availability without malnutrition. Since its discovery several decades ago (McCay, 1935), many

effectors of CR have been isolated and the associated gene-environment interactions were established, unravelling evolutionary conserved mechanisms of aging (Bishop & Guarente, 2007). Perception and integration of CR is mainly mediated through nutrient sensitive pathways such as insulin/IGF-1, target of rapamycin (mTOR), AMPK, sirtuins, which regulate themselves numerous lifespan determining cellular processes including stress response, mitochondrial respiration, oxidative stress, genome stability, autophagy, energy and fat metabolism (Alvers et al., 2009; Schulz et al., 2007; Madia et al., 2008; Hansen et al., 2008; Yuan et al., 2012; Weinberger et al., 2007; Wei et al., 2008). These findings have opened the way to the first pharmaceutical interventions extending lifespan in model organisms (Martin-Montalvo et al., 2013; Harrison et al., 2009; Eisenberg et al., 2009). One of the most promising drugs, the TOR inhibitor rapamycin, is already clinically approved and used in several applications as an antifungal agent or in chemotherapy (Loewith, 2011). Rapamycin was found to extend lifespan in yeasts, flies, worms, and even in mice, and is thought to do so by, at least partly, mimicking CR (Blagosklonny, 2010). However associated undesirable side effects and lack of treatment optimisation currently prevents its usage for humans (Kaeberlein, 2014). Similarly, human anti-aging therapies based on CR are still restricted by our limited knowledge and understanding of related downstream mechanisms and data interpretation are made even more complicated by several studies pointing to inefficient, or even life shortening, CR outcome (Mattison et al., 2012; Liao et al., 2010). Thus, a significant amount of work

remains to be done in unravelling the genetics of aging and environmental interactions before interventions improving healthspan and delaying age-related diseases are made available on the market.

The budding yeast *S. cerevisiae* has been pivotal in elucidating genes and mechanisms regulating aging. Yeast aging can be studied through two distinct approaches: the replicative lifespan (RLS) and the chronological lifespan (CLS). Replicative lifespan is based on the number of mitotic divisions a single yeast cell can perform in its life before undergoing senescence and is used as a paradigm to study aging of proliferative tissues, such as stem cells (Mortimer & Johnston, 1959; Steinkraus et al., 2008), while chronological lifespan is based on the time duration yeasts can survive in non-dividing conditions and is a paradigm to study aging of post-mitotic cells, such as neurons (Longo et al., 1996; Longo & 2012). Taking the advantage of the yeast deletion collection (Giaever et al., 2002), multiple genome-wide studies have contributed to identify hundreds of genes regulating CLS (Powers et al., 2006; Fabrizio et al., 2010; Garay et al., 2014) and replicative lifespan (Kaeberlein et al., 2005; McCormick et al., 2015). Some similar studies were also performed under CR to identify genes involved in its modulation (Matecic et al., 2010; Gresham et al., 2011; Campos et al., 2018). However, a certain lack of consistence remains between all these studies and our understanding of molecular processes associated with aging and their subsequent modulation by CR remains uncomplete and elusive (Smith et al., 2016). An approach that remains largely unexplored in the field is the study of

natural variations. The classical laboratory strain S288C, and its derivatives, have been the work horse of pretty much all published work on yeast aging the past two decades. However, laboratory strains have long ago been isolated from their natural environment and extensively genetically engineered. They can be maintained as stable haploids while natural yeasts are usually diploids and have been made auxotrophic which is known to alter lifespan (Gomes et al., 2007; Boer et al., 2008). Moreover S288C was found to be a phenotypic outlier compared to the rest of the species (Warringer et al., 2011) and its laboratory niche cannot encompass the whole diversity of life history strategies established in natural habitats, ranging from wine fermentation vats to tree barks (Peter & De Chiara et al., 2018). Another limitation that comes along with classical genetics is the systematic use of gene deletions. Although it has effectively contributed to settle down the foundations of aging genetics and to identify conserved regulatory networks from yeasts to mammals, it might fail to recapitulate more subtle mechanisms, including protein gain of function or expression regulation. It also makes difficult the study of essential genes, which account for approximately 20% of the yeast ORFeome. Genome-wide linkage analysis represents a valuable alternative to classic genetics, with the advantage to tackle the points raised above (Brem et al., 2002; Steinmetz et al., 2002). While it has successfully been used to map quantitative trait loci (QTLs) associated with various complex traits in yeast, it remains poorly used in the aging field (Kwan et al., 2011; Kwan et al., 2013; Stumpferl et al., 2012; Jung et al., 2018). These previous studies

have narrowed down natural polymorphisms in the rDNA and in SIR2 (Kwan et al., 2013; Stumpferl et al., 2012), established a regulatory link between telomere maintenance and CLS (Kwan et al., 2011), and identified a role of serine biosynthesis in lifespan regulation (Jung2018). However, except Jung et al., all other approaches have used F1 crosses with a laboratory strain, limiting the discovery of new QTLs due to lower genetic diversity and lack of resolution. To overcome these issues, new methodologies were developed using advanced intercrossed lines (AILs) derived from natural crosses, allowing high resolution QTL mapping in unexplored genetic backgrounds (Parts et al., 2011, Cubillos et al., 2013; Liti & Louis, 2012).

In this present work, we performed state-of-the-art genome-wide linkage analysis to map QTLs regulating yeast CLS with an unprecedented resolution. We run our analysis using a F12 outbred AIL derived from a natural domesticated West African palmwine strain (DBVPG6044) and a natural wild North American oak tree strain (YPS128) (Liti & Carter et al., 2009; Parts et al., 2011). A subset of segregants were sequenced and crossed with each other to generate prototrophic diploids with phased genomes (Hallin & Märtens et al., 2016), before being individually phenotyped for their CLS in three different environments (control, calorie restricted, and rapamycin supplemented). We mapped a total of 30 unique QTLs, shared or not within environments. Among these QTLs, the two strongest peaks were pointing to the cell wall glycoproteins *FLO11* and *HPF1*, which were experimentally validated. Both genes contain intragenic tandem repeats, which

got remarkably expanded in the West African lineage. Extension of internal tandem repeats was sufficient to generate a detrimental dominant effect on lifespan, which was buffered by rapamycin only. We observed that this long *HPF1* allele was inducing flotation and adhesion during exponential phase. This characteristic was required to impair CLS and was found to alter several cellular processes including redox homeostasis, proteostasis, hexose transport, along with methionine, ribosome and lipid biosynthesis.

Results

Outbred segregants display high CLS variability and different response to calorie restriction and rapamycin treatment

To identify natural genetic variants regulating yeast CLS, we performed a genome-wide linkage analysis using a highly outbred advanced intercross line (AIL) between two natural strains (Parts et al., 2011). The wild North American (NA) oak tree bark strain (YPS128) was crossed for twelve generations with the domesticated West African (WA) palm wine strain (DBVPG6044) and a subset of F12 haploid segregants was isolated, sequenced, and genotyped (Liti & Carter et al., 2009; Illingworth et al., 2013). Parental strains differ at 0,53% of their nucleotides and a total of 52466 genetic markers were called and used to run the linkage analysis. Segregants of opposite mating types were then randomly crossed with each other to generate a total of 1056 unique diploids with phased genomes, termed Phased Outbred Lines (POLs) (Hallin & Märtens et al.,

2016). Each of the 1056 isolate was subsequently phenotyped for its Chronological Lifespan (CLS) in different environments, using high throughput cytometry coupled with cell viability stainings (Propidium iodide and YO-PRO-1), in 96 well plates layout including the strain BY4743 used as a control for normalisation (see methods). While it is

known that complex traits such as CLS are regulated by complex gene-gene and gene-environment interactions, direct comparison of how environment affects and shapes QTLs was almost never done (Jung et al., 2018). We performed CLS in three related environments that differ by a single variable which has broad implications in cellular aging. We chose to run our experiments in

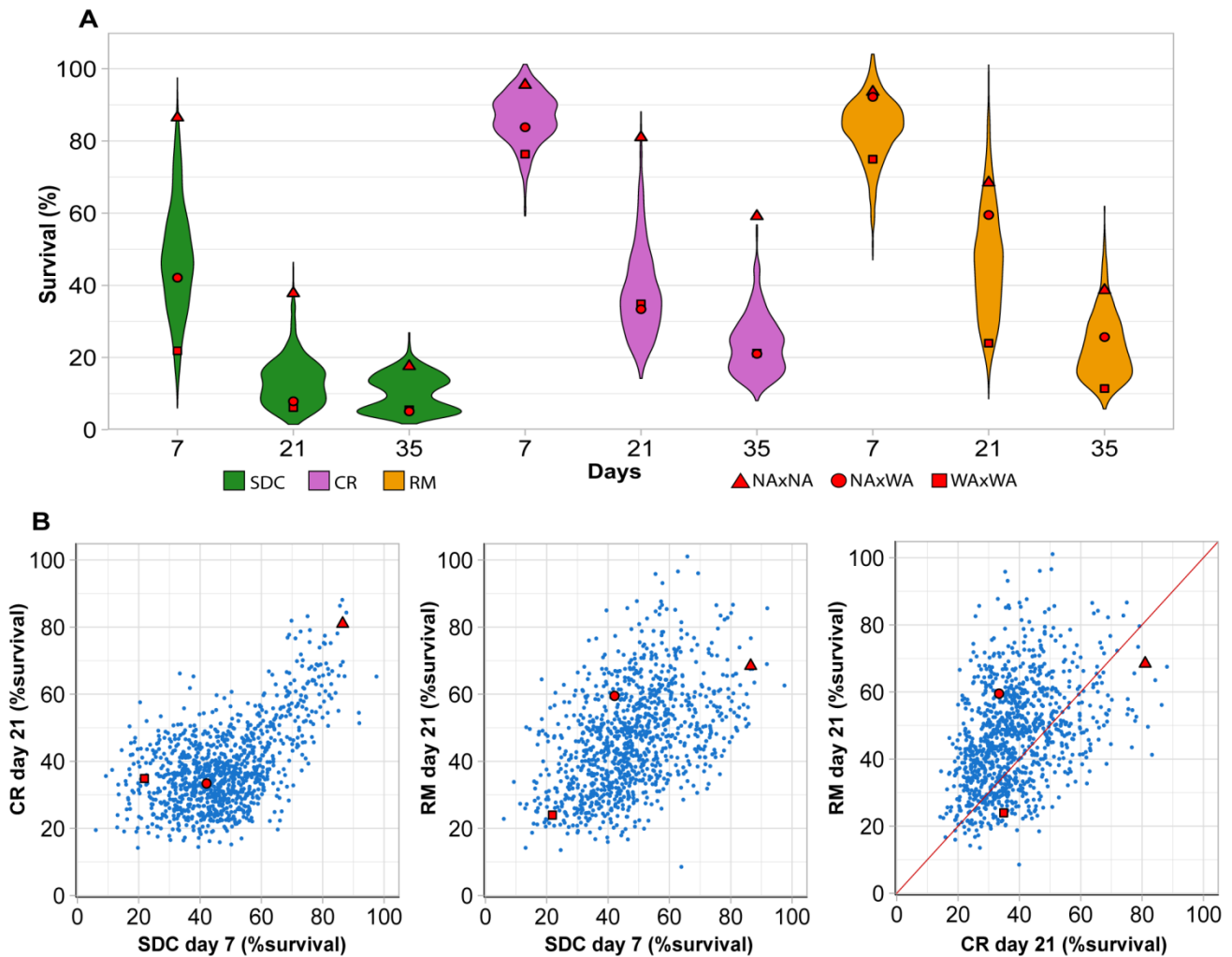


Figure 1. Genetic and environmental variation in survival rates. (A) Violin plot CLS distribution of 1056 F12 diploid segregants from the NAxWA. CLS was monitored at three different aging time points (days 7, 21, 35), and in three different media conditions (SDC, CR, RM). The percentage of living clones at a given time point for each strain is plotted on y-axis. The viability of the founder homozygote parents (NAxNA and WAxWA) and the F1 hybrid (NAxWA) are indicated with red symbols. (B) Scattered plots of CLS of selected environments and time points (reported in panel A) underlie CLS correlation across conditions. In the right panel, the same time points were plotted for CR and RM for a direct comparison between these two environments. Parental strains and F1 hybrid are indicated with red symbols.

the most frequently used synthetic media (SDC) with or without calorie restriction, and with or without addition of rapamycin, a drug inhibiting the TOR pathway. The choice of synthetic media over YPD was motivated by the observation that survival rate in the latter environment is highly fluctuating due to frequent regrowth episodes induced by nutrients released by dead cells (Fabrizio & Longo, 2007). Calorie restriction (CR) was applied by simply decreasing the glucose concentration from 2% to 0.5% (Jiang et al., 2000; Lin et al., 2000; Smith et al., 2007). Although further reduction of glucose concentration kept improving lifespan, we selected 0.5% concentration, which led to a more similar life expectancy compared to the rapamycin condition for both parental strains, while still exhibiting a strong effect on CLS (Fig. 1A and Fig. S1B). Rapamycin based CLS assay was done by growing our cells in synthetic medium supplemented with 0.025 μ G/mL rapamycin. This concentration was found to be sufficient to support cell growth, while substantially increasing CLS (Vazquez-Garcia et al., 2017). Doubling this concentration did not improve further the CLS of parental strains (Fig. S1). Given the scale of the experiment, cell viability was only monitored for three timepoints (7, 21, and 35 days of stationary phase which is assumed to start 72 hours post inoculation (Fabrizio & Longo, 2007)), which were highly informative of the CLS dynamic of the three different environments (Fig. 1A and Fig. S1B).

The overall phenotypic distribution reveals remarkable lifespan diversity among the 1056 isolates with a survival rate ranging from 6 to 97% after 7 days in SDC (47%

mean viability). As expected, the survival rate was significantly higher when cells were subjected either to CR (86% mean viability at day 7) or to RM (83% mean viability at day 7). Similarly to SDC, CR and RM conditions also displayed a broad diversity among isolates with a survival rate oscillating between 14 and 88% after 21 days in CR (38% mean survival), and between 9 and 100% after 21 days in RM (47% mean survival) (Fig. 1A). The continuous phenotypic distribution of our isolates indicates that multiple QTLs regulate variation in CLS, in concordance with what is expected for a complex trait. However, unlike Kwan et al., we did not observe much heterosis within our POLs. This result might be explained by the extreme CLS divergence of the parental strains we used. Indeed, the NA strain, which has a robust lifespan, might already carry an optimized allelic combination, by opposition to the WA strain, which is quite short living. Therefore, it becomes statistically rare to generate an allelic combination that would transgress parental phenotypes. Interestingly, some conditions have a partial bimodal distribution (SDC day 21 and 35, CR day 7 and 35), suggesting the existence of a single allele strongly impacting CLS. We then checked the correlation between the different environments. We obtained overall a fair correlation between them (Pearson's $r = 0.62$, $p < 2.2e-16$ for SDC_D7 versus CR_D21 and $r = 0.53$, $p < 2.2e-16$ for SDC_7 versus RM_D21) (Fig. 1B). These results indicate that in general the fittest individuals in SDC are also the fittest in CR and RM (and vice versa), implying that POLs lifespan is partly determined by the genetic background. Nevertheless, the absence of a strong correlation indicates that

a certain amount of genetic contributors is driven by genetic background-environment interactions. This is well illustrated by looking at the viability ratios between SDC, CR, and RM. When dividing survival at day 21 in CR by survival at day 21 in SDC, ratios range from 1.02 to 18.97, meaning that lifespan enhancement induced by CR, depending the genetic background, can range from no benefit to almost twenty-fold improvement. Likewise, ratios range from 0.75 to 25 for the RM environment. No isolate had a negative effect of CR on CLS, and only one isolate had a slightly lower value in RM compared to SDC (Fig. S1C). The direct comparison of CR and RM show a modest correlation between these two environments (Pearson's $r = 0.43$, $P < 2.2 \times 10^{-16}$, day 21), suggesting that the molecular mechanisms involved in CR and RM lifespan improvement are moderately redundant. Overall, POLs had a better lifespan benefit from RM compared to CR (733/1056 isolates had a superior survival in RM at day 21, Fig. 1B), although both parental strains had a better CLS in CR.

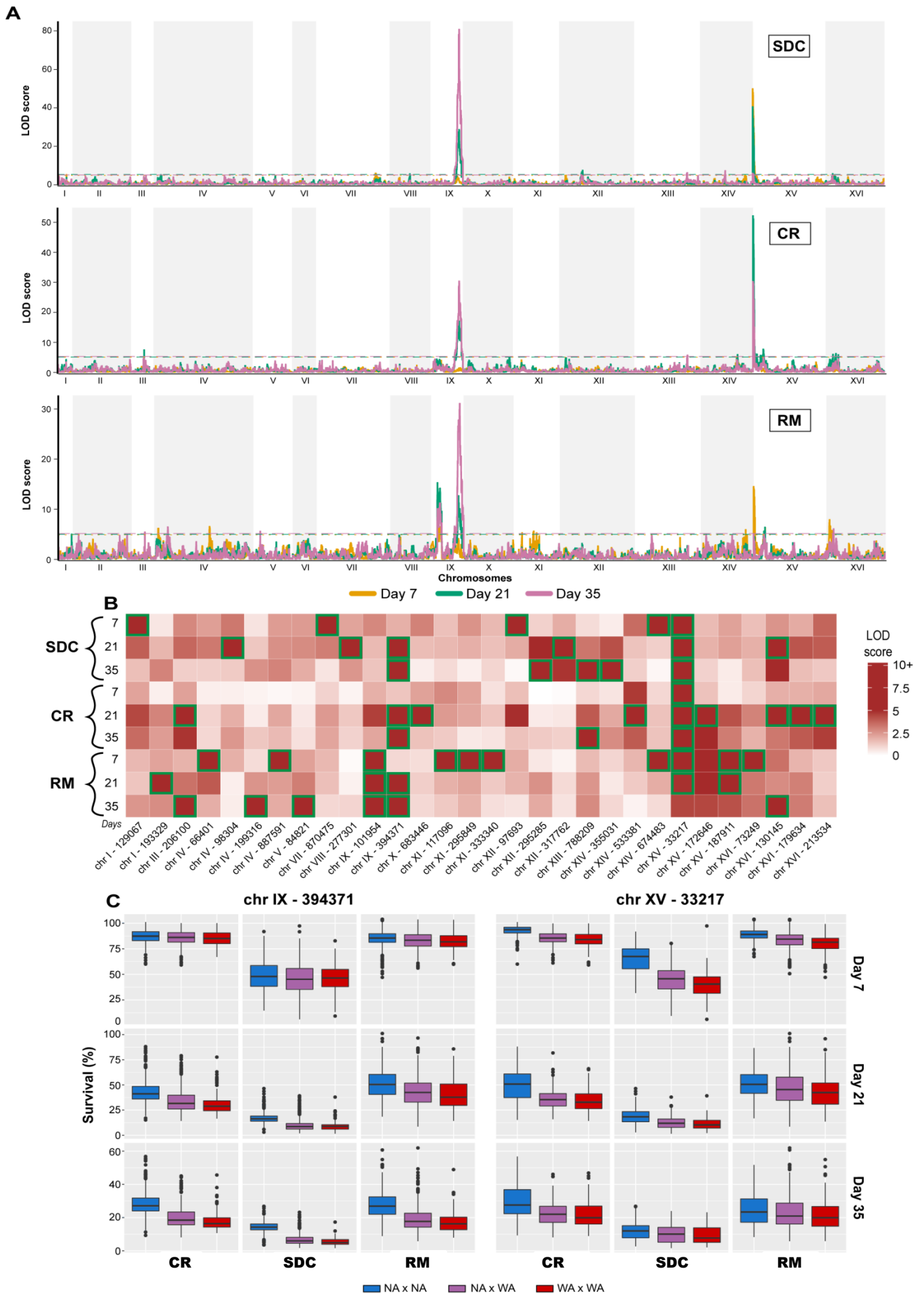
Chronological lifespan is regulated by environment dependent and independent QTLs

We used a genome-wide linkage analysis to map QTLs regulating CLS in the three environmental conditions tested in this

study. We called QTLs using the software R coupled with the dedicated package R/qtl. The genotype of each of the 1056 descendants was determined based on the repartition of the 52466 single polymorphisms existing between the parental strains (Illingworth et al., 2013), and the logarithm-of-the-odds (LOD) scores were calculated with 1000 permutations to call QTLs with a significance level of 0.05. In total, we identified 13 QTLs in SDC, 10 in CR, and 16 in RM (Fig. 2A and 2B), for a total of 30 unique QTLs with an average confidence interval of 15kb. The large number of QTLs mapped is consistent with the fact that CLS is a complex trait. Interestingly, only 3 QTLs were present in all three environments, 1 QTL was common between SDC and CR, 1 QTL was common between SDC and RM, and 1 QTL was common between CR and RM. On the other hand, we found 8 QTLs specific to SDC, 5 specific to CR, and 11 specific to RM, a result that emphasizes the importance of environmental cues in modulating genetic networks (Fig. 2B and Table S3). We also noticed that most of the QTLs we mapped were dynamic in time (only 2 QTLs remained significant for all time points in a given environment), pinpointing the fact that yeast cells go through different phases of cellular processes all along the course of CLS and these phases are regulated by different genetic determinants. The total genetic contribution of all the QTLs we

Figure 2. Time and environmental dependent chronological lifespan QTLs. (A) Linkage analysis plots reveal multiple QTLs regulating CLS. The three panels represent the different conditions (from top to bottom SDC, CR, and RM) while line colours represent time points (yellow = day 7, green = day 21, purple = day 35). The y-axis indicates the LOD score while genomic coordinates are displayed on the x-axis. Dashed lines represent significance thresholds ($p < 0.05$). (B) Summary heat map of the 30 distinct QTL regions that were mapped. Green frames indicate conditions for which QTLs were significant ($p < 0.05$). (C) CLS distribution of the 1056 segregants according to their genotype at the two strongest QTLs detected in chromosome IX and XV.

- RESULTS -



present in all environments and at all the time points, except in RM in which it was only significant at day 7 and had a lower LOD score (15 compared to 22 in CR and 50 in SDC at day 7) (Fig. 2A and 2B). The phenotypic distribution of our POLs shows that individuals with WA homozygotes at chrIX-394371 and chrXV-33217 had a significantly lower CLS compared to homozygote NA isolates (Fig. 2C). Heterozygote isolates at these positions had the same shortened CLS than WA homozygotes, indicating a dominant detrimental effect of the WA allele. Also, the viability distribution did not appear bimodal anymore when POLs were sorted according to their genotype at chrIX-394371, an observation in agreement with the strength and the impact of this QTL (Fig. 2C).

Natural genetic variations in *HPF1* and *FLO11* genes affect CLS

We investigated candidate genes within the QTL intervals mapped by linkage analysis mapping. We initially focused on the two main QTL peaks with a LOD score substantially higher than others. The chrIX QTL mapped within the *FLO11* gene, whereas the chrXV QTL peaked within the *HPF1* gene. Interestingly, these two genes are secreted cell wall glycoproteins, and none of them is known to regulate CLS. *HPF1* is a poorly characterized gene whose only known function is to prevent wine haziness (Brown et al., 2007). On the other hand, *FLO11* has been extensively studied for its role in regulating yeast adhesion, pseudohyphal growth, and biofilm formation (Guo et al., 2000; Douglas et al., 2007;

Vachova et al., 2011). To confirm the effect of *HPF1* and *FLO11* on CLS we performed a reciprocal hemizygosity assay (RHA), an approach that is based on direct allelic comparison within an isogenic background (Steinmetz et al., 2002) (Fig. 3A). We built F1 NA/WA hybrids in which we deleted either the NA or the WA allele of either *HPF1* or *FLO11* (see methods). The reciprocal hemizygous confirmed the role of *HPF1* and *FLO11* in regulating CLS. Deletion of *HPF1* NA allele did not impact at all CLS compared to the wild type F1 NA/WA hybrid (mean CLS = 5.09 and 4.74 days in SDC, respectively) (Fig. 3A). However, deletion of *HPF1* WA allele significantly enhanced CLS compared to the wild type hybrid (mean CLS = 8.49 days in SDC), indicating that *HPF1* WA allele has a detrimental effect on CLS while NA allele seems neutral. Moreover, deletion of NA *HPF1* allele did not improve further CLS compared to the single deletion of WA allele (mean CLS = 8.53 days), confirming that the WA allele has a dominant detrimental impact on CLS and that the NA allele has no effect. The reciprocal hemizygous results are fully consistent with the phenotypic distribution of the POLs partitioned for the *HPF1* genotypes (Fig. 2C). This trend was also observed in CR (mean CLS = 28.65 and 12.55 days for *HPF1*-WA Δ and *HPF1*-NA Δ respectively, Fig. 3B). However, we observed no significant CLS difference in RM for both *HPF1* alleles (mean CLS = 13.97 and 16.68 days for *HPF1*-WA Δ and *HPF1*-NA Δ respectively, Fig. 3B). Repeating this experiment multiple times confirmed the ability of RM to rescue the detrimental effect exerted by the WA allele, although the recovery was not complete every time. This result fits well with the

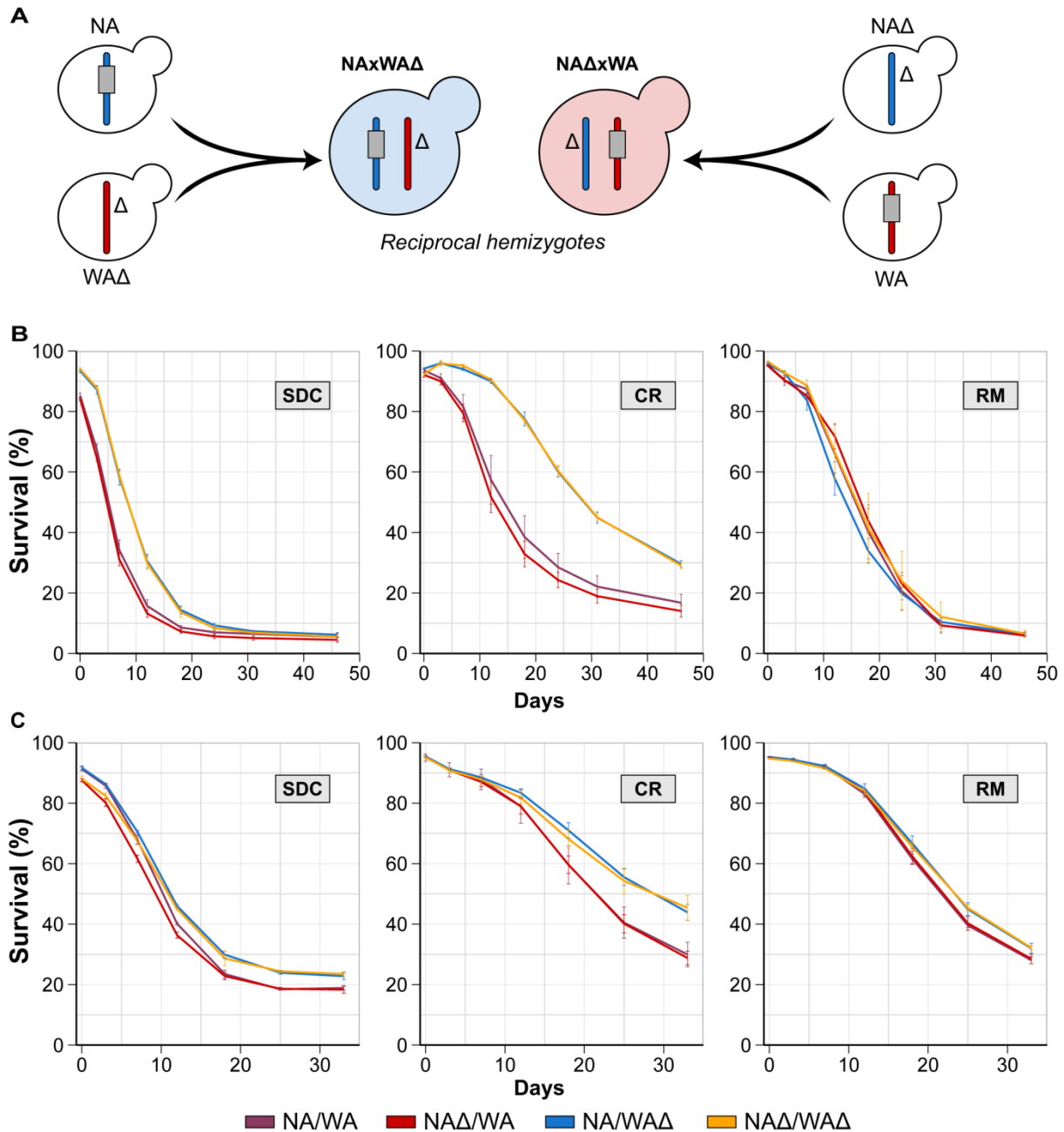


Figure 3. *HPF1* and *FLO11* allelic variants regulate chronological lifespan. (A) Reciprocal hemizygosity was used to experimentally validate the role of *HPF1* and *FLO11* on CLS. Blue and red chromosomes indicated NA and WA background, respectively. The candidate gene is represented by the grey rectangle and Δ indicates deletion of the candidate gene. (B) Reciprocal hemizygosity analysis of the *HPF1* gene. CLS dynamic in the reciprocal hemizygous (red NA Δ and blue WAA), WT (purple) and double deleted hybrids (yellow). Environmental conditions used are indicated in the top right grey box. (C) Reciprocal hemizygosity analysis of the *FLO11* gene (labelled as panel in B).

QTLs predictions that we previously described since the chrXV QTL had a much lower LO D score in RM and was only significant at day 7 during the early phase of CLS (Fig. 2A and 2B). The results obtained for the reciprocal hemizygosity assay of

FLO11 followed a very similar pattern compared to *HPF1*. Deletion of the WA allele of *FLO11* (*FLO11*-WAA) was also beneficial for CLS while deletion of the NA allele of *FLO11* (*FLO11*-NA Δ) was neutral (Fig. 3C). Once again, the presence of the

WA allele was sufficient to impair CLS and the complete deletion of *FLO11* did not improve further the lifespan. However, unlike *HPF1*, the detrimental effect of *FLO11* WA allele was not rescued by RM (mean CLS = 21.93 and 23.37 days for *FLO11*-NA Δ and *FLO11*-WA Δ , respectively). The CLS shortening induced by *FLO11* WA allele was more severe at late time points (day 35) in agreement the QTLs map which predicted this locus not to contribute to CLS at day 7 (Fig. 2A and 2B). However, the effect of *FLO11* WA allele was surprisingly mild compared to *HPF1* WA allele, given the LOD score of this QTL (deletion of *FLO11*-WA allele improved mean CLS by 11% in SDC, whereas deletion of *HPF1*-WA allele improved it by 67%). This could potentially be explained by the presence of other unidentified causative genes linked to *FLO11* that jointly contribute to the variance explained of this locus.

In addition to *FLO11* and *HPF1*, we tried to identify the candidate gene linked with a robust RM specific QTL found on chrIX (LOD = 15 at day 21, position 101954, 37392bp of confidence interval). We tested by RHA several genes located in this region (*FKH1*, *ASG1*, *RPL16A*, *RPII*), but none of them displayed significant CLS allelic difference (Fig. S3). Only *RPII* had a decreased CLS when both alleles were deleted and was suffering haplo-insufficiency when only one allele was kept. Although it confirms the already known role of *RPII* in CLS regulation (Marek et al., 2013), this gene could not be the one mapped in our study since there was no allele dependent phenotypic difference (Fig. S3). Moreover, we took the advantage of using a linkage

analysis approach to look for essential genes regulating CLS, a task that is difficult to access with classical gene deletion approaches. We found two promising candidates, the genes *TAF2* and *TFG1*, respectively associated with QTLs on chrIII (206100) and chrVII (870475). Both genes are involved in RNA polymerase II dependent transcription initiation. Unfortunately, the RHA for these genes did not show any effect on CLS.

Massive tandem repeat expansions within *FLO11* and *HPF1* impairs CLS

To understand why the WA allele of *HPF1* and *FLO11* is detrimental for CLS, we analysed the genetic sequence of the parental alleles. Interestingly, both genes display important structural variations that might strongly alter the protein structure, and that we could decipher in detail taking the advantage of long read Pac-Bio sequencing availability (Yue et al., 2017). Both *FLO11* and *HPF1* contain a prominent amount of internal tandem repeats, which are massively expanded in the WA allele of both genes. The NA allele of *HPF1* is relatively close to the reference lab strain S288C (*Saccharomyces* Genome Database: <http://www.yeastgenome.org/>). It shares 94.5% identity with S288C and has a total ORF size of 3033bp (versus 2904bp for S288C) due to minor repeat expansions. However, the WA allele of *HPF1* is remarkably divergent. It only shares 47.6% identity (MUSCLE alignment, including repeats) with its NA counterpart and is twice longer with a size of 6006bp due by massive expansions of internal repeats. Two distinct blocks of internal repeats can be

distinguished within *HPF1*. The first block of repeats (N-terminal) is the largest and begins 100bp downstream the start codon and covers approximately a third of the NA allele (900bp) and half of the WA allele (3000bp) (Fig. 4A). The second block of repeats (C-terminal) is closer to the end of the protein and is shorter. The C-terminal repeats span approximately a fifth of the NA allele (600bp) and a quarter of the WA allele (1500bp). Both blocks were drastically expanded in the WA parental background

with the Nterminal repeats expanded by 2100bp and the Cterminal repeats by 900bp compared to the NA allele. In terms of content, Nterminal repeats are serine rich (49%), while Cterminal repeats are enriched in threonine (32%), consistent with high content of serine and threonine reported in cell wall proteins (Verstrepen & Klis, 2006). Delimiting the boundaries and quantifying the exact motifs of both blocks with is difficult due to partial degeneration of the repeats. Nonetheless we estimated that

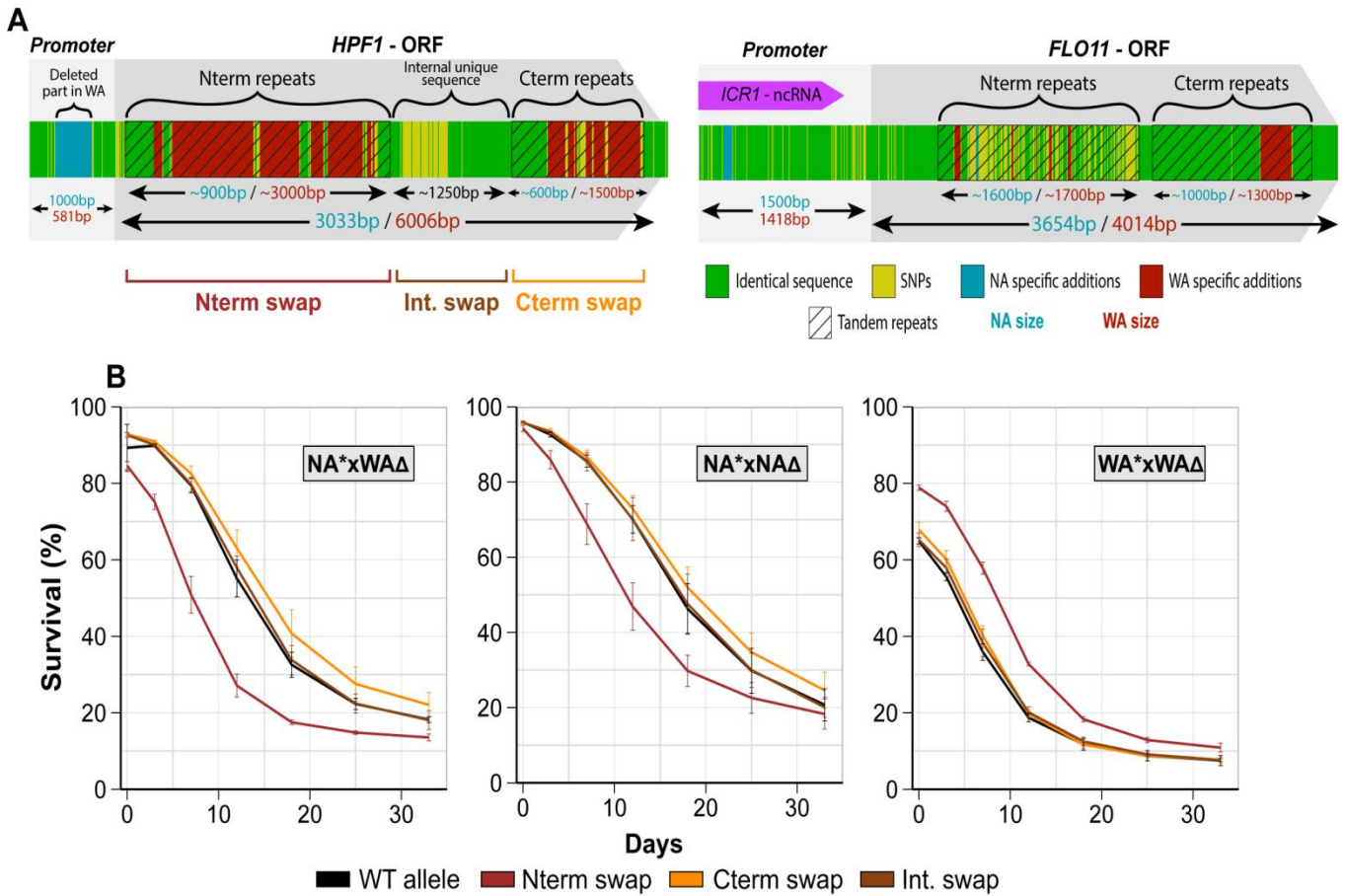


Figure 4. Intragenic repeats expansion shorten yeast chronological lifespan. (A) Schematic representation of *HPF1* and *FLO11* sequence structural variation in the two parental backgrounds. Segments that were engineered in the sequence swapping experiment are indicated underneath the *HPF1* scheme. (B) Allele swapping of different *HPF1* parts effect on CLS. The top right grey boxes indicate the background used in the experiment. Δ refers to the complete *HPF1* deletion: left panel WA-*HPF1* was deleted, middle panel one copy of NA-*HPF1* was deleted and right panel one copy of WA-*HPF1* was deleted. The remaining *HPF1* allele, indicated with a star (*), was either unmodified (WT allele), or the indicated subpart was substituted with its NA or WA counterpart (Nterm refers to the exchange of the intragenic N-terminal repeats block, Cterm to the exchange of the C-terminal block of repeats, and Int. to the exchange of the unique part in between the two repeat blocks).

Nterminal repeats are mainly constituted of a 21 amino acid long motif that would be repeated approximately 40 times in the NA allele, and 140 times in the WA allele. On the other hand, the Cterminal repeats are made of a longer 71 amino acid motif that would be repeated approximately 8 times in the NA allele and 20 times in the WA allele. In between the two blocks, both alleles share a non-repeated region of the same length (1250bp) that we named internal unique domain. Surprisingly, this region is also highly divergent between NA and WA alleles with only 90.8% of identity compared to 99.47% genome-wide. The discrepancy between the NA and WA alleles of *FLO11* was less staggering than *HPF1* with a sequence identity of 82.5% (including repeats), and a total length of 4014bp for the WA allele, versus 3654bp for the NA one. It also contains two main blocks of repeats, even though the C-terminal block in this case is more diffused and almost seamlessly linked with the Nterminal block (Fig. 4A). A modest WA specific repeat expansion of around 100bp can be found in the Nterminal repeats, while another 300bp expansion are found within Cterminal repeats. *FLO11* repeat content was also enriched in serine and threonine similarly to the *HPF1* repeats. However, most of the existing polymorphisms between NA and WA alleles *FLO11* were located within tandem repeats and not in the unique sequence. Reciprocal hemizygosity analysis confirmed the dominant detrimental effect of *HPF1* and *FLO11* WA allele on CLS but did not give us further information to narrow down the causative genetic variants within these genes. To determine whether repeat expansion is truly the driving factor, we have performed localized allele swapping

within the F1 NA/WA hybrid and homozygous parents, focusing our efforts on *HPF1*. For this, one allele of *HPF1* was deleted, while the remaining allele was partly engineered with a given piece of the other allele (see methods). We divided *HPF1* in three chunks, the Nterminal repeats, the Cterminal repeats, and the internal unique domain. Substitution of *HPF1* NA Cterminal repeats and internal domain with its WA counterpart had no effect on CLS compared to the long living reciprocal hemizygote F1 hybrid only harbouring the NA allele (mean CLS = 15.50 and 13.97 days for Cterminal repeat and internal domain substitutions, respectively, compared to 13.37 days for the native NA allele. Fig. 4B). However, substitution of NA with WA Nterminal repeats was sufficient to impair CLS to the same extent than the native WA allele (mean CLS = 7.17 days). We obtained the same result doing the reciprocal experiment; swapping WA Nterminal repeats with the NA ones in the reciprocal hemizygous only carrying the WA allele was sufficient to increase lifespan similarly to the full deletion of the WA allele (Fig. 4B). To confirm the causative effect of WA Nterminal repeats, we then inserted them into the NA homozygote parental strain. Doing this was sufficient to significantly decrease CLS mean from 17.06 days to 11.28 days. On the other hand, replacement of WA with NA Nterminal repeats in the WA homozygote parental strain was increasing CLS similarly to the full deletion of *HPF1*.

Transcription of WA *HPF1* allele is required to impair CLS and is regulated by rapamycin

Given the massive repeat expansion found in WA *HPF1*, we explored whether the simple presence of this allele within the genome could be sufficient to shorten CLS by forming secondary DNA structures or by generating replication stress (Moruno-Manchon et al., 2017; Malousi et al., 2018). To test this hypothesis, we deleted the promoter (including the first hundred coding

bases) of WA *HPF1* with an antibiotic cassette carrying a strong terminator in our reciprocal hemizygous strains (see methods). Deletion of WA *HPF1* promoter increased CLS comparable to the full *HPF1* deletion, suggesting that transcription of *HPF1* is required to exert its effect (Fig. 5A).

Analysis of the regulatory regions of WA and NA *HPF1* alleles revealed 419bp deletion in the WA promoter, 252bp upstream of the initiating methionine. This

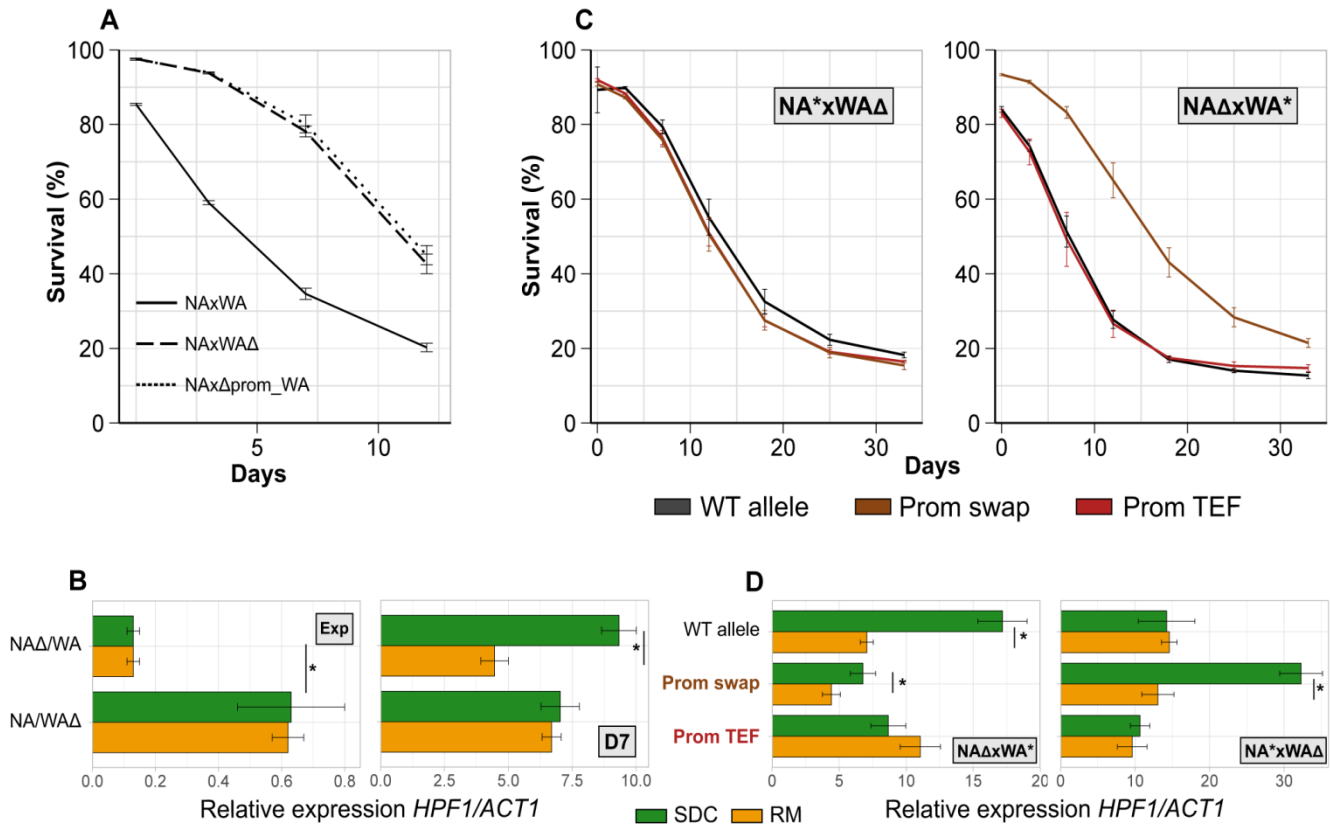


Figure 5. Rapamycin regulated expression of WA *HPF1* caused by partial promoter deletion. (A) Effect of *HPF1* promoter deletion on NAXWA F1 hybrid CLS. The deletion of WA-*HPF1* promoter (NAXΔprom_WA, dotted line) was sufficient to extend lifespan similarly to the full deletion of WA allele (NAXWAΔ, dashed line). (B) RT-qPCR data of *HPF1* expression in reciprocal hemizygote NAXWA strains. RNA was extracted either at mid-exponential phase (Exp, 7 hours of growth), or after 7 days of aging (D7, 3 days of pre-growth followed by 7 days of aging). Cells were grown either in SDC (green) or in RM (yellow). *HPF1* expression was normalized with *ACT1*. (C) *HPF1* promoter swapping effect on CLS. The grey boxes indicate the background used in the experiment. Δ refers to complete deletion of *HPF1* allele: deletion of WA-*HPF1* (left panel) and deletion of NA-*HPF1* (right panel). The promoter of the remaining *HPF1* allele, indicated with a star (*), was either kept unmodified (WT allele), or substituted with its NA or WA counterpart (Prom swap), or substituted with the strong constitutive promoter TEF (Prom TEF). (D) RT-qPCR of *HPF1* expression after 7 days of aging, in reciprocal hemizygote strains engineered with promoter swapping as described in (B) and (C). **p* < 0.05 (Wilcoxon test).

observation led us to hypothesize whether such a deletion could have a regulatory effect on the WA allele. To quantify *HPF1* expression we performed RT-qPCR experiments using RNA extracted either during exponential growth phase or after 7 days of aging, from our NA/WA reciprocal hemizygous strains. We found that WA *HPF1* was 5 times less expressed compared to the NA allele during exponential growth in SDC (0.13 versus 0.63 relative expression compared to *ACT1*, respectively, Fig. 5B). This result might sound surprising since WA-*HPF1* detrimental effect requires its expression. However, after 7 days of aging in SDC, we found that WA-*HPF1* was 1.33 times more expressed than NA-*HPF1* (9.33 versus 7.03 relative expression compared to *ACT1*, respectively). These data suggest that WA-*HPF1* expression level is maintained low during exponential growth before being activated during chronological aging, possibly through the deletion of regulating transcriptional binding factors located in the promoter. Interestingly, *HPF1* expression level during exponential growth was exactly similar in SDC and RM for both alleles. However, after 7 days of aging, while the expression of the NA allele remained identical in SDC and RM, the expression of WA allele was twice lower in RM compared to SDC (4.46 versus 9.33 relative expression compared to *ACT1*, respectively, Fig. 5B), implying that RM might rescue the deleterious effect of the WA-*HPF1* allele by limiting its over-expression during the chronological aging.

To validate the effect of the *HPF1* at the protein level in regulating lifespan, we swapped the WA and NA promoters and investigated whether it recapitulated the

observed effect on CLS with or without RM treatment. When the NA promoter was inserted upstream of the reciprocal hemizygous hybrid carrying the WA allele, the detrimental effect of WA-*HPF1* was lost (mean CLS = 16.11 days compared to 7.28 when WA is expressed under its native promoter, Fig. 5C). Expression profiling of this construction by qPCR confirmed what was found previously. When WA-*HPF1* allele was under control of NA promoter, its expression was 3.41 fold increased in SDC and 3 fold increased in RM, in exponential phase, compared to its wild type counterpart (Fig. 5D). Confirming the above observation, after 7 days of aging, expression of WA-*HPF1* under control of NA promoter was significantly lower compared to when it is regulated by the WA promoter (2.53 fold decrease). Reversely, NA-*HPF1* allele impact on CLS remained neutral when expressed under control of WA promoter, despite having an expression signature similar to the WA allele, including a substantial transcription boost during aging in SDC but not in RM (Fig. 5D). We next tested whether a further overexpression of WA-*HPF1* could worsen its effect. For this we swapped its native promoter with the constitutive TEF promoter. According to RT-qPCR data, TEF driven expression profile was close to the one driven by NA promoter (higher in exponential phase and lower at day 7), although slightly higher (1.21 and 1.28 fold higher in exponential phase and during aging, respectively) (Fig. 5D). Unlike our trial with the NA promoter, when expressed under control of TEF, WA-*HPF1* was as detrimental as it is when regulated by its native WA promoter (Fig. 5C). This difference could be explained by the slightly increased level of expression

induced by TEF compared to the NA promoter, which could be sufficient to get over an effective threshold.

We then looked for transcription factor binding sites within the 419bp deleted in the WA *HPF1* promoter to identify the causative factors using Yeastract (www.yeastract.com). We found several potential interesting binding sites for transcription factors known to regulate CLS, such as *FKH1* (mammalian *FOXO1* orthologue) (Postnikoff et al., 2012) and GIS1 (Pedruzzi et al., 2000), including some directly regulated by the TOR pathway like *TEC1* (Bruckner et al., 2011) and *GCN4* (Steffen et al., 2008) and also a key oxidative stress response regulator, *YAP1* (Okazaki et al., 2007). Nevertheless, explaining a scenario in which the full size NA-*HPF1* promoter would be unsensitive to RM treatment, while the truncated WA-*HPF1* promoter gets overexpressed in SDC and inhibited in RM, remains elusive at the moment.

***HPF1* intragenic repeat expansions alter oxidative stress resistance in an age dependent fashion**

According to the disposable soma theory, most genes identified to regulate lifespan exert a trade-off on organism fitness and fecundity (Kirkwood, 2005). This is the case in yeasts where several pro-longevity mutants, especially associated with mRNA translation, were found to slow down growth rate (Delaney et al., 2011). On the other hand, genes associated with lifespan shortening could be due to cellular defects leading to death instead of accelerated aging. Thus, we investigated whether *HPF1*

and *FLO11* have an impact on growth rate in the three environments used in this study. Neither the WA allele, nor the NA allele of both genes (in the NA/WA hybrid background) influenced growth in SDC, CR, and RM (Fig. 6A). This result implies that the detrimental lifespan effect of the WA allele is not due to unsustainable stress.

To check whether intragenic repeat expansions in *HPF1* and *FLO11* could have an adaptive advantage to compensate their effect on lifespan, we measured growth rate of the reciprocal hemizygous strains in 97 different environments. The environments tested include various carbon sources (both fermentable and non-fermentable), various nitrogen sources (amino acids, ammonium sulphate, GABA), various stresses (heavy metals, osmotic, pH stresses), and various drugs and chemicals (Table S4). Growth rate was monitored using a recently developed method based on colony size on solid agar plates (Zackrisson et al., 2016). However, we did not find any significant and reproducible environment in which allelic variation of *HPF1* or *FLO11* had an effect.

Since increased longevity is often correlated with increased resistance to heat and oxidative stresses (Fabrizio et al., 2001), we monitored if our reciprocal hemizygous strains were performing differently in these conditions. While *FLO11* did not display any allelic related change, *HPF1* alleles had a strong phenotypic difference on oxidative stress resistance (H₂O₂), but not on heat sensitivity (growth at 40°C) (Fig. 6B). Surprisingly, the presence of the sole WA allele was sufficient to increase oxidative stress resistance during exponential phase, while the presence of the NA allele, or the

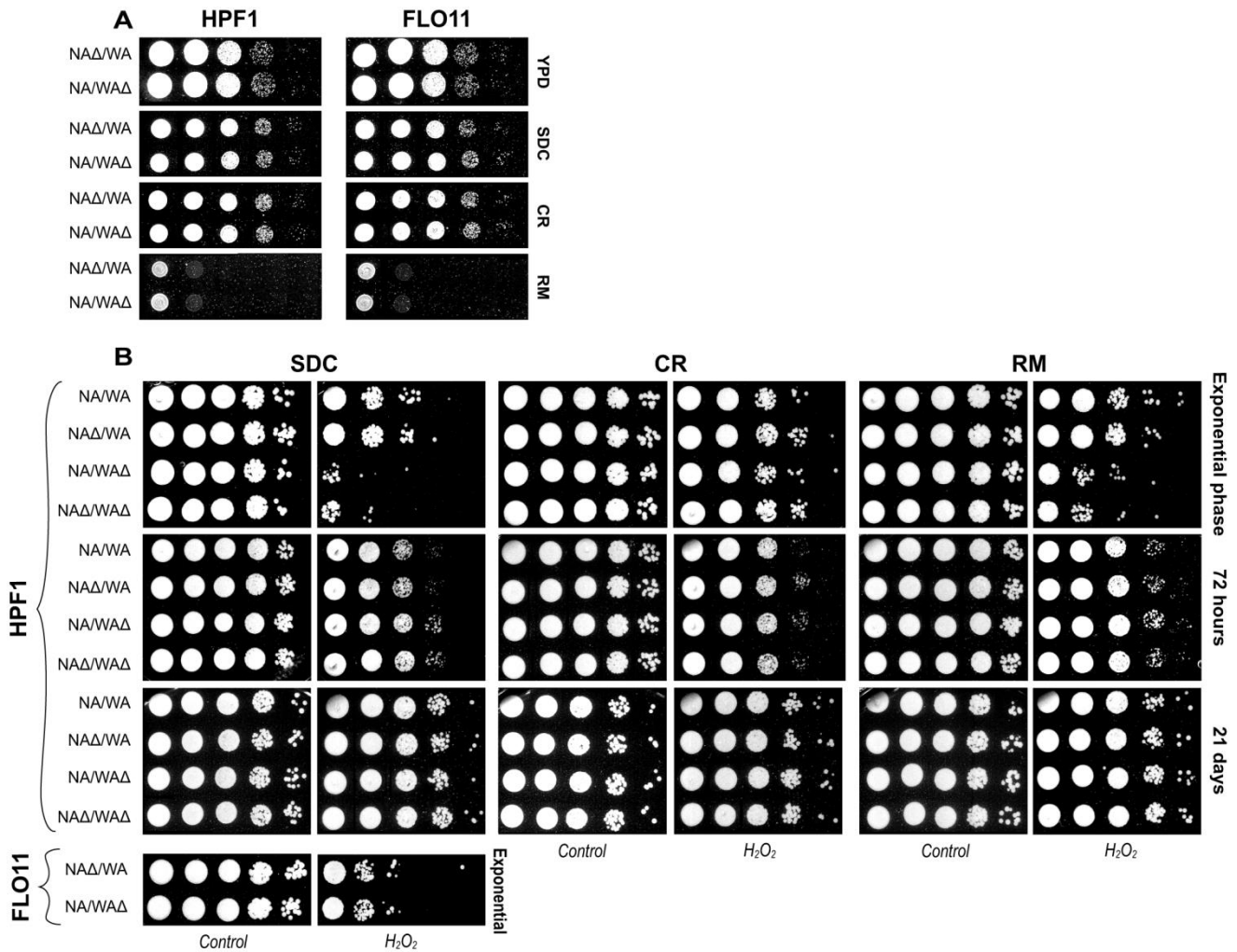


Figure 6. *HPF1* intragenic repeat expansions alter oxidative stress resistance. (A) Spotting assay of *HPF1* and *FLO11* reciprocal hemizygote strains on various media (Δ indicates which allele was deleted). Cells were grown to mid-exponential phase before being serially diluted and spotted. Plates were incubated 24 hours at 30°C (to observe growth differences before saturation). (B) Spotting assay of *HPF1* or *FLO11* reciprocal hemizygote strains. Cells were grown to mid-exponential phase (7 hours), or kept 72 hours in the indicated media (SDC, CR, or RM), before being serially diluted and spotted on SDC plates (Control), or SDC plates supplemented with either 1.5mM H₂O₂ (Exponential phase), or 2mM H₂O₂ (72 hours). Plates were incubated 48 hours at 30°C.

total deletion of *HPF1*, conferred lower resistance to H₂O₂. This result is contradictory with our expectations since higher oxidative stress resistance is generally synonym of longer lifespan. This oxidative stress resistance was also observed when cells were collected from exponential growth in RM. However, we observed a substantially higher oxidative stress resistance and no *HPF1*-WA and NA allelic differences in cells collected from exponential growth in CR. Interestingly, after 72 hours

of growth in any of the three environments (equivalent to day 0 in the CLS assay), oxidative stress resistance robustly increased as expected due to entry into stationary phase (Gray et al., 2004), but the advantage of the WA-*HPF1* allele during exponential phase disappeared. In contrast, a mild advantage was seen for the NA allele when cells were collected from exhausted SDC (Fig. 6B).

Thus, intragenic repeat expansions in WA-*HPF1* allele seem somehow to confer an early advantage during exponential phase that is lost, and even reversed, with cellular aging. In our screen of 97 environments, we did not observe such an effect when cells were grown on paraquat. This discrepancy is likely due to cells not being in exponential phase when exposed to the drug or the oxidative stress in paraquat and H₂O₂ being different.

Cell flotation triggered by *HPF1* expansion reduce chronological lifespan

It was previously found that media acidification, along with the release of ethanol and acetic acid, were potent inducers of cell death during yeast CLS assays, at least partly through the induction of mitochondrial activity and the generation of oxidative stress during stationary phase (Fabrizio et al., 2005; Burtner et al., 2009; Pan et al., 2011). To check if the effect of WA-*HPF1* could be mediated by alteration of external cues such as media acidification or ethanol and acetic acid production, we switched cells to water at the beginning of the CLS assay and we also monitored pH during aging in saturated media. Switching cells to water significantly increased lifespan but did not suppress the detrimental effect of WA-*HPF1* on CLS (Fig. 7A). The media acidity during CLS was not different between both alleles of *HPF1* (Fig. 7B). Therefore the detrimental effect of the WA-*HPF1* was not modulated by these external factors.

Interestingly, we noticed that the presence of WA-*HPF1* allele was inducing flotation and adhesion of the culture during exponential phase, but not anymore in stationary phase (Fig. 7C). This is a characteristic of ale beer strains and is usually associated with flocculation and cell wall hydrophobicity (Verstrepen & Klis, 2006; Palmieri et al., 1996). In the case of *HPF1*, we did not observe any sign of cell aggregation in exponential or stationary phases, implying that WA allele of *HPF1* is inducing flotation and adhesion independently of flocculation. Nevertheless, to verify if flotation during exponential phase could alter respiration rates and ROS production due to higher availability of oxygen in our non-shaking cultures, we performed the CLS assay in highly ventilated conditions (shaking flasks). In these conditions, while the overall lifespan was twice shorter compared to cells aged in 96 well plates, we did not observe anymore a lifespan difference between our reciprocal hemizygous strains (Fig. 7D). Thus, we conclude that flotation and higher oxygen exposition during exponential growth due to the cell physical properties conferred by the WA *HPF1* is enough to substantially shorten CLS. To further investigate that high ventilation of the culture was also sufficient to suppress the previously observed oxidative stress sensitivity, we spotted cells grown in erlenmeyers on H₂O₂ plates. Similarly to what we observed for CLS, the allelic difference in oxidative stress sensitivity was suppressed by these growing conditions (Fig. 7E).

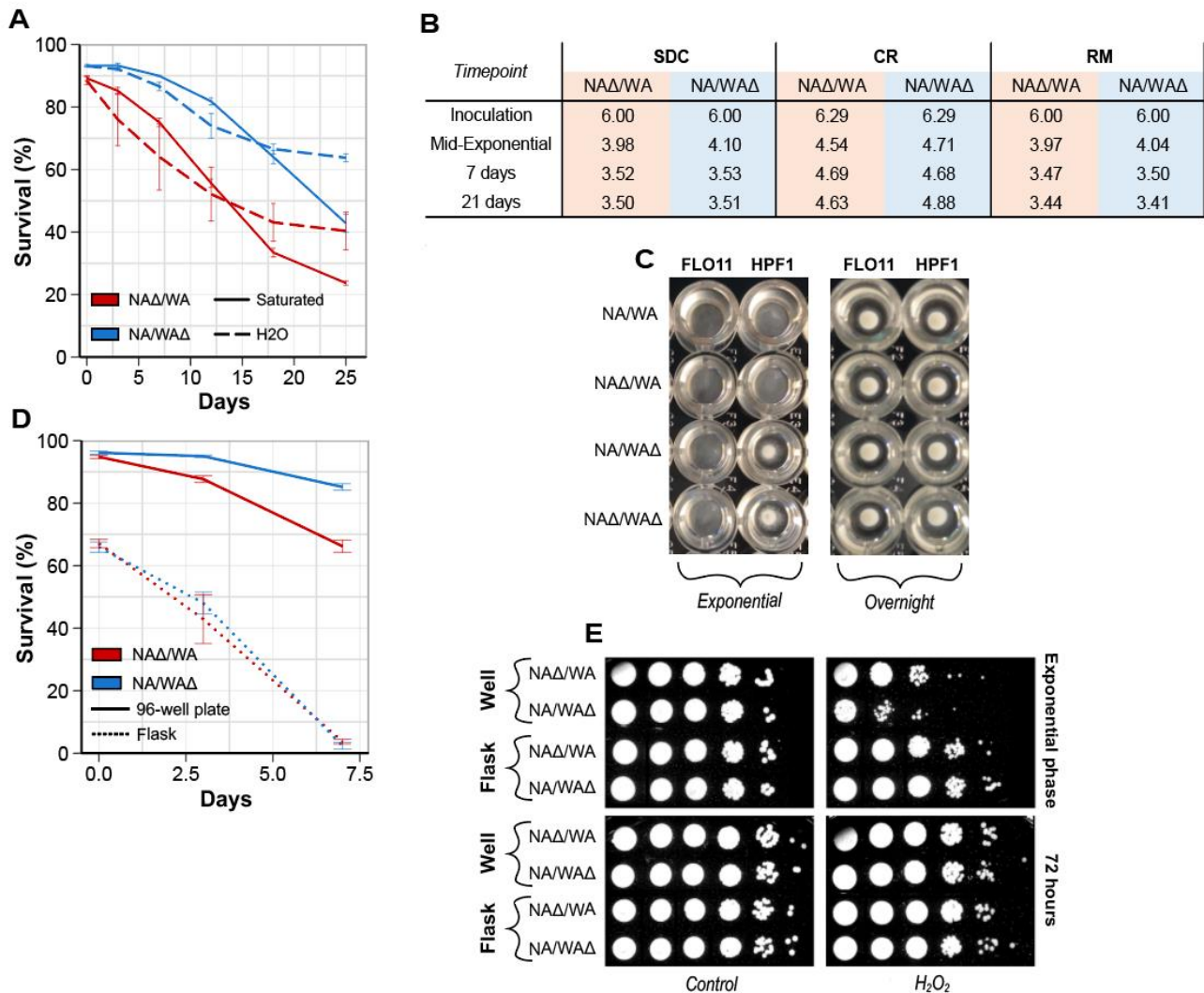


Figure 7. Expanded HPF1 induces cell flotation. (A) CLS of *HPF1* reciprocal hemizygote strains in water (Δ indicates which *HPF1* allele was deleted). Cells were pre-grown 3 days in SDC before washed and resuspended in water, or kept in saturated media. Error bars represent standard deviations. (B) pH of *HPF1* reciprocal hemizygote cultures during CLS. Cells were grown and aged either in SDC, CR, or RM, as indicated. Inoculation refers to the pH at the time of initial incubation, mid-exponential corresponds to 7 hours post-inoculation. (C) Flotation profile of *HPF1* and *FLO11* reciprocal hemizygote strains. Cells were grown 7 hours in SDC in a 96-well plate before pictures were taken. (D) CLS of *HPF1* reciprocal hemizygote strains in flasks compared to 96-well plates. The same ratio of cells was incubated either in both containers. Flask cultures had a 5:1 volume/medium ratio, and were shaken at 220rpm, while 96-well plates were filled with 200 μ L without shaking. (E) Spotting assay of *HPF1* reciprocal hemizygote strains pre-grown either in flasks or in 96-well plates for the indicated period (exponential phase = 7 hours). Cells were then spotted on SDC plates (Control), or SDC plates supplemented with either 1.5mM H_2O_2 (Exponential phase), or 2mM H_2O_2 (72 hours). Plates were incubated 48 hours at 30°C.

Higher oxygen exposition during exponential phase alters methionine and lipid biosynthesis

To understand the molecular mechanisms associated with CLS shortening through oxygen exposition and redox perturbation during exponential growth, we compared the transcriptomes of the *HPF1* reciprocal

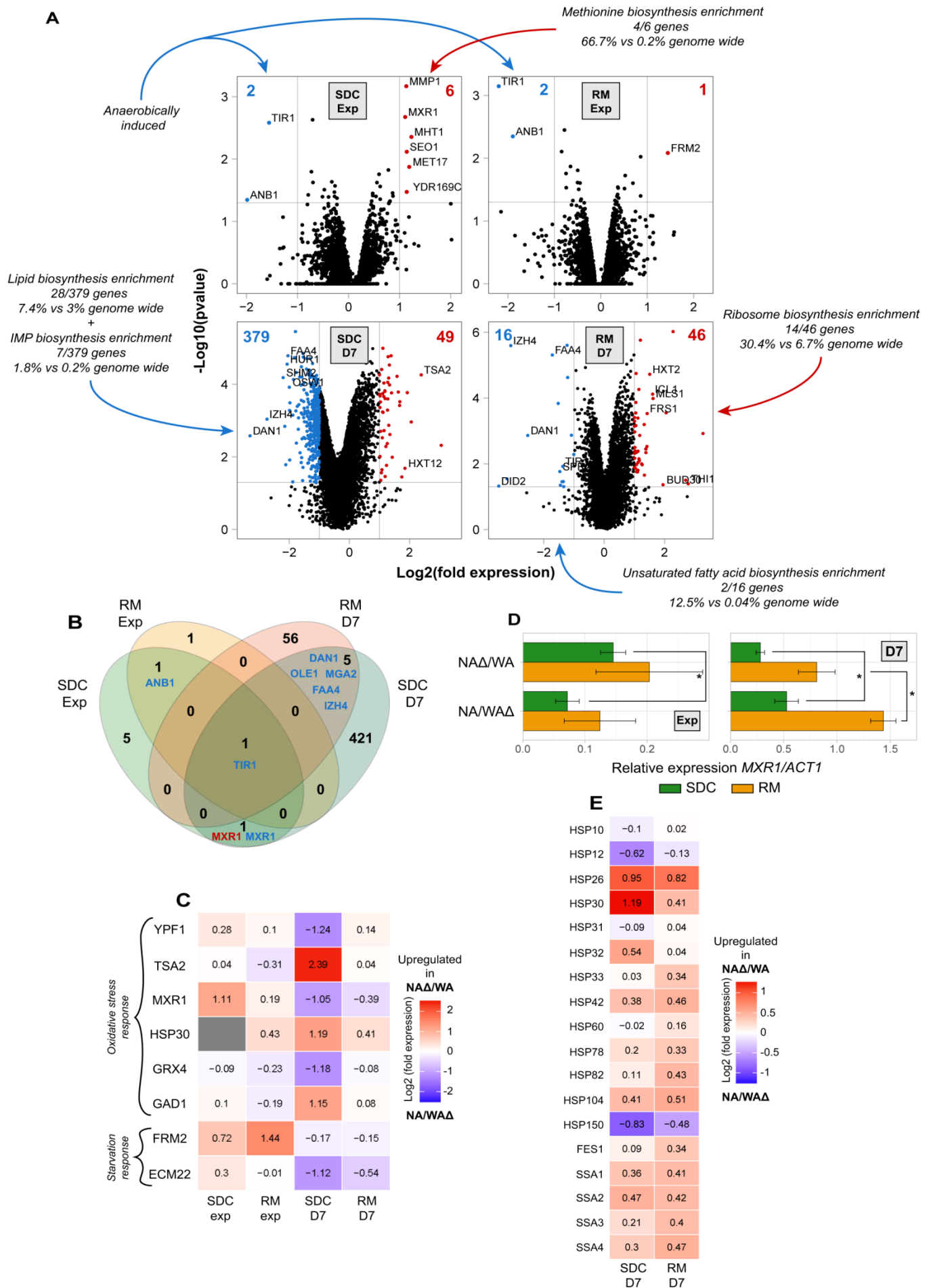
hemizygous NA/WA hybrids by RNA-seq. Total RNA was extracted from two biological replicates either during exponential growth or after 7 days of aging in SDC or in RM. Global expression level was analysed by pairwise comparison of the two reciprocal hemizygous strains within

each condition to determine genome-wide differentially expressed transcripts due to the *HPF1* genotype. We detected 8 genes with more than two-fold expression change during exponential growth in SDC, 3 genes during exponential growth in RM, 428 genes after 7 days of aging in SDC, and 62 genes after 7 days of aging in RM (Fig. 8A). These results are consistent with reciprocal hemizygous strains being essentially isogenic, apart from the *HPF1* allele, therefore we expected to find few genes with significant expression difference during exponential phase. In contrast, after 7 days of aging the alterations induced by *HPF1* generated a cascade of downstream events with a pronounced effect of the overall transcriptional profile.

Only two genes (*TIR1* and *ANB1*) were upregulated in the NA-*HPF1* hemizygote during exponential phase, both in SDC and RM (Fig. 8A). Interestingly, these two genes are both anaerobically induced, reinforcing the hypothesis that deletion of WA-*HPF1* limits oxygen exposition. The cell wall protein *TIR1* was the only gene differentially expressed in all conditions, including after 7 days of aging, while the translation elongation factor *ANB1* was only found in exponential phase (Fig. 8B). The gene *DAN1*, which presents similarities with *TIR1*, is also anaerobically induced but was found only at aging time points. None of these genes has been linked with CLS so far, consequently their contribution to lifespan extension in the NA-*HPF1* hemizygote is uncertain. Among the genes upregulated in NA-*HPF1* hemizygote during aging, we observed a strong enrichment for genes involved in lipid biosynthesis (using SGD ontology search tool), despite some

candidates not being shortlisted by SGD, such as *FAA4* and *IZH4* (Fig. 8A). We also mapped the essential fatty acid desaturase *OLE1* whose enzymatic activity is tied with lipid levels and upregulated by hypoxia. These results are interesting since lipid metabolism and storage have been associated with long CLS (Arlia-Ciommo et al., 2018; Handee et al., 2016). In addition, we identified enrichment for genes involved in purine biosynthesis (Fig. 8A), a pathway required for durable CLS (Garay et al., 2014).

Reversely, when looking at the genes upregulated in the WA-*HPF1* hemizygote, we found a striking enrichment (4/6 genes) linked with methionine metabolism (Fig. 8A). Since methionine depletion is usually associated with shorter lifespan, the upregulation of this subset of gene possibly contributes to the lower performance of WA-*HPF1* hemizygote (Ruckenstuhl et al., 2014). Moreover, the presence of the methionine sulfoxide reductase *MXR1* among the WA-*HPF1* upregulated genes is unlikely to be random. Methionine and cysteine, due to their thiol group are highly sensitive to oxidation. Hence, it is possible that increased oxidative stress in the WA-*HPF1* hemizygote specifically damages methionine and cysteine residues, causing increased expression of the detoxification protein *MXR1* and of related biosynthetic proteins. Upregulation of methionine biosynthetic genes was inhibited by RM during exponential phase, constituting a possible way by which RM prevents *HPF1* lifespan shortening. Interestingly, the only gene upregulated in RM during exponential phase was *FRM2*, an inhibitor of fatty acid synthesis, also involved in redox homeostasis



(De Oliveira et al., 2010). During aging, we found enrichment in genes linked with ribosome biogenesis (Fig. 8A). While induction of ribosome biogenesis is shortening lifespan (Powers et al., 2006) in agreement with the phenotype of WA-*HPF1* hemizygote, we could not explain why this enrichment was found in RM (which should inhibit ribosome production through TOR inhibition) and not in SDC.

Since we previously found variation in oxidative stress sensitivity within our reciprocal hemizygous strains, we looked for differently expressed candidate genes associated with oxidative stress according to SGD database. We also searched for genes linked with resistance to starvation, which could directly impact CLS. We obtained a list of 6 genes related to oxidative stress, and 2 genes related with starvation resistance (Fig. 8C). Only *MXR1*, which was upregulated in the WA-*HPF1* hemizygote, had a significant expression difference in exponential phase and could be responsible for higher H₂O₂ resistance in this strain. Interestingly, after 7 days of aging, *MRX1*

was this time upregulated in the NA-*HPF1* hemizygote (Fig. 8C and 8D), which is consistent with the observation that this strain becomes more resistant to oxidative stress during aging (Fig. 6B). However, in RM exponential phase, WA-*HPF1* was still found more resistant to H₂O₂ (Fig. 6B) although *MXR1* was not differently expressed (Fig. 8C). In this case, *FRM2* seemed to be the only candidate gene that could be linked with oxidative stress resistance, although it was found to negatively impact H₂O₂ resistance (De Oliveira et al., 2010). More generally, after 7 days of aging in SDC, all the shortlisted oxidative stress associated genes were significantly up or down-regulated, implying that the subtle differences induced during exponential phase culminate in a global alteration of redox homeostasis.

Moreover, given the overexpression of the heat shock protein *HSP30* in the WA-*HPF1* hemizygote after 7 days of aging (Fig. 8E), we investigated whether other chaperones could also be upregulated due to a global proteostatic stress in this strain. Indeed, we

Figure 8. Transcriptional signatures of *HPF1* intragenic repeat expansions

(A) Volcano plots summarizing the RNAseq data obtained for *HPF1* reciprocal hemizygote strains in 4 different conditions as indicated in grey boxes (SDC and RM during exponential phase and after 7 days of aging). The $-\log_{10}$ of the p-value was plotted in function of the \log_2 fold expression (x-axis). The fold expression was calculated by dividing NA Δ /WA by NA/WA Δ raw expression for each gene (see methods). Only genes which had a p-value < 0.05 and which were more than 2-fold differently expressed were retained. Blue dot represents upregulated genes in the NA/WA Δ hemizygote strain, while red dots represent upregulated genes in the NA Δ /WA hemizygote. The total number of upregulated genes is reported in the top corners of each plot (also in blue and red). All gene ontology enrichment identified are indicated with arrows (with blue and red colour code). (B) Venn diagram of genes over 2-fold differently expressed. Genes mapped in several conditions are in blue when upregulated in the *HPF1* NA/WA Δ hemizygote and in red when upregulated in the NA Δ /WA hemizygote. (C) Heat map displaying the \log_2 fold expression of differently expressed genes (see above) that were associated with oxidative stress or starvation resistance (according to SGD). Blue colour indicates upregulated genes in the *HPF1* NA/WA Δ hemizygote, while red refers to upregulated genes in the NA Δ /WA hemizygote. (D) RT-qPCR data of *MXR1* expression in reciprocal hemizygote *HPF1* NAxWA strains. RNA was extracted either at mid-exponential phase (Exp, 7 hours of growth), or after 7 days of aging (D7, 3 days of pre-growth followed by 7 days of aging). Cells were grown either in SDC (green) or in RM (yellow). *MXR1* expression was normalized with *ACT1*. *p < 0.05 (Wilcoxon test). (E) Heat map displaying the \log_2 fold expression of multiple chaperone encoding genes. Colour code is similar to (C).

found that multiple chaperones were reasonably over activated, including the disaggregase *HSP104*, during aging. Altogether, redox perturbations during exponential phase may eventually favour protein damage and misfolding contributing to lifespan shortening.

Spermidine rescues *HPF1* shortened CLS independently of autophagy

One of the main mechanisms by which rapamycin extends lifespan is mediated through autophagy activation following TOR inhibition (Alvers et al., 2009). Moreover, autophagy is activated by methionine restriction (Ruckenstuhl et al., 2014) and by specific lipids (Niso-Santano et al., 2015; O'Rourke et al., 2013), especially unsaturated. Given the phenotypic buffering

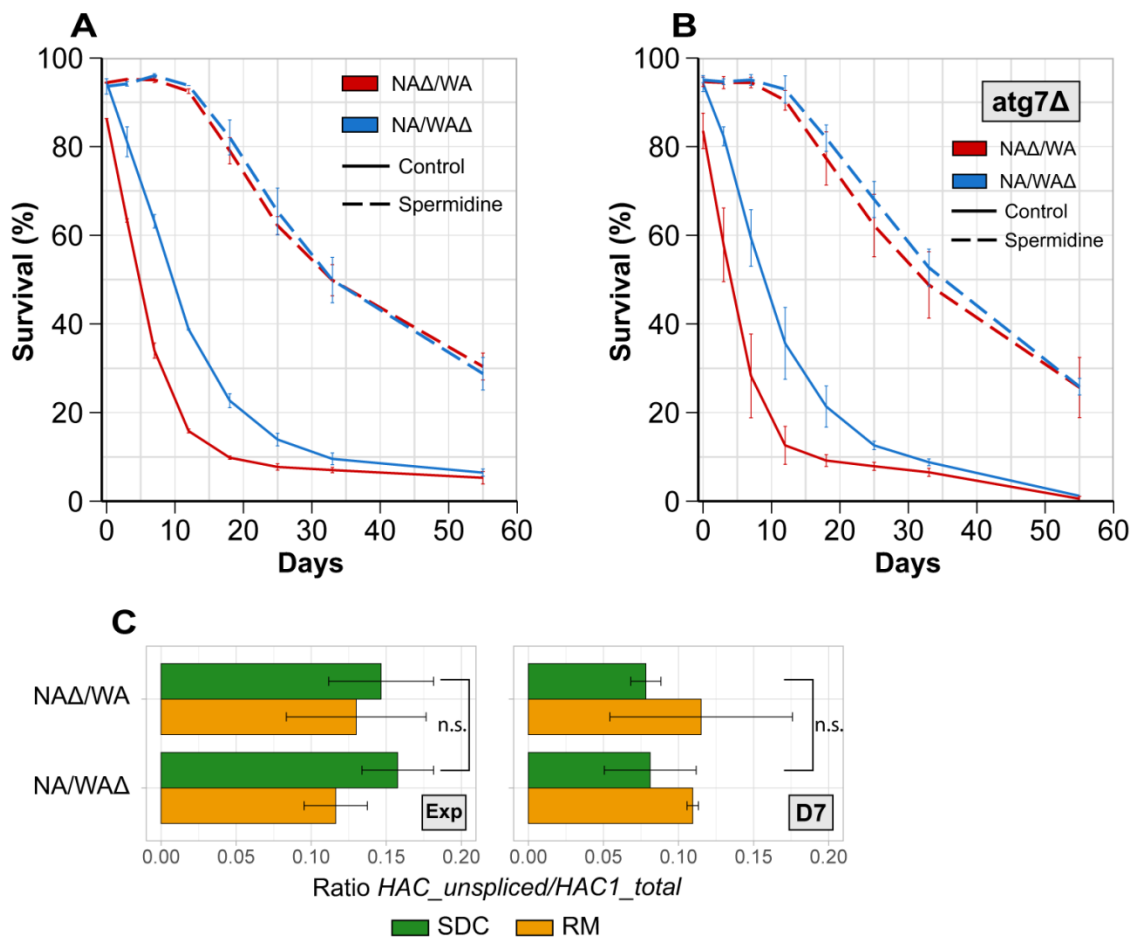


Figure 9. Spermidine rescues *HPF1* chronological lifespan independently of autophagy.

(A) CLS of *HPF1* reciprocal hemizygote strains in SDC supplemented with 5mM spermidine (dashed lines) or without (plain lines). Δ indicates which allele of *HPF1* was deleted. (B) CLS of autophagy mutant *HPF1* reciprocal hemizygote strains in SDC supplemented with 5mM spermidine (dashed lines) or without (plain lines). (C) Unfolded protein response activity determined by ratio of *HAC1_unspliced*/*HAC1_total* in the *HPF1* reciprocal hemizygote strains. The abundance of each form was determined by RT-qPCR using primer pairs that could amplify either the full RNA or the unspliced form. RNA was extracted either at mid-exponential phase (Exp, 7 hours of growth), or after 7 days of aging (D7, 3 days of pre-growth followed by 7 days of aging). Cells were grown either in SDC (green) or in RM (yellow). n.s. not significant.

of rapamycin and the RNAseq data, we investigated if the lifespan shortening due to *HPF1* expansion is linked with decreased autophagy. We performed CLS in SDC supplemented with the polyamine compound spermidine, known to increase lifespan by activating autophagy (Eisenberg et al., 2009). As expected, lifespan was substantially increased when we treated our reciprocal hemizygote strains with spermidine. In agreement with our hypothesis, spermidine fully recovered the detrimental effect of the WA-*HPF1* allele (Fig. 9A). Furthermore, we deleted the key autophagy gene *ATG7* to further validate the role of autophagy. Surprisingly, deletion of *ATG7* did not prevent RM and spermidine to rescue CLS loss (Fig. 9B), suggesting that autophagy is not involved in lifespan shortening by WA-*HPF1*. However, in disagreement with previous work, we noticed that our *ATG7* mutants were not short lived compared to the wild-types (Alvers et al., 2009). Possible explanation could be that *ATG7* loss is compensated by another gene, or that autophagy is not required in this given genetic background. Thus, the involvement of autophagy remains uncertain.

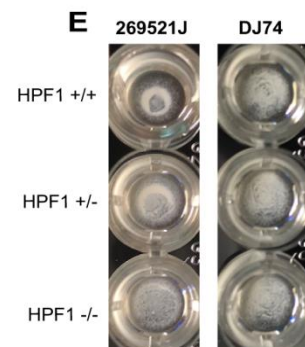
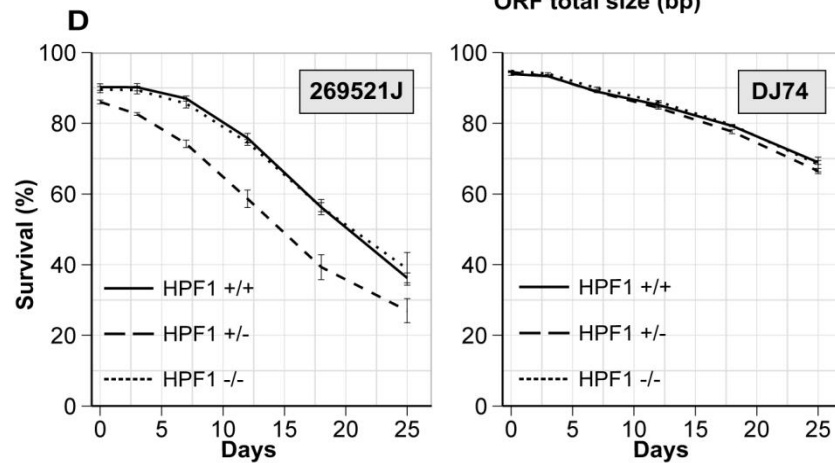
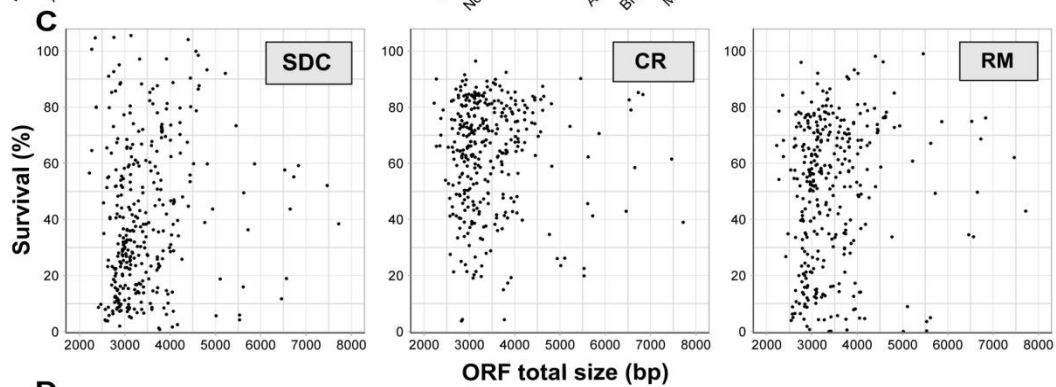
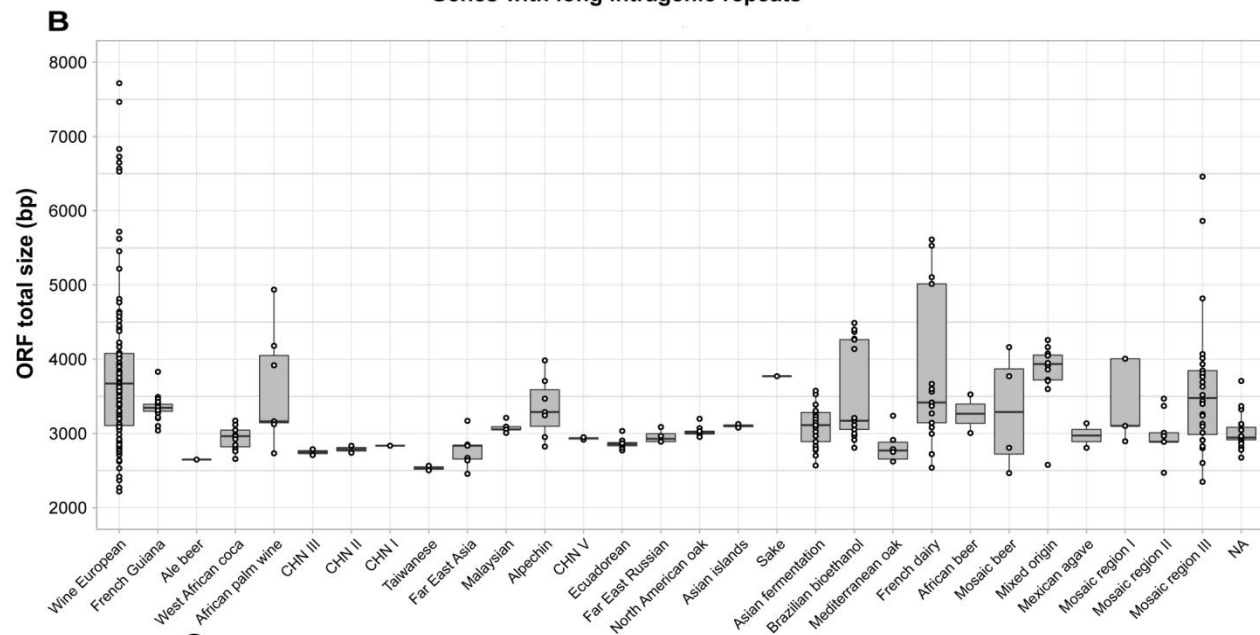
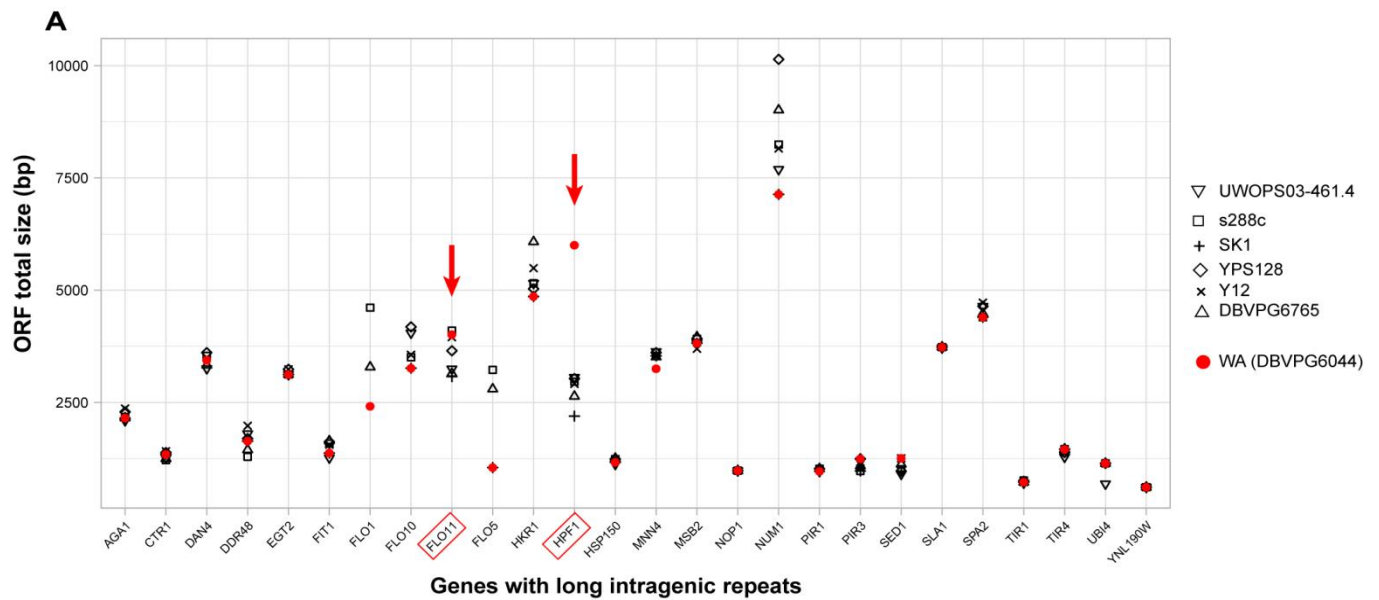
Since we found an activation of chaperone proteins during aging of WA-*HPF1* hemizygote, we also tested if this could correlate with the activation of other pathways involved in proteostasis, such as the unfolded protein response (UPR). Activation of this pathway relies on splicing of the protein *HAC1*, which we monitored using RT-qPCR approach. We did not find any difference in UPR activation between our reciprocal hemizygote strains, implying that UPR is not involved despite

upregulation of chaperone proteins (Fig. 9C).

HPF1* length and effectiveness are highly variable throughout *S. cerevisiae

Ectopic recombination events drive sequence expansion and contraction in genes containing tandem repeats (Hartl, 2000). Given that both *HPF1* and *FLO11* alleles were greatly expanded in the WA genetic background, we investigated if the intragenic repeat expansion was a hallmark of this strain background. We analysed 26 genes known to contain long tandem repeats and 15 genes known to contain short tandem repeats (Verstrepen et al., 2005). We compared their length using 7 different *S. cerevisiae* strains with high quality complete genome assemblies (Yue et al., 2017). Results showed widespread gene size variation across this strain panel, confirming intragenic repeats are highly dynamic (Fig. 10A). Nevertheless, the WA did not display a generalised intragenic repeat expansion compared to the other strains. Interestingly, while WA-*FLO11* had a similar length of other two strains, WA-*HPF1* was clearly an outlier compared to the other strains. Thus, we conclude that the WA strain does not have a general tandem repeat overextension mechanism and the extreme repeats expansion is restricted to *HPF1*.

To determine how *HPF1* size varies species-wide, and whether WA-*HPF1* extreme expansion is a unique case, we measured *HPF1* length by PCR in 365 natural strains. This panel included both strains related to the WA clade as well as samples all the major species clades (Peter & De Chiara et al., 2018) (Fig. 10B and Table S6). We



detected large variation in *HPF1* repeat size resulting in total length of the gene ranging from 2200bp to 7700bp. We observed a weak clade influence on *HPF1* size; for instance the West African Cocoa strains had an average *HPF1* size of 2937bp compared to 3828bp for Mixed Origin strains. Interestingly, the intra-clade variation was nearly similar to the inter-clade variation. This implies that the repeat dynamics is a continuous process that occurs at high rate and shape *HPF1* length also among closely related strains. The majority of strains were had an average size of 3400bp with some outliers with extremely long or short *HPF1* alleles. The Wine/European clade, which contained both the smallest and the largest *HPF1* alleles (2200 and 7700bp respectively), was the most variable clade. It is difficult to determine whether such intra-clade variability is also shaped by adaptation. In order to investigate the phenotypic effect of *HPF1*, we tested if the allele length has an impact in the CLS (using data obtained in chapter 5). However, we did not observe any correlation between *HPF1* size and CLS (Fig. 10C).

We previously showed that WA_*HPF1* exerts a dominant detrimental effect on CLS due to its N-terminal intragenic repeat expansion, which could be recapitulated both in WA and NA genetic backgrounds (Fig. 3B, 4B and S4B). On the other hand,

NA_*HPF1* allele, which is close to the referenced S288C allele, did not have any effect on CLS. To check whether the *HPF1* phenotype is solely determined by the repeat size or there is an interaction with the genetic background, we deleted the full *HPF1* ORF in two strains related to the WA strain. DJ74 was isolated from palm wine industry from Djibouti Republic, while the 269521J is a clinical isolate belonging to the Mosaic R3 group, which has abundant ancestry from the African palm wine clade. Both strains have a moderate *HPF1* length (3260bp for DJ74, and 3800bp for 269521J). Deletion of *HPF1* in S288C did not have any effect on CLS, which confirmed the neutrality of this allele also in the laboratory strain background (Fig. S2A). Similarly, deletion of *HPF1* from the DJ74 strain did not impact CLS. However, *HPF1* deletion in the clinical 269521J isolate shortened CLS in contrast to our expectation based on the observation in the WA homozygote (Fig. 10D and S2A). A similar trend was also observed in CR and RM with both conditions that did not rescue the CLS reduction due to the *HPF1* loss in 269521J. Surprisingly, the CLS of the wild type 269521J strain was not extended by the RM treatment (Fig. S5A). Interestingly, like the NA strain, deletion of *HPF1* in DJ74 did not change the cell sedimentation profile during cellular growth (Fig. 10E). On the other hand, deletion of *HPF1* in the clinical

Figure 10. Species wide *HPF1* size variation and chronological lifespan. (A) Size variation of genes containing long intragenic repeats across seven diverged *S. cerevisiae* strains. Red dots indicate the WA strain. Arrows indicate the *HPF1* and *FLO11* genes. (B) *HPF1* length determined by PCR across 365 natural diploid isolates (Table S6). Band size was estimated with GelAnalyzer software. (C) Correlation between *HPF1* length and CLS in SDC. CLS data were obtained from the results of chapter 5, following a similar experimental procedure. (D) CLS of the natural strains 269521J and DJ74 WT (plain line) or *HPF1* deleted (dotted lines = 1 copy deleted, dashed lines = both copies deleted). CLS of the 269521J strain was performed in SDC, while CLS of DJ74 was performed in CR due to its extremely short lifespan in SDC. (E) Flotation profile of 269521J and DJ74 (WT and *HPF1* deleted). Cells were grown 7 hours in SDC in a 96-well plate before taking pictures.

269521J strain induced cellular flotation while wild type cells were sinking in this strain background. This result confirms the implication of the *HPF1* gene in cell-cell interactions and in modulating the floating phenotype, although the effect is opposite to what we observed in the WA parental background. Therefore, it seems *HPF1* effect on CLS is mainly mediated by flotation which has a negative impact on lifespan (Fig. S5B and S5C). The relationship between *HPF1* and flotation remains to be determined at the structural and cellular levels and seems to be dictated by the genetic background.

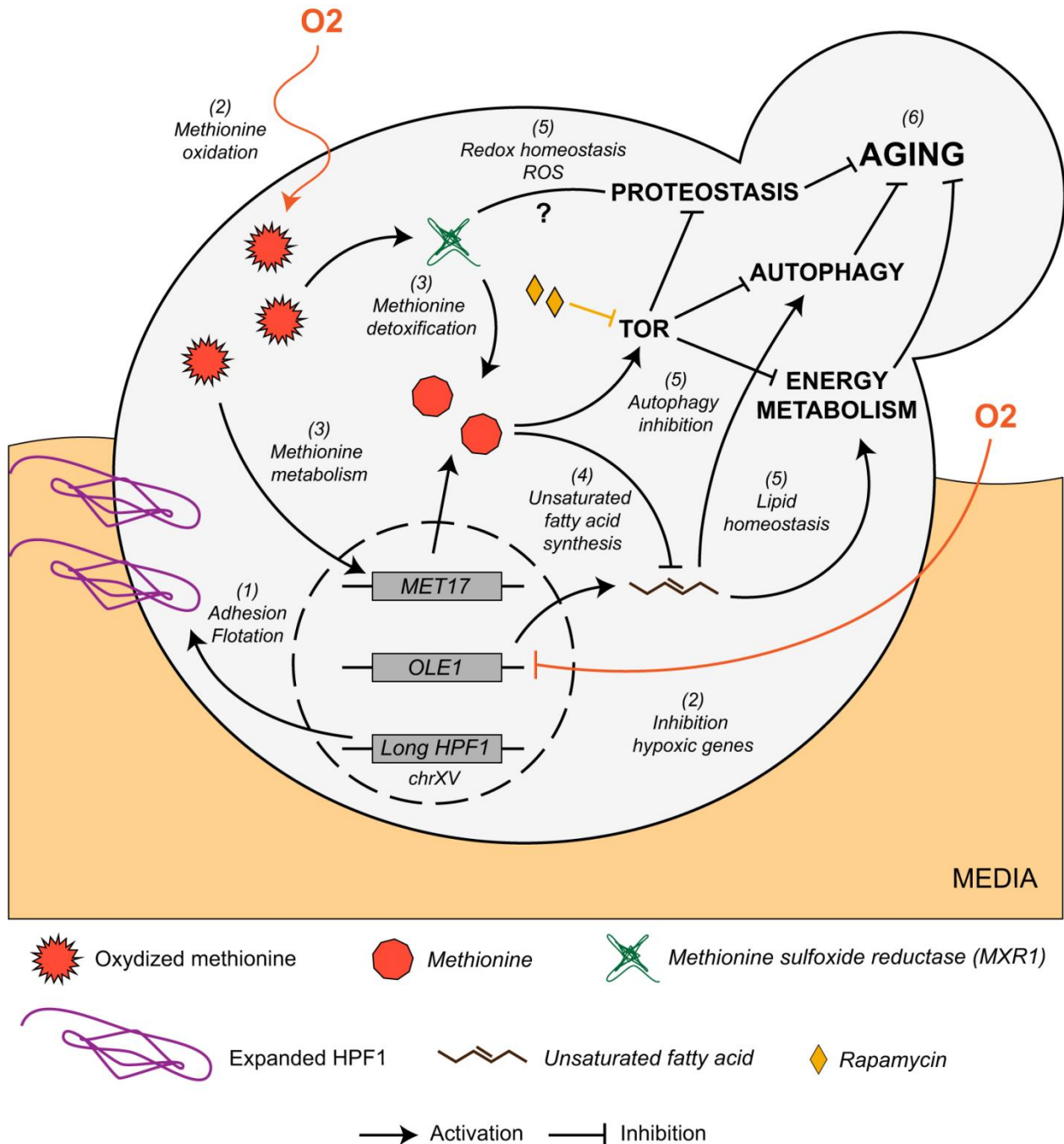
Discussion

While genetic studies in yeast RLS are still limited by the scalability of the model, several high-throughput approaches of yeast CLS have been developed and routinely used. Researchers have taken advantage of yeast CLS tractability to perform genome-wide gene deletion screens, leading to the identification of over a thousand genes regulating CLS (Powers et al., 2006; Fabrizio et al., 2010; Garay et al., 2014; Matecic et al., 2010; Gresham et al., 2011; Campos et al., 2018), making yeast CLS one of the best characterized aging model. Although such achievement has undoubtedly contributed to our knowledge of aging biology, this approach fails to elucidate subtler mode of regulations and make it difficult the study of essential genes, due to the dominant use of non-essential gene deletion collections. Alternatively, forward genetic strategies have been developed to map QTLs by linkage analysis using outbred pool of segregants.

Paradoxically, the few studies that have applied this approach are making use of outbred population derived from a cross with the classic laboratory strain S288C (Kwan et al., 2011; Kwan et al., 2013; Stumpferl et al., 2012), which segregate multiple auxotrophic mutations and was shown to be a phenotypic outlier (Warringer et al., 2011). Recently, Jung and colleagues have run a linkage mapping on a F1 segregating population derived from two natural strains (Jung et al., 2018) and mapped loss of function polymorphisms in the genes *RIM15* and *SER1*. While *RIM15* lifespan effect was already known due to its role in quiescence entry upon starvation, *SER1* had never been identified to impact CLS. Interestingly, both genes were mapped as QTLs only in given environments, emphasizing the importance of gene-environment interactions in shaping the complex architecture of the CLS trait.

In this work, we performed state of the art linkage analysis that enables high resolution and sensitivity QTL mapping. We used haploid segregants from an F12 outbred advanced intercross line derived from the two natural parents YPS128 (NA) and DBVPG6044 (WA) (Liti & Carter et al., 2009; Parts et al., 2011; Hallin & Märtens, 2016; Liti & Louis, 2012) and combined them into 1056 unique diploid phased outbred lines (POLs). The POLs approach has several advantages that include the characterisation of yeast CLS in diploid cells, the study of heterosis, dominance, and recessivity and it does not suffer of the issues associated with auxotrophy.

POLs segregants were aged in three different environmental conditions relevant to aging



(SDC, CR, and RM), and their survival was monitored at three different time points (7, 21, and 35 days). As expected for a complex trait, viability diversity among segregants was continuous, ranging from very short to very long living individuals. We observed few heterotic descendants, due to the extreme lifespan difference of the original parents. All isolates benefited from lifespan improvement induced by CR or RM treatments, but the quantitative CLS response varied greatly across backgrounds. Our highly recombinant POLs allowed us to map QTLs with an average resolution of 15kb, while using over a thousand individuals increased our detection sensitivity. In total we identified 30 unique QTLs distributed among the different environments and time points we monitored. Most QTLs were environment dependent with only 3, which were shared across all conditions. In addition, except 2 QTLs, all the others show a dynamic response across the time points. As previously observed, these results suggest that yeasts are going through different stage during chronological aging and that environmental interventions can completely remodel the CLS genetic architecture (Kwan et al., 2011; Jung et al., 2018).

As the QTLs validation is a lengthy process, we decided to focus our efforts on the two regions that explained most of the phenotypic variation. These regions were mapped with high precision on chromosomes IX and XV with a confidence interval of 4364 and 5246bp respectively. Identification of genes associated with these QTLs appeared rather straightforward since the candidate genes *HPF1* (chrXV) and *FLO11* (chrIX), located just below the peaks,

displayed quite peculiar genetic features. Indeed, both genes contain long intragenic tandem repeats. Strikingly, those repeats were massively expanded in the WA allele of *HPF1* and *FLO11*. Intragenic tandem repeats are known to be highly dynamic in size due to ectopic recombination events occurring during DNA replication or repair (Hartl, 2000) and their uncontrolled expansion was found to be the cause of multiple human diseases, such as Huntington's chorea (Orr & Zoghbi, 2007). Nonetheless, intragenic tandem repeat size variation has also been found to have a functional adaptive role (Verstrepen et al., 2005; Gemayel et al., 2015). In yeasts, the vast majority of genes containing intragenic tandem repeats encode for cell wall proteins (Verstrepen et al., 2005). Cell wall is the first layer of interaction between yeasts and their environment, and cell wall proteins, which are involved in biofilm formation, flocculation, and adherence, are essential features for micro-organisms to cope with their surrounding environments (Verstrepen & Klis, 2006). Intragenic tandem repeat variations within yeast cell wall proteins were found to generate dynamic functional variability and to have an adaptive value (Verstrepen et al., 2005). While *FLO11* is known to be an important regulator of cell adhesion, pseudohyphal growth, and biofilm formation (Guo et al., 2000; Douglas et al., 2007; Vachova et al., 2011), *HPF1* is largely uncharacterized (Brown et al., 2007), and none of these two genes have been associated with aging so far. By reciprocal hemizygosity (Steinmetz et al., 2002), we confirmed that the expanded WA alleles of *HPF1* and *FLO11* had a dominant detrimental effect on CLS. In the case of *HPF1*, localized expansion of N-terminal

repeats was sufficient to reproduce this phenotype in the NA background. Interestingly, *FLO11* was mapped only at late aging time points, while *HPF1* was mapped at all age time points and suppressed by rapamycin, suggesting different mode of actions despite similar protein profiles. We noticed that the expanded form of *HPF1* was enough to trigger cell flotation and adhesion during exponential growth, but not anymore in stationary phase. Cells deleted for *HPF1*, or harbouring the short allele, were simply sinking at the bottom of the well. On the other hand, we did not identify any observable cell sedimentation phenotype in the case of *FLO11*. Intriguingly, we found that flotation and adhesion during growth phase were responsible of CLS shortening since cells that were vigorously shaken in flasks did not show any lifespan difference between long and short versions of *HPF1*. This constitutes a nice example of the antagonistic pleiotropy theory of aging according to which genes with beneficial effect early in life could have a detrimental effect on the long run (Williams, 1957). In the present case, flotation and adhesion during exponential phase could confer growth advantage by helping cells to stick on surfaces or to reach new nutrient rich niches, while shortening lifespan at term. Since this feature has evolved in a palm wine strain proliferating in man-made environments, it is plausible that the consequences of decreased CLS capacity on strain fitness are minors. Moreover, not all cells within the clonal culture were displaying this phenotype, suggesting that while some cells could sacrifice some of their life expectancy to try to optimize their growth chance, the rest of the population

have a longer lifespan. Thus, this phenotypic heterogeneity could constitute a potential advantage.

The link between flotation, adhesion, and aging was less straightforward to establish. One of the main characteristics of floating yeasts is their higher exposition to oxygen. Indeed, we found that floating cells had a better oxidative stress resistance during growth phase, presumably as an adaptation to higher oxygen insults, compared to the ones with short *HPF1* that were sinking. When cells were grown in shaking flasks, oxidative stress resistance was even higher, and we did not observe any difference between strains with short or long *HPF1*. Regarding life expectancy, this result contrasts with data from the literature since mild oxidative stress during growth has been associated with longer lifespan, according to the virtue of hormesis (Mesquita et al., 2010; Pan et al., 2011). However, we noticed that short living strains with expanded *HPF1* grown in CR had an equivalent resistance to oxidative stress during exponential phase compared to strains with short *HPF1*, despite floating. Moreover, strains with long *HPF1* grown in RM were floating and had a better oxidative stress resistance compared to strains with short *HPF1*, as observed in SDC, while this time their lifespan was rescued by RM. Altogether, early activation of oxidative stress response seems not to be linked with premature aging in our floating strains harbouring expanded *HPF1*.

Using RNAseq profiling, we found that 4 of the 6 genes upregulated during exponential phase in the floating strains were directly linked with methionine biosynthesis. Interestingly, among the two remaining

genes, one was the methionine sulfoxide reductase *MXR1*, involved in detoxification of oxidized methionine residues. Since methionine is particularly sensitive to oxidation due to its thiol group (Vogt, 1994), we deduced that higher oxygen exposition upon floating aerobic growth increased methionine oxidative damage and led to *MXR1* activation. *MXR1* was the only gene linked with oxidative stress resistance, which was upregulated in floating strains during exponential phase, presumably responsible of increased H₂O₂ resistance, even though, as stated above, this might not impact CLS (Moskovitz et al., 1998). As methionine restriction has been linked with lifespan extension in multiple organisms (Orentreich et al., 1993; Hansen et al., 2005; Troen et al., 2007; Johnson & Johnson, 2014), we hypothesized that oxidative methionine stress could eventually lead to higher methionine levels via activated biosynthesis and enhanced detoxification, and eventually shorten lifespan. Beyond being a protein constituent, methionine is a pleiotropic amino acid that is involved in nutrient and redox signalling, and in methylation, having downstream incidences on countless cellular processes, including autophagy and lipid metabolism (Ruckenstuhl et al., 2014; Hasek et al., 2013). Interestingly, we found that sedimenting strains with short *HPF1* had specific upregulation of genes associated with lipid biosynthesis. Lipid metabolism has been tightly associated with aging, and several intermediates were found to have either pro or anti-aging effect (Mohammad et al., 2018; Handee et al., 2016). Moreover we found that higher oxygenation during growth inhibited hypoxic sensitive genes such as *OLE1*, involved in unsaturated fatty

acids synthesis, which were found to activate autophagy (O'Rourke et al., 2013; Niso-Santano et al., 2015). Altogether, increased methionine rates and lower fatty acid synthesis are likely to deeply alter cellular metabolism and energy balance, which are essential to sustain longevity (Bonawitz et al., 2007; Ocampo et al., 2012; Arlia-Ciommo et al., 2018; Chaudhari & kipreos, 2018). Autophagy could also constitute an appealing converging target for all these processes, especially given the rescuing effect of spermidine, however our experiments to test this hypothesis remained inconclusive.

The implication of proteostasis on the other hand was unclear. We found activation of multiple chaperone encoding genes in the floating strains with long *HPF1*, suggesting higher proteostatic stress during aging. Usually, protein damage and aggregation during aging is associated with higher oxidative stress (Stadtman, 1992). However, cells floating during growth phase displayed better resistance to oxidative stress, and while this resistance became similar to the one of short *HPF1* strains at stationary phase, it did not get worse with time. Interestingly, *MXR1*, which was the only upregulated gene linked with oxidative stress during exponential growth, became downregulated in floating cells during aging. Intriguingly, after 7 days of aging, multiple genes involved in oxidative stress resistance were differently expressed. Three genes, including *MXR1*, were upregulated in sedimenting strains with short *HPF1*, while three other genes, including *TSA2*, were upregulated in the floating strains with long *HPF1*. This observation echoes recent findings according to which recruitment of

chaperones to misfolded proteins requires the guidance of redox-regulated enzymes such as *TSA1* (Hanzen et al., 2016). Thereby this could be a mechanism leading to altered proteostasis, although apparent oxidative stress resistance seems unchanged.

More globally, lifespan impairment due to *HPF1* induced flotation could be rescued by the TOR inhibitor RM. Inhibition of TOR presumably antagonizes the effect previously described of methionine increase and lipid metabolism alteration by directly improving autophagy, energy metabolism and proteostasis (Loewith, 2011). On the other hand, while CR could improve CLS of both strains with long and short *HPF1*, the lifespan difference between them was still substantial. Although RM is commonly considered to increase lifespan by mimicking CR, it appears evident that, at least in yeast, the two interventions increase lifespan through distinct mechanisms and might be determined by a different sugar and amino acid sensing mechanisms in yeasts.

Conclusion

The work done in this study describes lifespan variation in more than 1000 diploid descendants from a two-way cross. The continuum of lifespan variation obtained by simply reshuffling two parental genomes underlies how complex is aging regulation. Lifespan was also determined in two environmental conditions known to promote longevity. While all descendants responded somehow positively, the variation of response to these interventions and the quasi absence of common QTLs cross environments underline how complex is the

gene-gene and gene-environment interactions network. This may explain the discordant data of the effect of CR on rhesus monkeys (Balasubramanian2017), and reminds how much additional work is needed before such interventions can be established in human medicine.

While functional intragenic tandem repeat variations are now well described from yeasts to humans (Verstrepen et al., 2005; Gemayel et al., 2015; Orr & Zoghbi, 2007), discovery of the role of *HPF1* repeat expansions in longevity control constitutes, to our knowledge, the first gain of function described for CLS regulation. This result underlines the strength of forward genetic approaches for exploiting natural diversity resources and uncovering new aging regulation mechanisms from genes that were so far considered neutral.

At the first glance, the link between a yeast cell wall protein causing flotation and human aging may not appear straightforward. However, it is known that oxygen rate strongly impacts longevity, and that even mammalian cell replicative senescence is affected by oxygen levels (Campisi, 2001; Rascon & Harrison, 2010; Leiser et al., 2013). Thereby, understanding the molecular mechanisms associated with lifespan shortening induced by oxygen exposition in yeasts can be a proficient paradigm to get insights into mammalian cellular senescence. Moreover, mammalian cells are maintaining complex relationships with the surrounding extracellular matrix and with neighbouring cells. For instance, it was found that exposition of satellite cells from old mice to young serum was sufficient to promote their rejuvenation (Conboy et al., 2005). Thus, mutations altering the

glycocalyx and more generally cell-environment interactions may have stronger effects than core regulatory proteins.

Materials and methods

Strains and media

Phased Outbred Lines (POLs) were derived from a two way cross between a North American (NA) oak tree strain (YPS128) and a West African (WA) palm wine strain (DBVPG6044) (Liti & Carter et al., 2009) (see details below). Reciprocal hemizygoty (Steinmetz et al., 2002) strains were built in the NAXWA F1 hybrid background, using genetically tractable NA and WA haploids previously described (Cubillos et al, 2009). Allele swapping was made using the same NA and WA haploids, and in the laboratory strains BY4741 and BY4742. The set of natural strains used to estimate the size of *HPF1* and to perform the *HPF1* deletion were selected from The 1002 Yeast genome project (Peter & De Chiara et al., 2018) and were restricted to euploid diploids.

YPD (1% yeast extract, 2% peptones, 2% dextrose, 2% agar (MP Biomedicals)) was used to revive strains from glycerol stocks and to enable mating to generate diploid cells. Synthetic minimal (SDM) medium (2% dextrose, 0,675% yeast nitrogen bases (Formedium), pH buffered to 6.0 with 2.5M NaOH) was used to select for successful mating due to the requirement of auxotrophic complementation to grow in this media. Synthetic complete dextrose (SDC) media (2% dextrose, 0.675% yeast nitrogen base (Formedium), 0.088% complete amino acid supplement (Formedium), pH buffered to 6.0 with 2.5M

NaOH), calorie restricted (CR) media (SDC with 0,5% dextrose instead of 2%), and rapamycin (RM) media (SDC supplemented with 0,025µG/mL rapamycin (Sigma)) were used to perform CLS experiments. A summary of strains used in this study can be found in table S1.

Generation of phased outbred lines

Outbred segregants used to perform linkage analysis were derived from a two-way advanced intercross between the YPS128 North American (NA) strain and the DBVPG6044 West African (WA) strain (Liti & Carter et al., 2009). Both ancestor strains were made heterothallic and engineered with different selectable markers at the same locus (Cubillos et al, 2009). They were then crossed into a F1 hybrid and forced going through 12 rounds of sexual cycles (Parts et al, 2011). From this pool, 86 F12 haploid segregants of each mating type were isolated, sequenced and their genotype was inferred using a set of 52466 markers (Illingworth et al., 2013). Phased Outbred Lines (POLs) were obtained similarly to what is described here (Hallin & Märtens et al., 2016) with some modifications. A set of unique diploids was generated by randomly crossing 80 sequenced F12 segregants of each mating type. For this, haploid strains were arrayed in 96 well plates and manually mixed with each other in new 96 well plates containing 200µL of YPD media. Cells were incubated overnight at 30°C without shaking before being diluted 1:100 in 200µL of SDM media and incubated overnight again at 30°C, still in 96 well plates. Dilution in SDM was repeated 3 times. Due to the presence at the same locus of a different selectable marker for each mating type, only cells that

successfully mated are prototrophic and grow in minimal media (3 rounds of selection). In total, 1056 unique diploids with phased genomes were generated and arrayed in 12 different 96 well plates. In addition, each plate included 8 internal controls used for the phenotypic normalisation (3 times ancestral diploid parents NAXNA and WAXWA, and twice the F1 hybrid NAXWA).

The linkage analysis method has been explained previously in (Hallin & Märtens et al., 2016). Shortly, to map QTLs in the POLs, the deviation of the POL from the estimated mean value of the two parents was used in order to correct for population structure issues (Hallin & Märtens et al., 2016). The mapping was done using the R/qtl package in R (Broman et al., 2003), using the marker regression method in the scanone function. Significance thresholds were calculated with 1,000 permutations and confidence intervals for the peaks were calculated using a 1.8-LOD drop using the lodint function in R/qtl.

Strain genetic engineering (reciprocal hemizygosity, allele swapping and deletions)

Yeast transformations were performed using the lithium acetate (LiAc) protocol (Cubillos et al., 2009). All strain constructions were validated by PCR. Crosses were made in YPD and diploids were selected on synthetic minimal medium plates. To build reciprocal hemizygote strains (Steinmetz et al., 2002), the gene of interest was first deleted for both mating types in haploid WA and NA strains (MATa; ura3::KanMX; ho::HygMX or MATa; ura3::KanMX; ho::HygMX; lys2::URA3) (Cubillos et al., 2009) by homologous recombination with a NatMX4

cassette (PCR amplified from pRED plasmid) and selected on synthetic medium supplemented with 100mG/L nourseothricin sulfate (Goldbio). Strains were then crossed with each other to generate the desired constructions. *HPF1* allele swapping was performed in two steps. First, part of *HPF1* (promoter, Nterm repeats, Cterm repeats, or internal part (Fig. 2)) were deleted in BY4741 (MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0), BY4742 (MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0) or in haploid WA and NA (MATa; ura3::KanMX; ho::HygMX) using homologous recombination with a URA3 cassette (PCR amplified from pRS316 plasmid) and selected on synthetic medium deprived of uracil. Then, the different *HPF1* parts were PCR amplified from the desired alleles and swapped back in the appropriate strains using homologous recombination (targeting consensus unique sequences for recombination) and selected on 5-FOA 1G/L (Sigma). Strains obtained were then crossed with *HPF1*::NatMX4 haploids to generate the indicated diploids. The PCR amplification of *HPF1* was difficult (especially the WA allele) due to the internal repeats (Hommelsheim et al., 2012), and was performed with LongAmp (NEB) or Platinum Superfi (Invitrogen) DNA polymerases. Our attempts to amplify and swap the whole gene were unsuccessful. Total deletion of *HPF1* in natural strains was performed in two steps. First, one copy of the gene was deleted by homologous recombination with a NatMX4 cassette, then, the second copy was deleted similarly using this time a KanMX4 cassette (amplified from pFA6a-KanMX4 plasmid).

Chronological lifespan assay

Cells grown overnight in SDC were diluted (1:100) in 200 μ L of either fresh SDC, or CR, or RM in a 96 well plate sealed with aluminium foil to prevent evaporation and incubated at 30°C for the whole duration of the experiment. Aging time points started 72 hours post inoculation at saturation of the culture. When CLS was performed in water, after 72 hours of growth in SDC, cells were centrifuged and washed 3 times before being resuspended in the same volume of water. When CLS was performed with spermidine, the compound was directly added in SDC, at concentration of 5mM.

Cell viability was determined by flow cytometry based on the uptake of the fluorescent molecules propidium iodide (PI) and YO-PRO-1 iodide (YP). At each time point, 5 μ L of cells were transferred in 100 μ L of staining solution (Phosphate Buffer Saline + 3 μ M propidium iodide (Sigma) + 200nM YP (Invitrogen)) in a 96 well plate and incubated 5min in the dark at 30°C. The samples were then analysed on a FACS-Calibur flow cytometer (Becton Dickinson) using a High Throughput Sampler (Becton Dickinson) device to process 96 well plates and detecting fluorescence with FL-1 (YP) and FL-3 (PI) channels. Propidium iodide and YO-PRO-1 are membrane-impermeable nucleic acid binding molecules that can only enter into dead cells whose cellular membrane is impaired. Therefore, non-fluorescent cells were counted as viable, whereas the bright ones were considered dead. Propidium iodide was previously shown to be a valuable choice for quick and reliable CLS assay (Ocampo & Barrientos, 2011). YO-PRO-1, unlike PI, is able to penetrate apoptotic cells, which are

encountered, along with necrotic cells, during the course of chronological aging (Herker et al., 2004, Wlodkowic et al., 2009). Experiments were run at least in triplicates, except for the POLs phenotyping, which were run in single replicate. To determine the final survival value of POLs, the viability percentage obtained was normalized using internal plate control strains. Each value was first normalised with the average value of control strains for every plate. Then, the normalised viability value obtained for each strain was multiplied by the global average value of all control strains of all plates.

Quantitative Real-Time PCR

Cells grown overnight in SDC were diluted 1:100 in fresh SDC or fresh SDC supplemented with Rapamycin (0.025 μ G/mL) in a 96 well plate and incubated either 7 hours or 10 days at 30°C before RNA was extracted. To perform RNA extraction, cells were first incubated in digestion buffer for 30 minutes at 30°C (sorbitol 2M, EDTA 10mM, Zymolyase 1mG/mL (Euromedex)), then, lysing, binding, DNase treatment and washing were performed according to the manufacturer's protocol (PureLink RNA mini kit, Invitrogen). Reverse transcription was done with a High-Capacity RNA-to-cDNA kit (Applied Biosystems) using 1 μ G of total RNA. Quantitative real-time PCR was performed with the FastStart Universal SYBR Green Master kit (Sigma) and run in a StepOnePlus (Life Technologies) cycler using a 1:50 cDNA dilution from the stock. We used primers 1038/1039 to monitor *HPF1* expression, primers 1281/1282 for *MXR1*, primers 867/868 for *ACT1*. All primers used are listed in table S8. *HPF1*

primers were manually designed in the internal region of the ORF, avoiding any repeated sequence and any polymorphisms between WA and NA alleles. Data were normalized with the constitutively expressed gene *ACT1*.

Spotting assay

Cells were incubated overnight in SDC before being diluted 1:100 in fresh SDC, CR, or RM as indicated, in 96 well plates. Cells were then incubated 7 hours (exponential phase), or longer at 30°C. At every time point, 5µL were collected from the cultures, serially diluted in water, and 5µL were spotted on the indicated type of media. Plates were left either 24 or 48 hours at 30°C and pictures were taken with a Epson Perfection V330 scanner. Peroxide hydrogen plates were made and used freshly with a final concentration of either 1.5mM or 2.0mM H₂O₂.

Growth rate

Growth rates were determined on solid agar plates based on colony formation speed, using the Scan-o-matic framework (Zackrisson et al., 2016). Four technical replicates and two biological replicates were run for every strain in 97 different environments. All results and conditions can be found in table S4.

RNAseq

We used the Kapa Biosystems hyperplus kit with Riboerase as per user's instructions at 500nG input. RNA integrity was assessed on an Agilent Bioanalyzer using the RNA pico kit to determine RIN scores. Library quality was assessed using Agilent Bioanalyzer using the DNA 1000 Kit. Libraries were quantified

using Kapa Biosystems library quantification kit. The libraries were normalized, pooled and loaded onto a NextSeq 500/550 High Output v2 kit (300 cycles) flow cell and sequenced on Next seq 500 instrument.

We performed transcript-level abundance quantification by pseudo-aligning RNA-seq reads to the coding sequences of SGD yeast reference gene set using Kallisto (v0.44.0) (Bray et al., 2016). In this way, we obtained the Transcripts Per Kilobase Million (TPM) value for each gene in each sample as its normalized expression level, which is directly comparable both among different genes and among different samples. Sleuth (0.30.0) (Pimentel et al., 2017) was further used to assess the statistical difference of the same gene between different samples by decoupling biological differences from experimental noise. False discovery rate (FDR) adjustment (Benjamini & Hochberg, 1995) was further applied for multiple test correction.

HPF1 length estimation by PCR

Amplification was performed with Platinum SuperFi polymerase (Invitrogen), according to manufacturer's instructions. PCR reactions were initiated with a 30sec 98°C denaturation step, followed by 35 amplification cycles (98°C for 10 seconds, 57°C for 10 seconds, 72°C for 6 minutes) and a final extension step at 72° for 10 minutes.

Due to intragenic repeats within *HPF1*, it was impossible to use the available short read sequencing data to determine *HPF1* size. We performed the *HPF1* amplification using the primers designed based on the WA-*HPF1* sequence (primers 857/975, see table S8), and retained only strains

producing a clear single band. Band size was estimated using the software GelAnalyzer.

Unfolded protein response (UPR)

The transcription factor *HAC1* is a master regulator of the UPR. Its mRNA contains an intron inactivating the protein until *IRE1* splices it upon proteostatic stress. To monitor UPR activation we have quantified the amount of spliced and unspliced *HAC1* by RT-qPCR (Navarro-Tapia et al., 2017). We used primers 1088/1099 to amplify both *HAC1* forms, and primers 1089/1098 to only amplify the unspliced form. Unspliced *HAC1* amount was then divided by total *HAC1* amount. All primers are listed in table S8.

Table S6. Length of *HPF1* determined by PCR among 365 *S. cerevisiae* natural isolates (Peter & De Chiara et al., 2018).

Table S7. DNA sequences of *FLO11* and *HPF1* ORFs.

Table S8. List of the primers used in this study.

Supplemental tables

(not provided)

Table S1. List of the strains used in this study.

Table S2. CLS of the 1056 POLs in SDC, RM and CR after 7, 21 and 35 days of aging.

Table S3. List of the QTLs found in this study.

Table S4. Growth rate of *HPF1* and *FLO11* reciprocal hemizygous strains in 97 different environments, measured by the Scan-O-Matic platform (Zackrisson et al., 2016).

Table S5. RNA-seq data obtained for *HPF1* reciprocal hemizygous strains either during exponential growth or after 7 days of aging in SDC and RM.

Supplemental figures

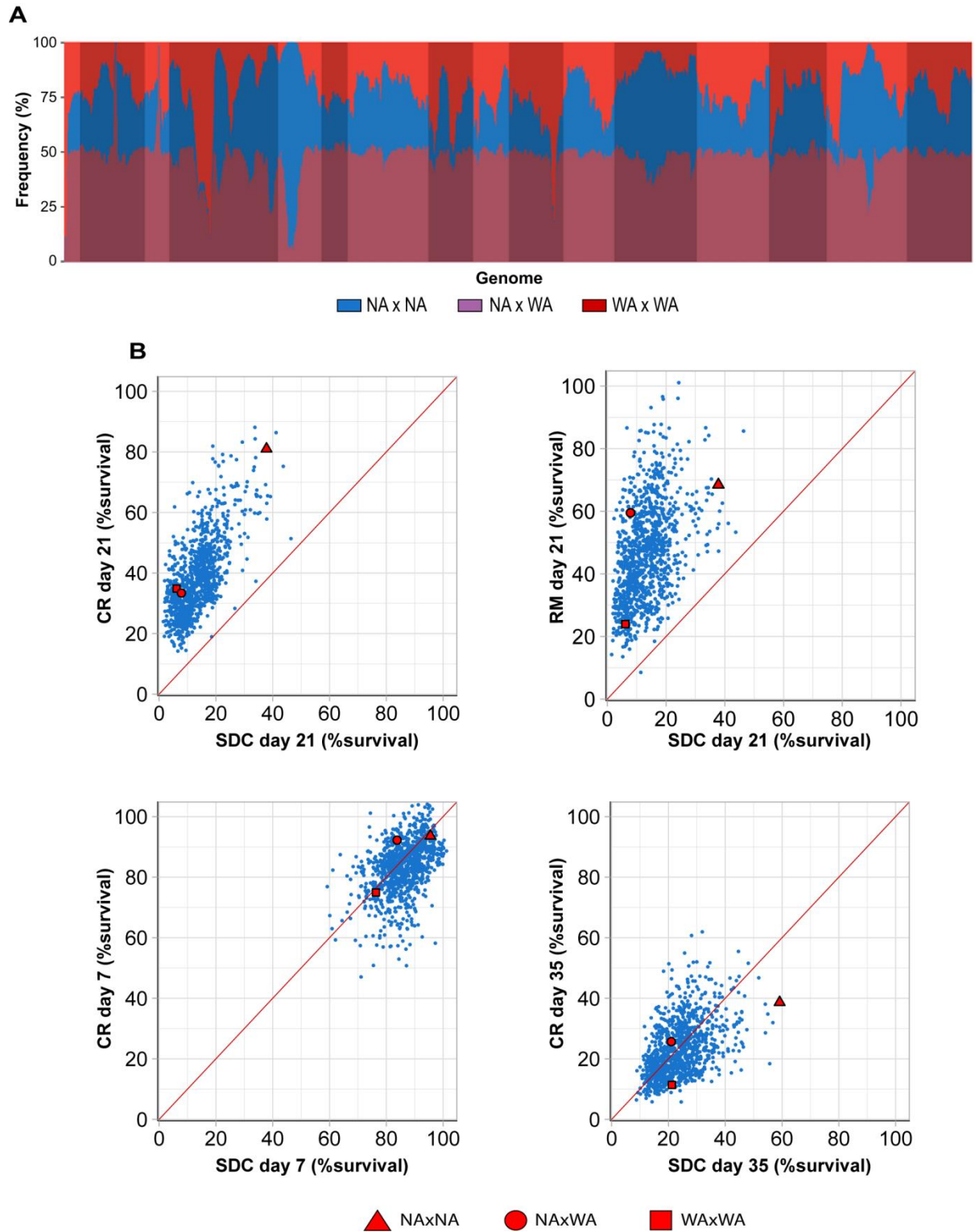


Figure S1. Segregants genotype distribution and chronological lifespan.

(A) Genome wide allele frequencies of homozygous and heterozygous sites across 52466 markers within the 1056 POLs.

(B) Viability correlation between the 1056 segregants in different conditions.

- RESULTS -

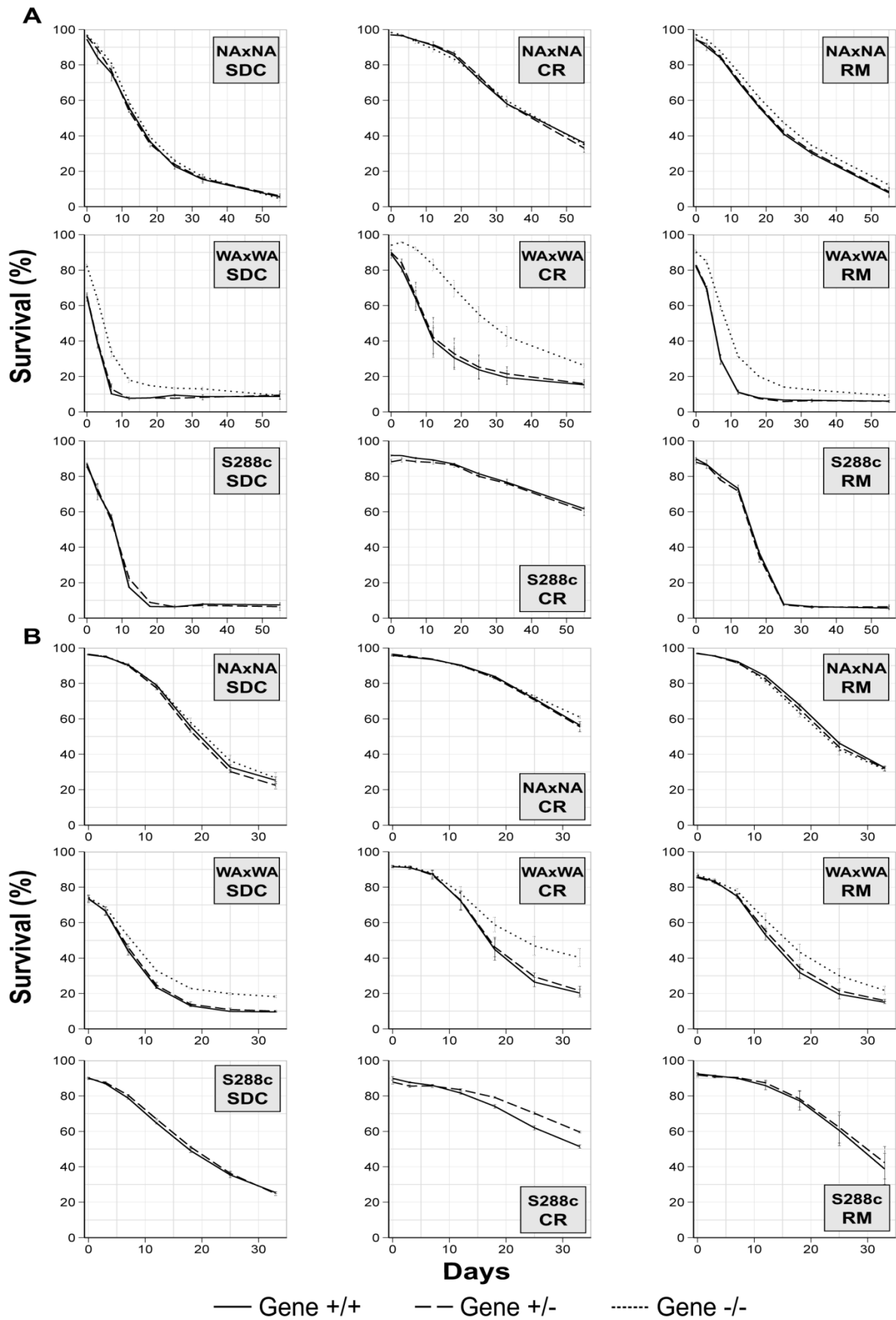


Figure S2. *HPF1* and *FLO11* deletions in homozygote parental and S288C strains.

Name of the genes and environments are indicated in grey boxes. Since S288C is haploid, dashed lines correspond to full deletion for this strain.

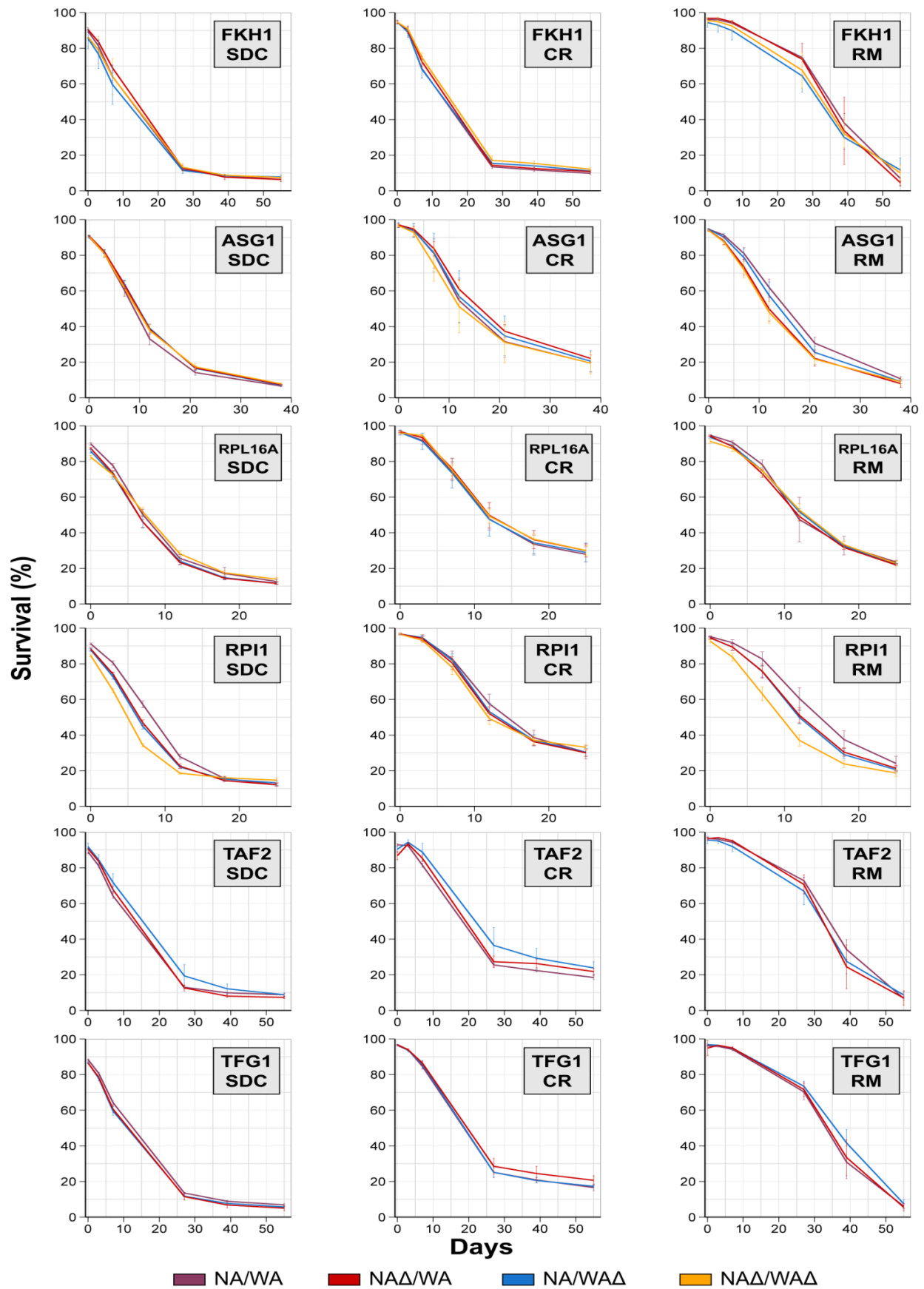


Figure S3. Additional candidate genes screening by reciprocal hemizyosity.

Reciprocal hemizyosity analysis of multiple candidate genes mapped with the linkage analysis. The genes *FKH1*, *ASG1*, *RPL16A* and *RPI1* were associated with the QTL localized on chrIX-101954, while the genes *TAF2* and *TFG1* were associated with the QTLs on chrIII-206100 and chrVII-870475, respectively. Name of the genes and environments are indicated in the grey boxes.

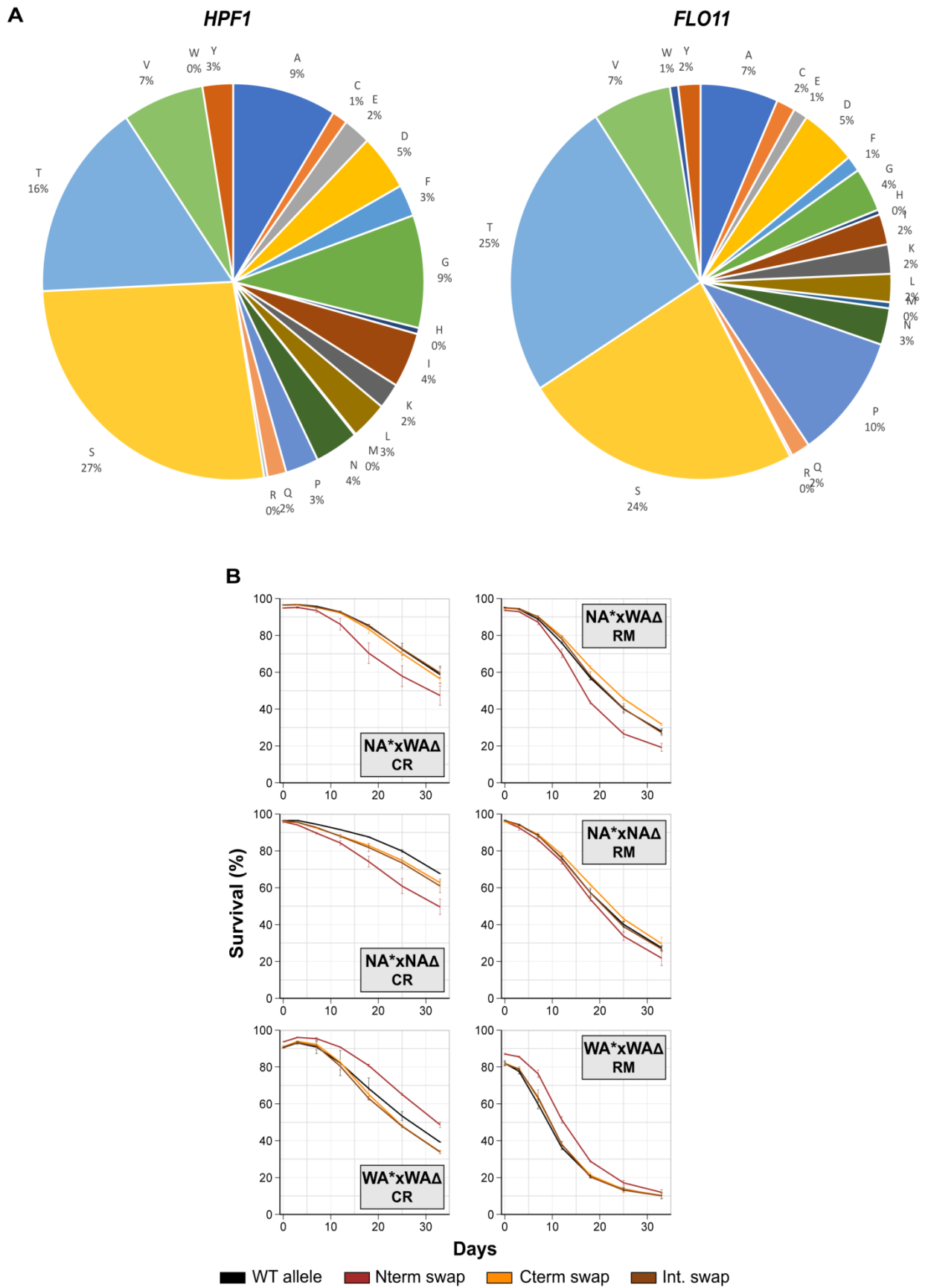


Figure S4. Amino acid composition of *HPF1* and *FLO11* and additional allele swapping results.

(A) Amino acid composition of *HPF1* and *FLO11*.

(B) Allele swapping of different *HPF1* subparts effect on CLS (construct are as reported in figure 4B).

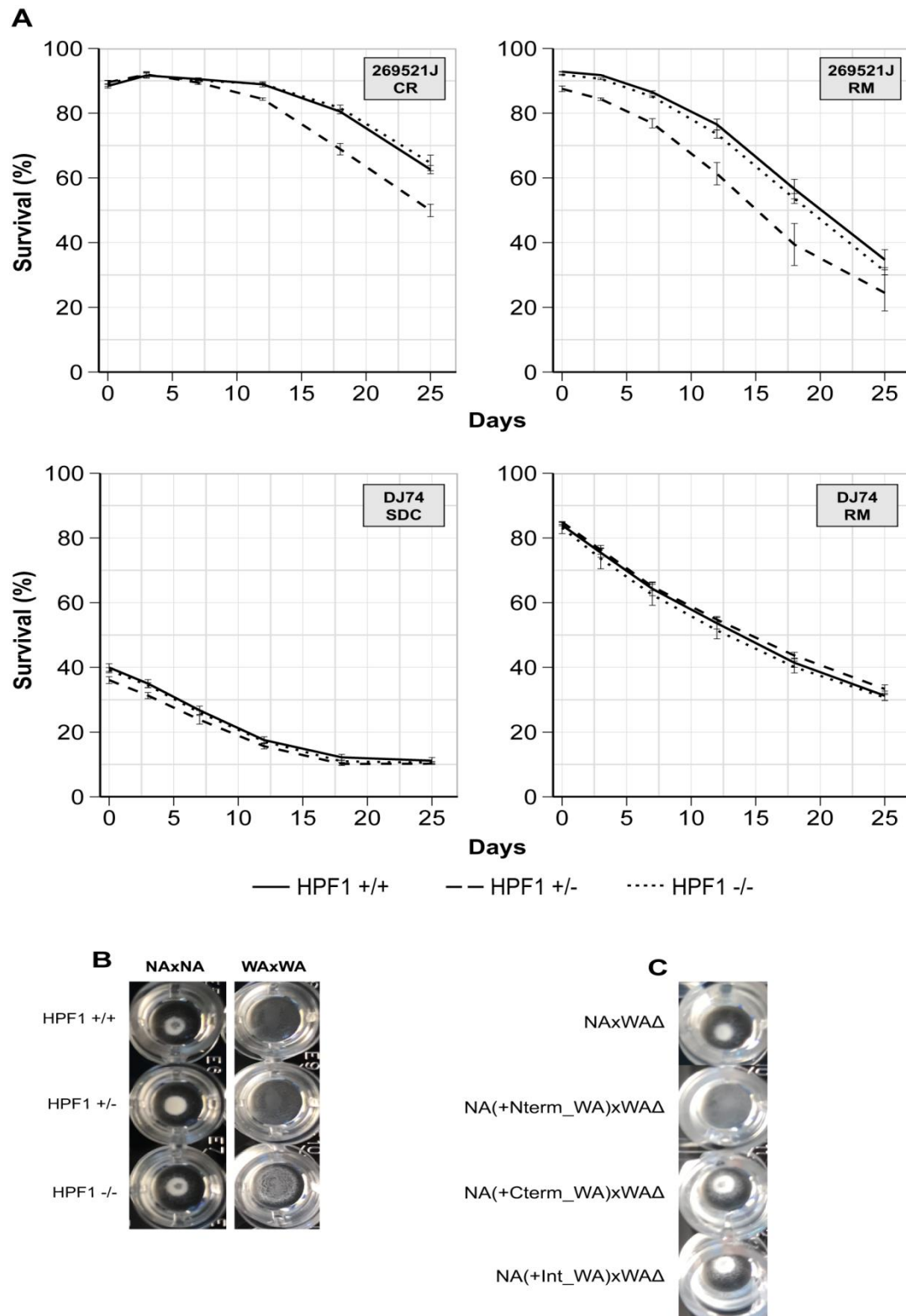


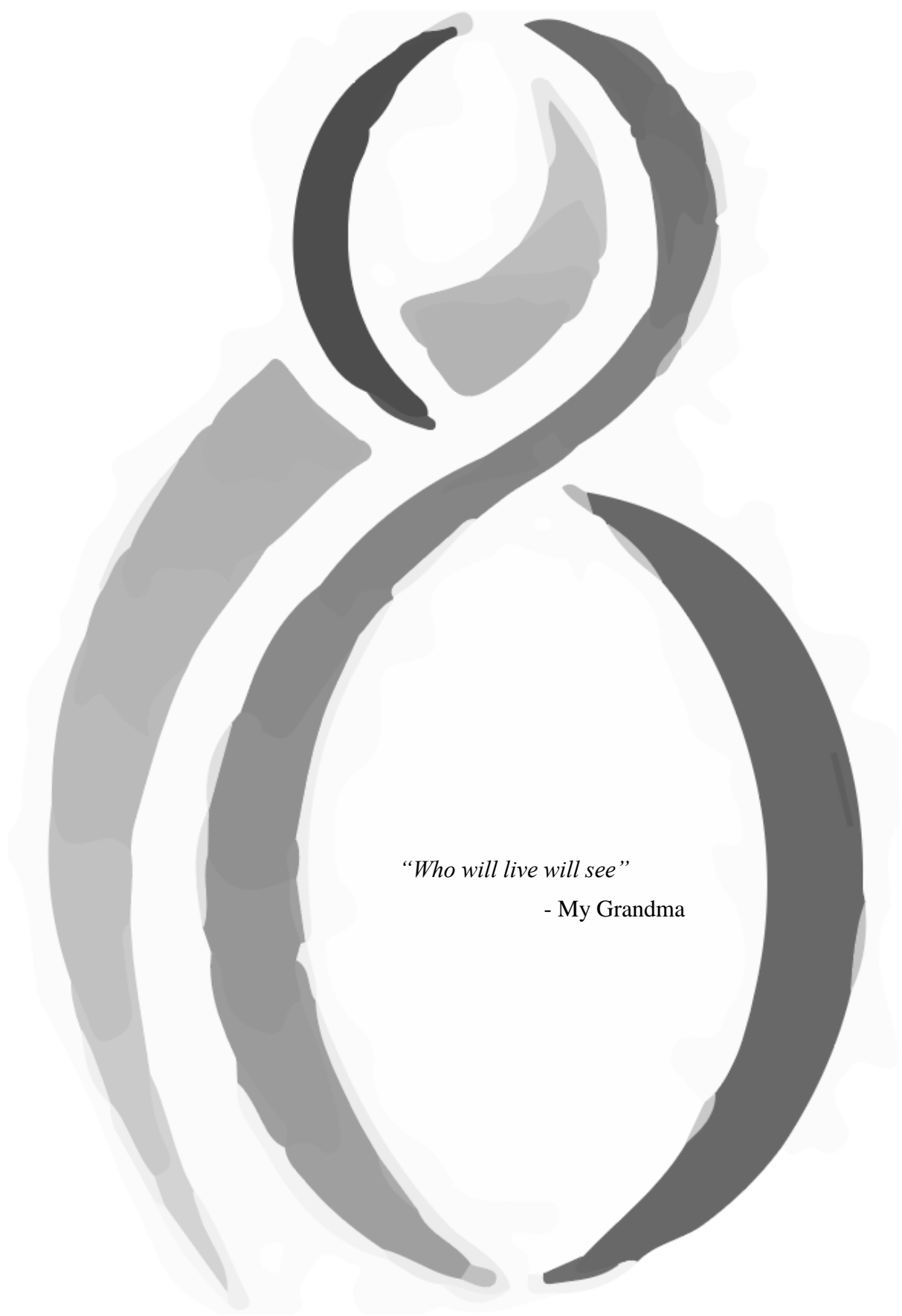
Figure S5. CLS of 269521J and DJ74 and additional flotation profiles.

(A) CLS of the natural strains 269521J and DJ74 with or without *HPF1* deletion. Environmental condition is indicated in the grey boxes.

(B) Flotation profile of NA and WA homozygotes with or without *HPF1* deletion.

(C) Flotation profile of the NAxWAΔ F1 hybrid deleted for the WA allele of *HPF1*. The remaining NA allele was either unchanged, or swapped with WA_*HPF1* Nterminal repeats (Nterm), Cterminal repeats (Cterm), or with the internal unique sequence in between repeat blocks (Int), similarly to what is described in figure 4B.

CONCLUSION



“Who will live will see”

- My Grandma

Chapter 7

Discussion & Perspectives

I recently attended to a conference focused on aging and associated diseases, with an audience both involved in fundamental and more translational research. During the breaks, I met two different researchers who expressed a certain surprise, tinted with a bit of scepticism, when I told them I was studying aging with yeasts. One of them actually politely asked if I was planning to switch to a “real” aging model for my possible post-doctoral training. While I tried to bravely defend my model system as a father would defend his children, it proved to be rather difficult to praise the strengths of this unicellular eukaryote to a more global audience.

Throughout this manuscript, I highlighted the power of *S. cerevisiae* to unravel the mechanisms of aging. Indeed, yeasts feature unrivalled genetic tractability and cost-efficiency, while being fairly conserved with higher eukaryotes and experiencing most of the aging hallmarks. Nevertheless, as good parents should do, I also have to keep a critic eye on my beloved model organism.

In this concluding chapter, I will review the strengths and weaknesses of this work along with the caveats of yeast aging.



Future perspectives. In Kubrick’s *Space Odyssey*, Knowledge came from mysterious extraterrestrial monoliths. This time will it come from budding microorganisms from the depths of beer?

A) About this work and forward genetics

What I like the most about this PhD project is its openness. Unlike reverse genetics, forward genetics is not based on any initial a priori. The overall idea is simply to pick up natural individuals and to try to explain what are the genetic factors driving their phenotypic diversity. In an era shifting toward personalised medicine, understanding the mechanisms of phenotypic variation constitutes a major challenge. In this regard, I think this PhD proposal met its goals. In chapter 5, I described one of the most comprehensive view of aging variation at the species scale, while in chapter 6, I further dissected the genetic and environmental bases of this diversity. Indeed, in the first project, data reveal an outstanding phenotypic variability among *S. cerevisiae* isolates, strongly subjected to environmental cues. In addition, candidate gene approach and GWAS have identified multiple causative genes regulating sporulation and CLS. In the second project, high resolution linkage analysis using outbred segregants have contributed to map 30 unique QTLs either conserved in time and across conditions, or very specific. The genes *FLO11* and *HPF1*, unknown to regulate CLS so far, were subsequently identified from the two largest peaks. Interestingly, both genes contain intragenic tandem repeats and become detrimental only when those repeats get expanded, revealing a new mode of aging regulation.

Nevertheless, the work accomplished remains primarily descriptive and will require more in-depth investigation in the

long run. The amount of data collected in the first study is gigantic, and consequently, is very complicated to handle. The candidate genes identified have not been experimentally validated and mechanistically investigated. Efforts must be prioritised intelligently, since validating each of them would constitute a tremendous task, although recent developments in genome editing enable to precisely engineer thousands of variants (Roy et al., 2018). In addition, the contribution of each causative variant, at the population level, is generally weak. In fact, the phenotype of each isolate results mainly from a myriad of minor effects. Also, because of epistasis, causative alleles might have a significative effect only in a given background and in a given condition. Surprisingly, the candidate approach failed to map loss of function in genes referenced as strong regulators of CLS such as *TOR1*, *SCH9* or *RIM15*, although the deletion of any of these genes substantially impact the lifespan of laboratory strains. This observation can be explained by 2 different scenarios: first, it is possible that among the 1000 natural isolates used in this study none or few of them harbour a deleterious mutation in these genes, second, deletion of these genes have no effect on CLS in other strain backgrounds. While the latter possibility cannot be ruled out, it seems the former option is the proper explanation. We know for instance the existence of a *RIM15* deleterious mutation present in some Sake strains (Jung et al., 2018). However, we were unable to map this polymorphism due to its rarity within the population. This absence of loss of function in key regulatory genes is actually intriguing. Since most of the genes promoting lifespan are

antagonistic with growth (Delaney et al., 2011), it seems plausible that genes like *SCH9* and *TOR1* were evolutionary protected. Conversely, a functional *RIM15* gene may be required to sustain in natural habitats because of its essential role in triggering sporulation and quiescence. Thus, besides extreme variability in yeast lifespans, it seems there is no major driver of CLS.

Interestingly though, one of the strongest candidates we identified from the GWAS corresponds to the gene *HPF1* which was also identified in the linkage analysis. This gene is extremely polymorphic because of the inherent instability of DNA tandem repeats. Consequently, it is not surprising that allelic variations in this gene are frequent within the population. Moreover, it is now established that dynamic fluctuation in tandem repeat size are contributing to rapid evolution and adaptation (Gemayel et al., 2015). Hence, one of the strongest aging modulators species-wide is not a gene involved in nutrient sensing or in stress response, but a gene encoding a cell wall protein which alters cell-environment interactions. In the second study, I started to unravel the underlying mechanism of *HPF1* and it seems clear that higher oxygen exposition during growth is responsible for CLS shortening. However, this oxygen exposition is not merely the result of damage accumulation. Expression profiling reveals that several genes involved in methionine and lipid biosynthesis are considerably altered. It is appealing to imagine a scenario in correlation with the process of methionine restriction, although for the moment there is no direct experimental evidence. Therefore, the

mechanism proposed in chapter 6 remains speculative and further work is needed to understand the underlying mechanisms of *HPF1*.

Altogether, understanding the genetic and environmental bases of complex traits remains an unprecedented challenge. Both forward and reverse genetics have contributed to identify hundreds of genes implicated in aging regulation. Nevertheless, our global understanding of the trait architecture remains elusive. Reverse genetics have been more successful than forward genetics in this quest. By taking advantage of the gene deletion collection, high throughput screens have led to the identification of 77% of the genes currently annotated to regulate CLS. By comparison, forward genetics in yeast CLS are limited to 2 published papers (Kwan et al., 2011; Jung et al., 2018) which, in the end, only identify a single new gene regulating CLS (the gene *SER1* involved in serine biosynthesis). At first glance, the contribution of forward genetics seems limited compared to the power of high throughput reverse genetics. In addition, forward genetics requires a certain effort to obtain a recombined natural or artificial population that subsequently needs to be phenotyped. Yeast forward genetics has not been extensively studied presumably because of these reasons. In fact, the strength of forward genetics mainly lies in the identification of more subtle effects, such as the gain of functions or change in expression. In my opinion, reverse and forward genetics are synergistic, and both approaches will be required to fully unravel the genetic basis of complex traits. I think that reverse genetics has been decisive in gauging the effect and function of each gene,

but once this task is done, it struggles to further understand gene-gene and gene-environment interactions. Moreover, reverse genetics experiments were quasi exclusively performed in the S288C background that appears as a phenotypic outlier compared to the rest of the species (Warringer et al., 2011). It is at this point that forward genetics is required to exploit the full potential of natural diversity and to specifically understand the nature of phenotypic variation in different genetic backgrounds.

B) About *S. cerevisiae* as an aging model

B1. RLS, budding considerations

I have a very limited personal experience with this approach that I tried only once by curiosity; unless it was masochism. From a technical point of view, the main limitation of RLS is the age-old microdissection technic that prevents large-scale experiments, which is one of the main strengths of the model. Screening the whole gene deletion collection has been a hectic task (McCormick et al., 2015), and this limitation does not encourage exploring multiple alternative environments. Fortunately, this problem is being addressed progressively thanks to alternative strategies such as microfluidics and the mother enrichment program (Lee et al., 2012; Lindstrom & Gottschling, 2009). Nevertheless, their scalability and flexibility are still a long way away from equalling the options offered in CLS studies.

From a biological point of view, RLS is the most popular of the two yeast models. It tries to directly understand why a simple unicellular organism cannot divide endlessly in optimal growth conditions. I think that it is indeed a very good paradigm to study the fundamental processes of aging and to identify the factors that prevent a cell to preserve its homeostasis with time. However, there are two caveats that should be taken into consideration.

First, RLS is based exclusively on cell division, and in my opinion, this is both its strength and its weakness. A certain number of interventions may prevent cell division without causing death. This fact may explain several discrepancies existing between RLS and CLS, especially regarding the role of oxidative stress (Fabrizio et al., 2004). In fact, while the replicative capacity of the mother cell is paralleled with the replicative capacity of somatic mammalian tissues, it is frequently forgotten that unlike ex-vivo cultivated fibroblasts, yeast mother cell are the whole organism. In this regard, I consider mother cells as entire independent individuals, equivalent to a single worm or a single human. Since human lifespan is based on the time elapsed between birth and cerebral death, and not on reproductive capacity, by extension, I think yeasts should be seen with the same consideration. For instance, as an extreme example of trade-off between growth and maintenance, if one manages to engineer a yeast strain which cannot divide while maintaining active metabolism and cellular homeostasis, should this yeast, with practically null RLS, be considered short-living or dead? Let's also consider a more likely example: two strains which have the same RLS but divide at two

different paces. One strain would reach on average 20 divisions in approximately 40 hours before dying, while the other strain would perform these 20 divisions in 30 hours before dying. In terms of pure RLS they would be considered equal, although in terms of life duration one strain lives 33% longer than the other one. Therefore, the question is: which parameter should be taken into consideration in regard with aging studies? The replicative capacity, or the life duration? In the future it may be needed to introduce the concept of chronological lifespan also for yeasts that are “replicatively aging”. This issue starts to be addressed in some studies that make the difference between reproductive potential, reproductive lifespan, post-reproductive lifespan, and total lifespan (Zadrag et al., 2008; Molon & Zadrag, 2016).

Second, because yeasts keep physically growing in addition to budding all along their life, it was proposed that cell size could reach a critical level and become the main limiting factor of RLS, known as the hypertrophy hypothesis. This hypothesis was tested with experiments using pheromone signals to promote cell growth without division, however, results were contradictory (Kennedy et al., 1994; Zadrag et al., 2005). As of today, cell size as an aging factor is still unclear. Some experiments show that daughter size at birth correlates with its lifespan, while some other studies show that the final size at death is quite variable from one cell to another (Yang et al., 2011; Lee et al., 2012). Nevertheless, this theory is worthy of thought. After all, yeasts probably never reach replicatively old age in the wild since they saturate their environments in few

generations in virtue of exponential growth. Therefore, reaching abnormal sizes is probably something that has not been tackled by evolution. Following this idea, aging appears as a continuum of indefinite growth which leads to the loss of cellular homeostasis. Although humans are considered to have a finite growth and cell division is limited by the available physical space, in practice, I think it is not implausible to imagine that some cell types, including stem cells could somehow keep growing a bit throughout life and progressively lose homeostasis.

B2. CLS, complex (t)issues

I started to work on CLS a long time ago, while I was an undergraduate student. At that time I was using the traditional colony forming unit (CFU) methodology to monitor CLS. Even though this technic is much less tedious than the RLS microdissection, it is still quite demanding and tedious. Moreover, the CFU technic suffers a certain inaccuracy due to a high stochasticity in the number of cells that are being serially diluted and spotted on agar plates. Since my project required accurate high throughput screening to perform linkage analysis, it became apparent that another technical approach was needed. I took the advantage of the high throughput cytometry facility available in the institute to develop a protocol based on fluorescent vital stainings (Ocampo & Barrientos, 2011) and to adjust it to 96 well plates. From this point, almost all the CLS experiments performed in this project were done according to this protocol. While this technic is as good as the others, it is

important to stress how much any methodology used impacts the results.

During the past years, I have had the opportunity to perform CLS in a multitude of conditions, including different media kept saturated or substituted with water, different containers or different temperatures. The result is that, every single methodological difference can drastically affect CLS. The lifespan of a given strain is completely different when grown in YPD, SDC, or with various carbon and nitrogen sources. Moreover, the container used to perform the experiment is of key importance. I have noticed that lifespan in 96 well plates is much longer compared to flasks. More surprisingly, lifespan in closed shaken tubes is even shorter than it is in flasks. The main reason for these variations lies probably in the exposure to different oxygen rates. For instance, deletion of the superoxide dismutase *SOD2* is only detrimental when cells are exposed to high doses of oxygen in shaking flasks (Fabrizio et al., 2003). In addition to the experimental conditions, the technic chosen to monitor viability also matters. Although the use of fluorescent vital stainings correlates rather well with CFU, some discrepancies were observed depending on the medium used during the experiment (Pereira & Saraiva, 2013). Since vital stainings are based on cell membrane permeability, while CFU is based on the ability to resume growth, it is fundamentally not surprising that both methods lead to distinct results. This being said, I do not think that one method is ideologically better than the other. It is more a philosophical question about whether senescent cells that

cannot divide anymore should be considered as dead or viable.

In conclusion, I think that all the different protocols are valuable. The problem mainly stems from the lack of consensus between all the labs working on CLS. Pretty much every team uses a distinct protocol, whether it is a different media, or a different incubation procedure. In the end, it has become extremely complicated to compare and analyse the various data from the literature since most of the time they use different protocols. The results of the second project, described in chapter 6, constitute a quite good example of this issue. The extended form of the protein *HPF1* is only detrimental when cells are grown in 96 well plates because it promotes floatation and high oxygen exposure. If this project was performed in flasks, this *HPF1* would not have been mapped as a QTL. Similarly, the CLS of the yeast deletion collection has been screened at least 6 times by different teams using different experimental strategies. Most likely, because of technical variations, there is little overlap in the candidate genes identified in these studies (Smith et al., 2016).

Besides technical aspects, there are a certain amount of biological concerns that are worthy of discussion. At the beginning, cells entering stationary phase were considered to be relatively synchronized and quiescent. However, when I got started with CLS, I remember I was often surprised to obtain two-phase survival curves with an initial rapid loss of viability, followed by a sort of plateau made of long living cells that could sustain for months. Given that CLS is determined with millions of clones growing in the exact same environment, I did not

understand how such heterogeneity could occur. This was until I read the paper from Allen and colleagues (Allen et al., 2006). Thanks to their work, it is clear that cells in stationary phase are extremely heterogeneous and that they differentiate either in long-living quiescent, or short-living non-quiescent cells when nutrients are exhausted. While this mechanism was observed for classic lab strains, it is also true for natural isolates. In fact yeasts have mainly evolved in harsh restrictive environments throughout their life history. Thus, besides quiescence, they have developed alternative strategies to survive extended starvation periods. Depending on the genetic background and the environment, yeasts can form spores, biofilms or pseudohyphae which will help to resist enzymatic digestion, survive external stressors or reach distant nutrients (Coluccio et al., 2008; Verstrepen & Klis, 2006). Altogether, through cellular differentiation, yeasts form primitive multi-cellular tissues aimed to cope with their surroundings. When determining the CLS of the strains derived from The 1002 Yeast Genomes Project, I have been facing all of these possible scenarios during experiments. In practice, there is nothing much I can do against it. I tried to withdraw from the assay cells that were obviously flocculating or sporulating, but I did not have much control regarding the content of what was in the well. While this phenomenon is particularly striking when working with natural strains, cell differentiation in stationary cultures is a global issue for any lab working on CLS. As of today, almost no lab, ours included, is quantifying the amount of quiescent cells during CLS. Thus, all the viability estimations obtained are the

result of an unknown mix of quiescent and non-quiescent cells dividing cells, and of possibly other cell types.

On the other hand, the choice of the medium must be cautiously considered. For the moment, the use of the rich media YPD is generally dismissed since it promotes periodic episodes of regrowth during stationary phase. Hence, the synthetic media SDC which maintains high metabolic rates and limits cell divisions is generally preferred over YPD (Fabrizio & Longo, 2007). However, the relevance of this media can be debated. It is well established that during growth in SDC the pH substantially drops and cells produce pro-longevity factors such as ethanol and acetic acid (Fabrizio et al., 2005; Burtner et al., 2009). It is still debated whether these factors accelerate aging or are merely toxic. Independently of the answer to this question, my personal feeling is that growth conditions in SDC prevent the entry into a proper quiescence state. Interestingly, strains that are prone to sporulate are able to initiate sporulation when kept in YPD or transferred to water. However, no sporulation is induced when cells are grown in SDC. Determining the CLS of pro-sporulating strains in water is actually tedious since a consequent fraction will turn into spores, while it is not a problem in SDC (see chapter 5). Although I have no proof, my assumption is that SDC may also block quiescence, similarly to sporulation.

In fact, my sentiment is that yeasts, which have evolved millions of years in harsh environments, have already fully tackled the problem of chronological survival. This is well exemplified with the sporulation process which generates immortal gametes. In my

opinion though, the quiescence program is as good as sporulation and can also lead to immortal quiescent cells when the proper conditions are encountered (probably not in SDC). This idea is fuelled by the observation that when cells are grown in non-fermentable carbon sources, or pre-grown in YPD before being switched to water they experience lengthy, if not endless, life durations. Especially when grown in 96 well plates and kept in water, after an initial drop in viability, the remaining cells can survive for more than 100 days without viability decline (unpublished data). If quiescent cells have not been considered immortal so far, it may be due to the existence of different quiescence stages ([Klosinska et al., 2011](#)) and to the fact that the most resilient ones are not easily visually distinguishable compared to spores.

Altogether, I am questioning what is the true nature of genes identified as CLS regulators. Do pro-longevity genes foster the entry into quiescence? Do they improve the survival of quiescent cells, or non-quiescent cells, or both? Do they promote resistance to external stresses and starvation? Or, do they promote alternative developmental programs? And, finally, which cells are supposed to mimic the best mammalian aging, the unstable short living non-quiescent cells, or the long-living and poorly active quiescent cells? Nevertheless, because the mechanisms of aging and quiescence strongly overlap with those of stress resistance, yeast CLS has contributed to and keeps contributing to the identification of fundamental cellular processes and genetic networks.

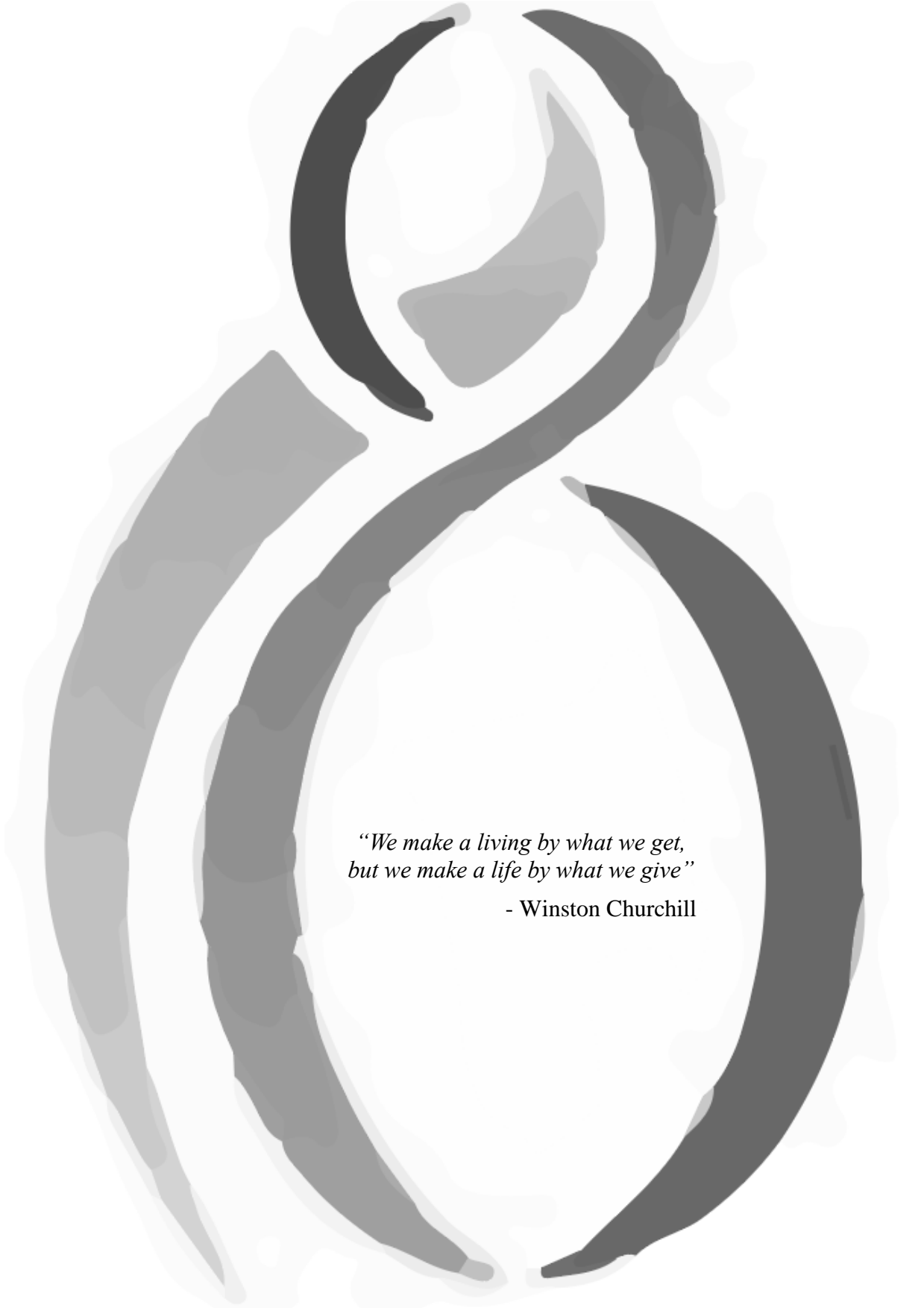
C) About the future of aging research

Aging is today one of the most active fields of investigation along with cancer research. Because of the impact of aging in the modern world and the upcoming challenges of human societies, aging research arouses a deep enthusiasm and receives a strong financial support. Substantial progresses were made during the past two decades regarding the identification of genetic pathways modulating aging and healthspan. Some scientists, like Craig Venter, estimate that the first human being who will live longer than a thousand years is already born. However, for the moment, the most promising short term interventions rely on pharmacological approaches. A certain number of drugs are currently undergoing clinical trials and will at some point enter into the market. Nevertheless, optimizing the dosage and the quality of these compounds will require time and efforts. Moreover, the real efficiency of these molecules is debatable. While pharmacological interventions were successful in extending the lifespan of model organisms, their effect have always been more contrasted in mammals. In the best case, drugs like spermidine or rapamycin barely improves mice lifespan by 10% in a sex and background dependent fashion.

Overall, I do not know how plastic human lifespan is. Worms and yeasts longevity can vary more than 10-fold in response to pharmacological treatment or genetic alterations. Conversely, humans do not experience drastic lifespan variations. Among a population rich of more than 7 billion individuals, human lifespan is rather

stable, progeria syndromes put aside. Human association studies have had only a mitigated success so far and have only identified two allelic variants of the genes *FOXO* and *APOE* that convincingly correlates with longevity. If human longevity could be substantially altered by simple genetic interventions, then we would expect the existence of a few individuals with extremely long lifespans, which is not the case despite the size of the human population. Another possibility is that mutations that could considerably extend human lifespan exert detrimental drawbacks on growth, development and reproduction. Thus, these mutations would have been cleared out by evolution, and similarly to yeasts, they might not arise in natural populations.

Even though we identify a couple of mutations that could double human lifespan, in practice it will be complicated to engineer them or to mimic their effect with pharmaceuticals. An alternative angle of aging research is focusing on regenerative medicine, including stem cell therapy, organ replacement or parabiosis. In the end, these approaches, that look extracted from science-fiction movies, may be the most promising interventions.



*“We make a living by what we get,
but we make a life by what we give”*

- Winston Churchill

Acknowledgements



Today's world has become extremely challenging and competitive, especially in Science. Everyone is striving to find its place. Nevertheless, life is mainly about being at the good place at the good moment, starting from birth. I think that everyone's career, ranging from singers to scientists, has at least a 50% chance component. Besides stochasticity, the main driver of personal and professional successes is for sure the environment to which I give a 40% influence. Finally, there is merely 10% remaining for genetics. Yes, Life does not have a strong heritability (if we put the social context aside). As I am concluding this manuscript, I am still uncertain regarding the extent of my genetic contribution, while on the other hand, I consider myself particularly lucky regarding the rest. Since I cannot acknowledge chance, I would like at least to render credits to all the Caesars who have contributed to this adventure.

I first met Gianni in October 2011. At this time, I was starting my Master's degree in the local university and I had to find a place to perform a scientific internship. While I was a totally unexperienced Master student barely speaking English, Gianni kindly gave me the opportunity to join the lab (well, the truth is that I was the only student applying!). Actually, I have to recognize that I almost signed up for the project without knowing what the meaning of the word "yeast" was. Since then Gianni has unswervingly supported me through all the impediments that one may encounter, ranging from the PhD application to the 4th year extension that he has been literally carrying. Besides his scientific support, he is an exceptional person, extremely open and caretaking. I would like to warmly thank him personally for his help and friendship throughout the past 7 years. I would certainly not have been able to complete this work otherwise, and probably would not even have started a PhD. I would also like to thank Gianni more generally, to remind us that we are all equal human beings and that a good leader is guiding friends and not managing collaborators.

Agnès is the only other French member of the team. Together we are leading the French Resistance against the ever-increasing Italian Mafia in the lab. Joke aside, the lab would not be the lab without her. She is flawlessly and meticulously organizing and taking care of everything. Her help these past years has definitely been very precious. Besides being a super lab manager, Agnès is a wonderfully positive and enthusiastic person, which is a quite remarkable achievement when you are surrounded all day long by grumpy PhD students! Thanks again for everything!

Of course I'm not forgetting Melania and Simone, my Pirates of Caribbean crew, with who I am sharing everyday pains and complains about this bloody PhD life! Thanks for being present, and sometimes patient. You are now the senior PhD students of the team (ie: the

next on the defense list, haha!). I leave you the responsibility to take care of the new upcoming generation!

I cannot write a paragraph for everyone, but I would like also to warmly thank all the members of the team that I did not quote yet (in the alphabetical order, so no one is jealous!): Agur, Jia-Xing, Jing, Lorenzo, Mateo. Thank you, you are making this group feeling like a family. It's been a real pleasure to be altogether.

I am also thinking about the previous members of the team. Francisco, who was the first member to join the team back in the past and who taught all the basics about yeasts. Anders, a.k.a. “Swedish Boy”, with you I am still travelling throughout Europe! And of course, Johan, my fellow PhD of the first generation, who became our first Doctor 100% Liti made. Lastly, I do not forget Matias and Jordi with who I had the chance to spend some great time!

I would also like to thank more globally everyone in the 8th floor. You all make this place colorful and living, in contrast with the greyness of our old tower!

More particularly, I would like to thank Eric Gilson who opened for me the gates of the IRCAN, and who always supported my work as well.

Aaron, my dear office neighbor since so many years, for being such a kind person, and for teaching me all the secrets of the qPCR, among others.

Julien, for his “bad” puns, but also for the discussions, the recommendations, and the reflections about experimental design, especially regarding cytometry!



Co-founders of the Liti team. Anders Bergström (left) discovering the power of yeast genetics, and Francisco Salinas (right) ready to join the Chilean football team. Johan Hallin (right background) meditating about his future career.

Marie-Jo, for sharing with me her precious experience and unravelling the mysteries of molecular cloning.

I would like to thank the sponsors which have brought this PhD to life (and also contributed to keep me alive in the meantime!). La Ligue Contre le Cancer, for the 3 first years, and la Fondation Recherche Médicale, for the 4th year. By extension, I am grateful to all the generous donators who are drawing on their own resources to give researchers the possibility to build a better world.

Jonas Warringer and Mikael Molin, for their enthusiastic external supervision along with all their ideas which have fueled this project.

I am also thanking the jury members, Angela Taddei, Paola Fabrizio, and Christian Braendle, for reviewing this work. I know how busy and precious time is, and I also know that revising a PhD is a demanding endeavor. I am glad and grateful you did embrace it.

Finally, a big thank goes to my family. Unlike many other students, I have had the chance to “play at home”. It has been undoubtedly a huge help. I am not even sure I would have succeeded otherwise. I have been literally baby-sited throughout this PhD, allowing me to allocate more time and energy to my work. They have tirelessly carried me through the storms. I owe them a lot. Even if the pill that makes people live over 1000 years old gets out tomorrow on the market, it won’t be enough time to pay back my debt. Thank you.



First star. The whole Liti team, looking for new Everest in Auron, March 2018.



"I know that I know nothing"

- Socrates

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