Title

High-throughput Screening Platform for Longevity Gene and Anti-aging Drug Discovery

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A High-throughput Screening Platform for Longevity Gene and Anti-aging Drug Discovery

by

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High-throughput Screening Platform for Longevity Gene and Anti-aging Drug Discovery

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ABSTRACT

Aging is a complex biological process that remains one of the greatest risk factors for numerous chronic diseases, including cancer, cardiovascular disorders, and neurodegenerative conditions. As global demographics shift towards an aging population, there is an urgent need to develop interventions that can delay the onset of age-related declines and promote healthy longevity. This thesis presents a groundbreaking high-throughput screening platform for identifying genetic and pharmacological interventions that extend lifespan in the model organism Saccharomyces cerevisiae (budding yeast). The foundation of this platform is the development of a novel genetic construct, the Daughter Arrest Program (DAP), which selectively arrests daughter cell division while allowing mother cells to continue dividing. By combining the DAP with innovative microfluidic devices and fluorescence-based assays, we have established a robust system for rapid and efficient measurement of yeast replicative lifespan. Leveraging this platform, we conducted comprehensive screenings of FDA-approved drug libraries and bioactive compound libraries, leading to the identification of several promising anti-aging compounds, including mTOR inhibitors, GSK-3 inhibitors, and topoisomerase inhibitors. Additionally, we performed genetic screenings using non-essential gene deletion mutants and the Decreased Abundance by mRNA Perturbation (DAmP) library, revealing the diverse roles of various genes in regulating aging processes, such as nutrient sensing, stress response, and cellular repair mechanisms. The findings from this research provide valuable insights into the genetic and molecular pathways underlying aging and longevity, paving the way for the development of targeted interventions to promote healthy aging.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Importance of Aging Research

Aging research has become increasingly crucial in recent years as global demographic shifts lead to a higher proportion of the population living into old age [United Nations, Department of Economic and Social Affairs, Population Division, 2019]. This shift presents profound public health, social, and economic challenges due to the rising prevalence of age-related diseases such as Alzheimer's, cardiovascular disorders, and cancer [Kennedy et al., 2014]. As life expectancy continues to increase, the burden of these diseases on healthcare systems and society as a whole is expected to grow substantially [Jaul and Barron, 2017]. Understanding the biological mechanisms that underlie the aging process is therefore essential not only for enhancing lifespan but also for improving healthspan, the period of life spent in good health [Kaeberlein, 2018]. By investigating the molecular pathways and cellular processes that contribute to aging and its associated diseases, scientists can develop targeted interventions that delay the onset of age-related decline and improve quality of life in later years [L'opez-Ot'ın et al., 2013]. Such interventions could include pharmacological treatments, dietary modifications, and lifestyle changes that promote healthy aging [Longo et al., 2015]. Moreover, insights gained from aging research may have broader implications for our understanding of biological systems and the fundamental mechanisms of life [Partridge et al., 2018]. As such, the field of aging research represents a critical area of scientific inquiry with the potential to transform public health and improve the lives of millions of people worldwide.

Yeast as a Model Organism to Study Aging

The budding yeast *Saccharomyces cerevisiae* has emerged as a foundational model organism in the study of aging due to its unique combination of advantages [Longo et al., 2012]. Firstly, this yeast species shares significant genetic similarity with humans, with about 30% of its genes having hu-

man homologs, making it highly relevant for translational studies [Botstein and Fink, 2011]. Many of these conserved genes are involved in fundamental cellular processes such as metabolism, DNA repair, and stress response, which play key roles in aging across diverse organisms [Janssens and Veenhoff, 2016]. Secondly, the genetic tractability of yeast allows researchers to easily manipulate its genome using well-established techniques such as gene deletion, overexpression, and mutagenesis [Duina et al., 2014]. This enables the systematic study of gene function and the identification of genetic pathways that modulate aging and longevity [Gershon and Gershon, 2000]. Thirdly, the short lifespan of yeast cells, which typically divide only 20-30 times before senescence, enables rapid experimental cycles and high-throughput screening of genetic and environmental interventions [Denoth Lippuner et al., 2014]. This accelerates the pace of aging research and facilitates the discovery of novel anti-aging strategies that can be subsequently tested in more complex organ- isms [Kaeberlein, 2010]. Finally, yeast offers a simple and inexpensive experimental system that is easily maintained in the laboratory, making it accessible to researchers worldwide [Karathia et al., 2011]. Taken together, these attributes have established yeast as a powerful model for elucidating the molecular basis of aging and identifying potential targets for therapeutic intervention.

Importance of Developing a High Throughput Screening Platform

The development of high throughput screening (HTS) platforms represents a transformative advance in the field of aging research, enabling the rapid and systematic evaluation of a vast array of genetic and chemical perturbations [Zimmermann et al., 2018]. Traditional approaches to studying aging have relied on low-throughput experiments that are time-consuming, labor-intensive, and limited in scope [Wasko and Kaeberlein, 2014]. In contrast, HTS platforms harness the power of automation, miniaturization, and parallel processing to test thousands of compounds or genetic

modifications simultaneously, vastly expanding the potential for discovery in the field of gerontology [Skwark et al., 2017]. For example, using HTS technology, researchers can quickly screen entire libraries of FDA-approved drugs, natural products, or synthetic compounds to identify molecules that unexpectedly delay aging or mitigate its effects in yeast models. These findings can then provide a basis for further testing in more complex organisms such as worms, flies, and mice, potentially leading to the development of new therapeutic strategies for age-related conditions in humans [Castillo-Quan et al., 2015]. HTS platforms can also be used to systematically explore the effects of genetic perturbations on aging, such as gene deletions or overexpression, providing insights into the molecular pathways that regulate longevity. Moreover, the high-throughput nature of these platforms enables the rapid generation of large datasets that can be mined using bioinformatic tools to uncover novel patterns and relationships in the biology of aging [Fabris et al., 2020]. As such, the development of HTS platforms for aging research represents a critical step towards accelerating the pace of discovery and translating basic science findings into clinical applications that can improve human health and longevity.

CHAPTER 1 METHOD DEVELOPMENT

Introduction

Aging research has witnessed significant advancements in recent years, with the development of novel technologies and methodologies to study the underlying mechanisms of the aging process. In this chapter, we present a groundbreaking high-throughput screening platform for measuring the replicative lifespan of yeast, a well-established model organism in aging research (**Figure 1.1**). This platform combines innovative genetic engineering techniques with state-of-the-art microfluidic device engineering to enable rapid and efficient analysis of multiple strains and drug treatments simultaneously. We have validated this technology using known longevity mutants and drugs and have applied it to perform preliminary screening, discovering new genes and drugs that extend yeast lifespan. Compared to previous studies, our system allows for high-throughput screening of small molecule drugs using lifespan as the direct readout, instead of relying on surrogate markers. We believe that this system will drastically accelerate the discovery of anti-aging drugs and conserved mechanisms of aging across species.

DAP Strain

The foundation of our high-throughput screening platform lies in the development of a novel genetically engineered yeast strain, which we refer to as the DAP (Daughter Arrest Program) strain. This strain is designed to halt daughter cell division upon a media switch while leaving mother cell division unaffected. This unique feature allows for the measurement of a mother cell's lifespan by simply counting the number of daughter cells surrounding it after its death, eliminating the need for time-lapse imaging and manual removal of daughter cells.

Microfluidics-based Assay

To complement the DAP strain, we have developed a microfluidic device that interfaces with a 96-well plate, containing 32 independent functional modules. Each module features an observational area with microfluidic channels that can trap single mother cells in regularly spaced microstructures, enabling automated high-throughput imaging. The combination of the DAP strain and this novel microfluidic device allows a single researcher to analyze approximately 130 strains or drug treatments per day using a microscope, representing a 100-fold increase in throughput compared to non-engineered strains and previous microfluidic devices, and a 500-fold increase compared to the traditional micro-dissection method.

Fluorescence-based Assay

To further accelerate high-throughput screening, we have developed a fluorescence-based assay that leverages genetically encoded fluorescent markers, a dual-labeling strategy for mother-daughter cell differentiation, flow cytometry-based classification of cell populations, and an automated data analysis pipeline for calculating lifespan ratios. The histone marker construct, consisting of a histone fused to a fluorescent protein, allows for more distinct classification of daughter cell counts. By employing a dual-labeling approach using RFP and GFP, we can effectively distinguish between mother and daughter cells.

The fluorescent markers enable rapid and efficient identification of cell populations, which is crucial for high-throughput analysis. Flow cytometry is then used to classify these cell populations based on their fluorescence signals. The automated pipeline for calculating lifespan ratios using the fluorescence signals streamlines the data analysis process, allowing for the rapid generation of results from high-throughput screens.

Results

Development of the Daughter-Arresting-Program (DAP) for Lifespan Measurement

We have developed a novel genetic construct called the Daughter-Arresting-Program (DAP) to measure the replicative lifespan of yeast cells. This innovative system selectively arrests daughter cell division while allowing mother cells to continue dividing unaffected (**Figure 1.2**).The DAP construct is integrated into a genomic locus and consists of two key components. First, the native promoter of the essential gene PMA1, which encodes a proton pump localized to the plasma membrane, is replaced with a glucose-repressible promoter. A fluorescent tag is also added to PMA1 for visualization. Second, the construct includes a mother-specific promoter (HO promoter) that drives PMA1 expression in the opposite direction (**Figure 1.2**). When cells are cultured in galactose media, Pma1 is expressed and properly localized to the cell membrane. However, upon switching to glucose media, Pma1 expression is repressed in daughter cells. Existing mother cells maintain Pma1 on their membranes due to its long-lived nature and continue to express PMA1 from the HO promoter (**Figure 1.2**). Daughter cells produced after the media switch do not inherit Pma1 from their mothers because of asymmetric cell division. As a result, these daughter cells immediately cease division. Importantly, we have demonstrated that the DAP system does not affect the lifespan of mother cells. Wild-type cells exhibit the same lifespan with or without the DAP construct integrated. Furthermore, using DAP strains, we can reliably identify both long-lived and short-lived mutants. This validates the utility and robustness of the DAP system for conducting yeast replicative lifespan studies. The development of the DAP system represents a significant advancement in the field of yeast aging research. By enabling precise control over daughter cell division while leaving mother cells unperturbed, the DAP construct allows for more accurate and high-throughput measurements of replicative lifespan. This innovative tool opens up

new possibilities for investigating the genetic and molecular mechanisms underlying cellular aging in yeast.

High-Throughput Microfluidic Device for Lifespan Assay

Building upon our previously designed microfluidic devices, the DAP construct, and a new concept for parallelization, we have developed a high-throughput device for reporter analysis and lifespan assays that is robust and easy to operate (**Figure 1.3**). This innovative device features a 96-well plate interfacing with a microfluidic device consisting of a two-dimensional array of modules, each with an inlet and outlet connecting to a well. The observational area of each module, containing microfluidic channels and microstructures, is strategically aligned to the middle well between the inlet and outlet wells. This novel design combines the advantages of a 96-well plate for automated liquid and cell culture handling with the microfluidic device's ability to trap mother cells and enable long-term time-lapse imaging. The modular nature of the device allows for optimization of each module's design for specific tasks. **Figure 1.3** showcases a specific chip design with microstructures (circles with open gates and two arcs inside) designed to trap mother cells, aiming for one cell per structure, and retain daughter cells for measuring the lifespan of mother cells with the DAP construct. We have also successfully addressed the challenge of high-throughput cell loading, a major bottleneck in existing microfluidic devices for yeast aging studies. By applying air pressure to the inlet wells using a custom-designed 96-well plate cover connected to a pump, cells can be loaded into the microfluidic channels within minutes (**Figure 1.3**). This innovative approach enables the loading of all 32 observational wells on a plate in a matter of minutes, significantly reducing the time and effort required for cell loading. Moreover, when working with yeast strains containing the DAP construct, there is no need for removing daughter cells, eliminating the requirement for pumps and tubes for media flow during the experiment. This simplifies the system setup and operation, making

it highly cost-effective as a drug screening platform. Compared to previously developed microfluidic devices, our system requires only 200 μL of liquid media per drug concentration, a significant reduction from the typical 300 μL/hour flow for 72 hours, resulting in a remarkable 100-fold decrease in the amount of drug needed for screening. The development of this high-throughput microfluidic device represents a major advancement in the field of yeast aging research.

By integrating the DAP construct, parallelization, and efficient cell loading techniques, our system enables rapid and cost-effective screening of drugs and other interventions that may modulate the aging process. This powerful tool has the potential to accelerate the discovery of novel therapeutic strategies for age-related diseases and promote a better understanding of the fundamental mechanisms underlying cellular aging.

Fluorescence-Based Assay for Accelerated High-Throughput Screening

To further accelerate high-throughput screening, we have developed a fluorescence-based assay that leverages genetically encoded fluorescent markers, a dual-labeling strategy for mother-daughter cell differentiation, flow cytometry-based classification of cell populations, and an automated data analysis pipeline for calculating lifespan ratios (**Figure 1.4**). The histone marker construct, consisting of a histone fused to a fluorescent protein, allows for more distinct classification of daughter cell counts (**Figure 1.4**). By employing a dual-labeling approach using RFP and GFP, we can effectively distinguish between mother and daughter cells. The fluorescent markers enable rapid and efficient identification of cell populations, which is crucial for high-throughput analysis. Flow cytometry is then used to classify these cell populations based on their fluorescence signals (**Figure 1.4**). The assay also features an automated pipeline for calculating lifespan ratios using the fluorescence signals (**Figure 1.4**), streamlining the data analysis process and allowing for the rapid generation of results from high-throughput screens.

In a representative experiment, the assay was able to analyze over 300 different drug treatment conditions (**Figure 1.5**), with over 10,000 yeast cells in each condition, within 3 hours using the dual-labeling strategy and flow cytometry-based classification. This rapid data acquisition and analysis significantly accelerates the pace for large numbers of samples, making the assay a valuable tool for identifying anti-aging interventions in a high-throughput manner.

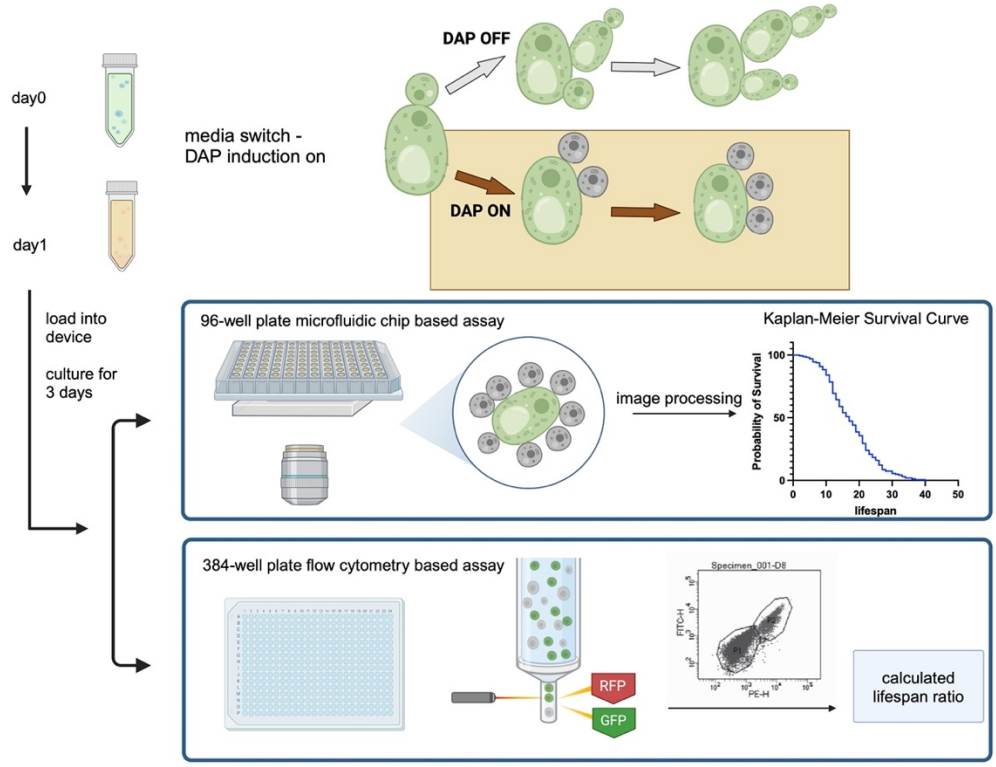


Figure 1.1 Overview of Research Rationale and Experimental Design The overall experiment spans from day 0 to day 3, utilizing both microfluidics-based and fluorescence-based assays to collect replicative lifespan data. On day 0, yeast cells are loaded into the microfluidic device or labeled for the fluorescence-based assay. The media is then switched to induce the Daughter Arrest Program (DAP) in the yeast cells. From day 1 to day 3, the microfluidics-based assay involves time-lapse imaging and analysis of replicative lifespan, while the fluorescence-based assay utilizes flow cytometry to determine the ratio of mother to daughter cells at different time points, serving as a proxy for replicative lifespan. The combination of these two methodologies enables high-throughput screening and analysis of potential anti-aging interventions in yeast.

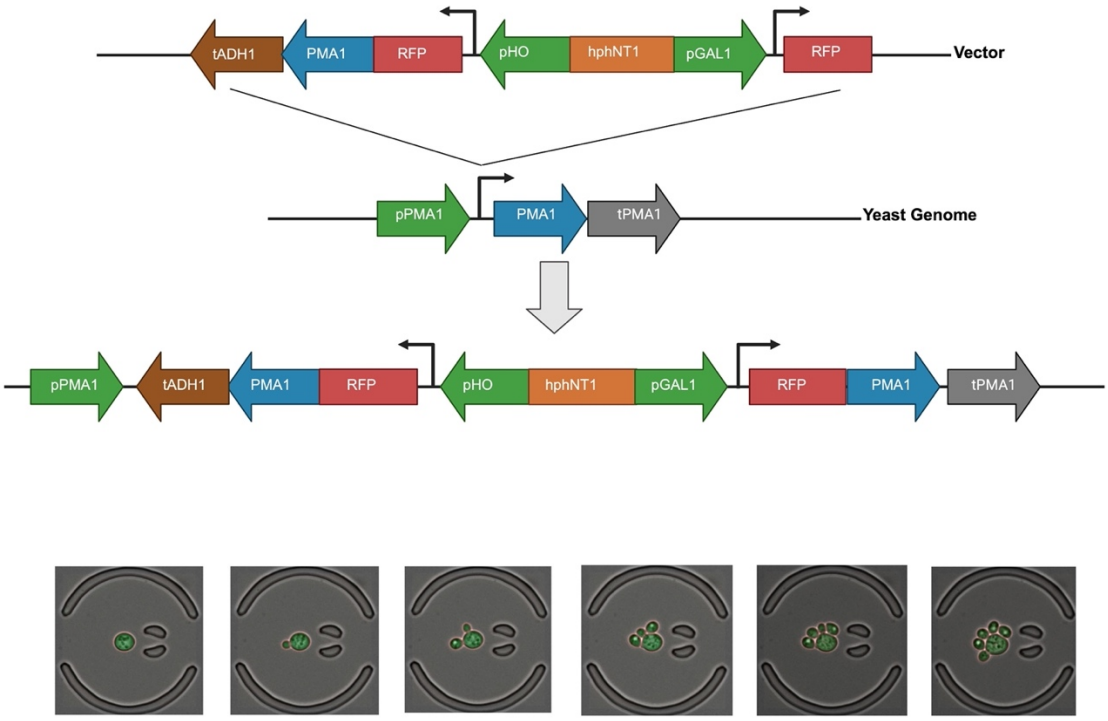


Figure 1.2 Daughter-Arresting-Program(DAP) A schematic of the genetic construct. pPMA1: promoter of gene PMA1; tADH1: terminator of Ashbya gossypii gene ADH1; FP: Fluorescence Protein (dTomato, GFP, etc.); pHO: promoter of mother specific gene HO; hphNT1: cassette of selection marker Hygromycin; PGAL1: yeast GAL1 promoter; PMA1: terminator of gene PMA1.

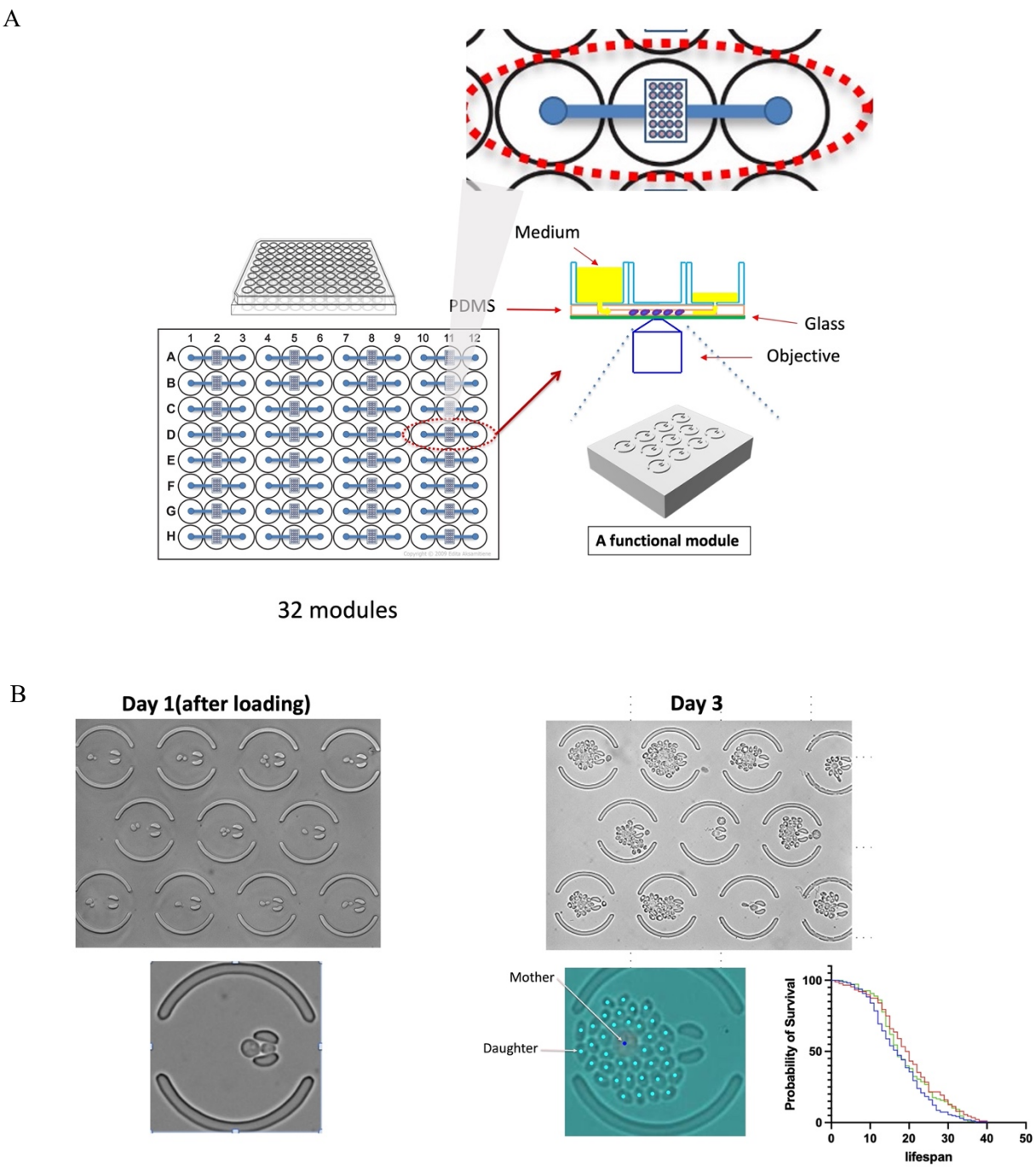


Figure 1.3 Microfluidics-based method for high-throughput lifespan analysis.
(A) Schematic diagram of the microfluidic device designed for yeast cell lifespan measurement. (B) Representative end-moment images of a single mother cell captured in a trap over the course of its lifespan. The number of daughter cells produced by the mother cell can be counted to determine its replicative lifespan. Image processing pipeline for automated detection and quantification of budding events.

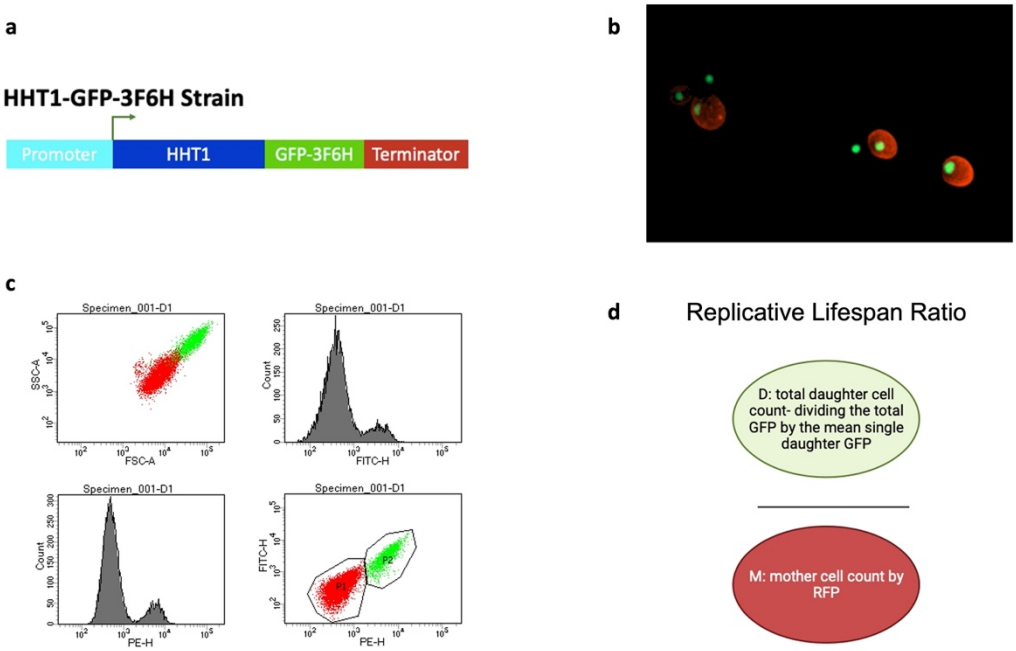


Figure 1.4 Fluorescence-Based Assay for Yeast Lifespan Measurement. (A). Illustrates the histone marker construct used in the fluorescence-based assay, including a diagram of the genetic construct and its functional role in the assay.(B). Depicts the use of RFP and GFP markers to differentiate between mother and daughter cell populations in yeast, including fluorescence microscopy images of the labeled cells.(C). Shows the methodology for classifying cell populations and calculating lifespan ratios using fluorescence markers, with graphical representations and example data plots. (D). Lifespan calculation based on fluorescence signal.

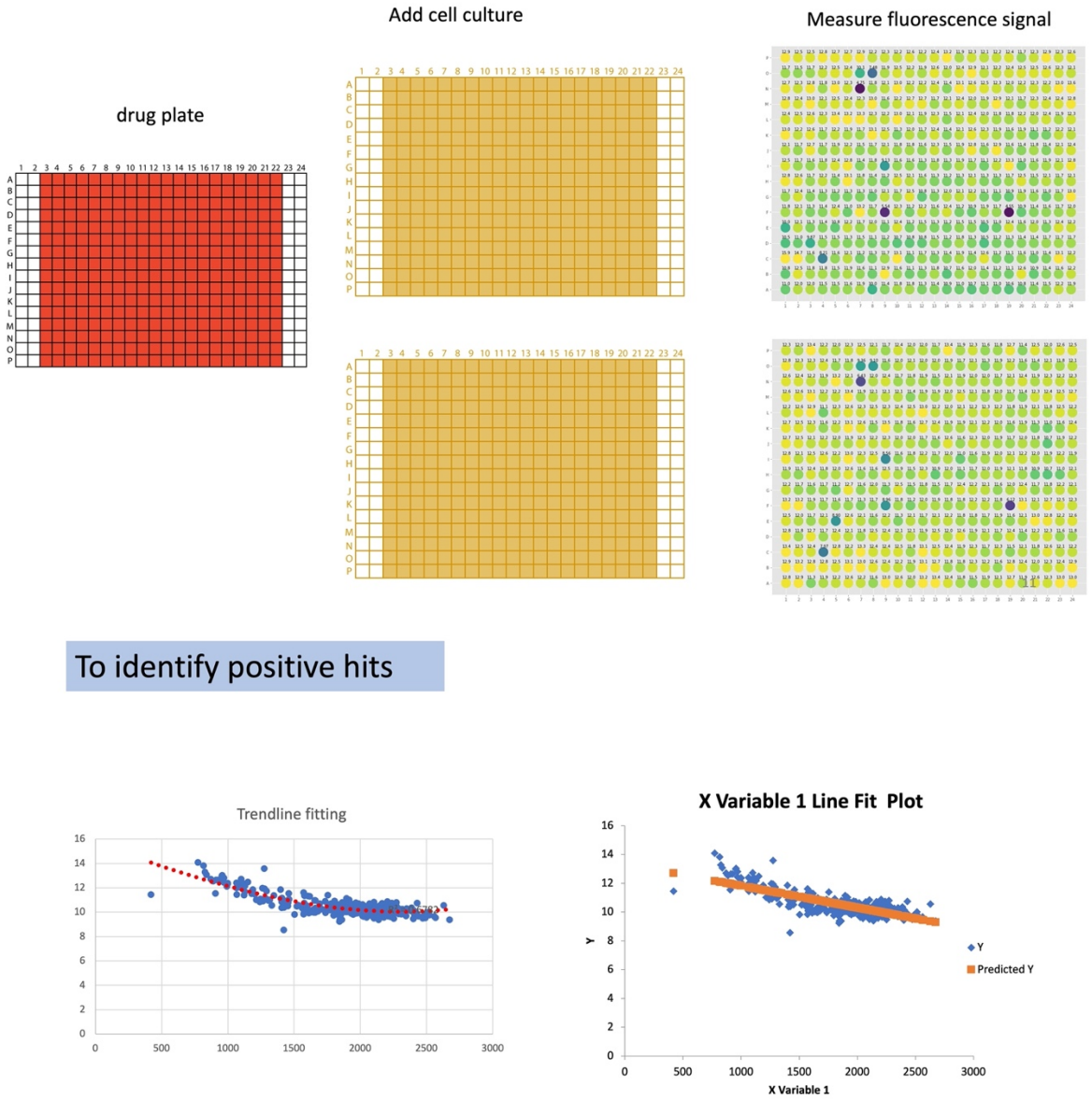


Figure 1.5 Fluorescence-Based Assay Data Analysis Pipeline. (A) Representative layout of a 384-well plate used in the high-throughput screening. Each well contains a unique yeast strain or treatment condition. (B) Scatter plot depicting the relationship between the number of mother cells and the lifespan ratio for each well. The data points are fitted with a correlation curve to identify outliers that deviate significantly from the expected trend. These outliers, represented by data points above the upper threshold or below the lower threshold, are considered positive hits and are selected for further validation and characterization.

CHAPTER 2 GENETIC SCREENING

Introduction

Yeast has emerged as a powerful model organism for studying the genetics of aging due to its rapid growth, ease of genetic manipulation, and conservation of many aging-related pathways with higher eukaryotes. Researchers can measure yeast lifespan using the replicative aging assay, which counts the number of daughter cells a mother cell can produce before senescence. By systematically deleting or overexpressing genes and assessing the impact on replicative lifespan, scientists can identify genetic modulators of aging. Genome-wide screens in yeast have uncovered numerous genes whose deletion extends lifespan. These genes cluster into pathways such as nutrient sensing, protein translation, and mitochondrial function. Importantly, many of these longevity pathways are conserved in worms, flies, and mammals, underscoring the value of yeast as a discovery platform for candidate anti-aging targets. [Kaeberlein, 2010].

Yeast Non-Essential Gene Deletion Mutant Screening

One effective approach for identifying genes that modulate aging in yeast is to systematically screen deletion mutants lacking individual non-essential genes and assess their impact on lifespan. By comparing the lifespan of these mutants to that of wild-type strains, researchers can pinpoint specific genes that either promote or suppress longevity. In this study, we conducted a comprehensive screen of the yeast deletion collection to identify mutants with altered replicative lifespans. Replicative lifespan, defined as the number of daughter cells a mother cell can produce before senescence, is a widely used metric for aging in yeast. Our results reveal a diverse set of genes involved in various cellular processes, such as nutrient sensing, stress response, and genome maintenance, that play critical roles in determining yeast lifespan.

Yeast DAmP Library for Essential Gene Mutant Screening

To extend our understanding of essential genes in yeast longevity, we utilized the Decreased Abundance by mRNA Perturbation (DAmP) library. This innovative tool allows for the partial suppression of essential genes, facilitating the study of genes whose complete deletion would be lethal. This method opens up new avenues for exploring how reduced expression of critical cellular machinery impacts yeast aging [Breslow et al., 2008]. A novel discovery from our screening of the DAmP library was the consistent lifespan extension observed in the PGI1-DAmP mutant. This mutant, characterized by partial suppression of the phosphoglucose isomerase gene, highlights the intricate link between glucose metabolism and aging. This finding not only broadens our understanding of metabolic control in aging but also suggests potential targets for modulating aging through metabolic pathways. In conclusion, genetic screening using both non-essential gene deletion mutants and the DAmP library has provided valuable in-sights into the genetic factors influencing yeast longevity. These discoveries have shed light on the diverse biological processes involved in aging, from nutrient sensing and stress response to cellular repair mechanisms and glucose metabolism. By identifying potential gene targets for lifespan extension, these findings pave the way for the development of targeted interventions to promote healthy aging.

Results

Validation of the DAP System Using Known Longevity Mutants

To validate our DAP (Daughter Arrest Program) system for measuring yeast replicative lifespan, we tested several non-essential gene deletion mutants that have been previously characterized using the traditional micro-dissection technique. We constructed these mutants in our parental strain containing the DAP construct and measured their lifespans using our microfluidic device. Our results showed a strong agreement with the lifespans obtained using the original mutants without DAP and the micro-dissection method. Two notable examples are the long-lived*fob1Δ* mutant and the short-lived *hom2Δ* mutant. *FOB1* encodes a nucleolar protein required for replication fork blocking at the rDNA locus. Deletion of *FOB1* is thought to extend lifespan by reducing the formation of toxic extra-chromosomal rDNA circles (ERCs) . ERCs are self-replicating circular DNA molecules that accumulate in aging yeast cells and are a major cause of replicative aging. Our measurements confirmed that the *fob1Δ* mutant has a 30% lifespan extension compared to the wild-type strain, consistent with previous reports(**Figure 2.2**) . These results demonstrate the reliability and accuracy of our DAP system in measuring yeast replicative lifespan. The ability of the DAP system to recapitulate known lifespan phenotypes of genetic mutants previously characterized by the labor-intensive micro-dissection method validates its utility for high-throughput lifespan screens. Furthermore, the strong concordance between lifespans measured with the DAP system in a microfluidic platform and the traditional micro-dissection approach highlights the robustness of the core molecular mechanisms governing replicative aging in yeast.

Integrating the DAP genetic system with microfluidic technologies enables rapid and efficient lifespan analysis while maintaining the key biological features of yeast replicative aging. In

summary, our validation experiments establish the DAP system coupled with microfluidics as a powerful tool for investigating the genetic and molecular basis of cellular aging in yeast. This innovative approach opens new avenues for large-scale genetic screens and holds great promise for uncovering novel longevity pathways and potential therapeutic targets for age-related diseases.

Screening non-Essential Gene Deletion Library for New Longevity Regulators

We selected 47 mutants from deletion library(**Figure 2.1**). Our results (**Figure 2.2**) shed light on the complex interplay between various genetic factors and cellular processes in determining yeast replicative lifespan. We found that the *gpr1Δ* mutant, deficient in a G protein-coupled receptor involved in glucose sensing and cAMP signaling , and the *mub1Δ* mutant, lacking a gene required for the spindle position checkpoint , both exhibit lifespan extension compared to the wild-type strain. The lifespan extension in *gpr1Δ* is consistent with previous reports showing that deletion of *GPR1* mimics calorie restriction and extends both replicative and chronological lifespan in yeast . The longevity effect of *MUB1* deletion is a novel finding and suggests a potential link between cell cycle regulation and aging. Similarly, the *rpl19bΔ* and *rpl21bΔ* mutants, which lack genes encoding ribosomal protein subunits, demonstrate significantly longer lifespans than the control. This observation aligns with the well-established role of reduced protein translation in promoting longevity across species, from yeast to mammals. Deletion of ribosomal protein genes is thought to extend lifespan by decreasing overall protein synthesis and mimicking the effects of dietary restriction .In contrast, the*rad6Δ* mutant, deficient in ubiquitin-conjugating enzymes involved in DNA repair pathways , the *skn7Δ* mutant, lacking a transcription factor that regulates the oxidative stress response , and the *fre8Δ* mutant, deficient in a protein required for metal ion homeostasis, all display severely diminished lifespans, with their survival curves dropping sharply at an early stage. These findings underscore the critical importance of maintaining genome stability,

mounting robust stress responses, and regulating metal ion levels for ensuring normal lifespan. The shortened lifespan of the*rad6Δ* mutant highlights the central role of DNA repair mechanisms in counteracting age-associated genomic instability and maintaining cellular viability . Similarly, the premature aging of the *skn7Δ* mutant emphasizes the vital function of the oxidative stress response in mitigating the deleterious effects of reactive oxygen species that accumulate during aging . The accelerated aging of the *fre8Δ* mutant suggests that metal ion homeostasis may also contribute to the aging process, possibly by exacerbating oxidative damage. Collectively, these findings highlight the diverse roles of different genes and cellular processes in regulating yeast replicative lifespan. Deletions of genes involved in nutrient sensing (*GPR1*), cell cycle regulation (*MUB1*), and protein translation (*RPL19B* and *RPL21B*) can lead to lifespan extension, while deficiencies in DNA repair (*RAD6*), stress response (*SKN7*), and metal ion homeostasis (*FRE8*) can drastically shorten lifespan. These results underscore the importance of maintaining a delicate balance between various cellular processes to ensure optimal longevity and provide valuable insights into the genetic basis of aging in yeast.

Screening Essential Gene DAmP Library for New Longevity Regulators

To further explore the potential of our DAP system, we turned our attention to essential genes, which have rarely been studied in the context of aging due to their requirement for cell viability. We utilized the DAmP (Decreased Abundance by mRNA Perturbation) library, which contains hypomorphic alleles of essential genes with reduced expression levels [Breslow et al., 2008].The DAmP approach involves disrupting the 3' untranslated region (UTR) of a gene with an antibiotic resistance cassette, thereby destabilizing the corresponding transcript and reducing mRNA levels typically two- to tenfold .We selected 51 DAmP strains from this library (**Figure 2.1**), focusing on functional categories known to influence lifespan, such as protein translation and glucose metabolism . We constructed these DAmP alleles in our parental strain containing the DAP

construct and measured their lifespans using our 96-well microfluidic device. Most of the DAmP strains exhibited lifespans similar to the wild-type strain, while some showed shortened lifespans. Notably, we identified one promising candidate, the *PGI1* DAmP strain, which displayed a strong lifespan extension of 30% (**Figure 2.2**). *PGI1*encodes the glycolytic enzyme phosphoglucose isomerase, which catalyzes the interconversion of glucose-6-phosphate and fructose-6-phosphate, a key step in glucose metabolism . The lifespan extension observed in the*PGI1* DAmP strain is likely due to an effect similar to glucose restriction, a well-known intervention that extends yeast lifespan . Reduced activity of Pgi1 may lead to a metabolic shift from glycolysis to respiration, which has been shown to mediate the longevity benefits of calorie restriction in yeast . Additionally, decreased glucose flux through glycolysis could activate stress response pathways and enhance cellular protection mechanisms, contributing to increased lifespan .The magnitude of lifespan extension in the *PGI1* DAmP strain is comparable to that of the classical *fob1Δ* mutant , suggesting a distinct and potent longevity mechanism. Given the known protein structure of Pgi1 , our findings highlight this enzyme as a promising target for the development of small molecule drugs to modulate glucose metabolism and potentially delay aging .Furthermore, the identification of*PGI1* as a longevity gene through our DAmP screen demonstrates the power of the DAP system to uncover novel aging pathways and therapeutic targets, even among essential genes. In conclusion, our results reveal that downregulation of the essential glycolytic enzyme Pgi1 extends yeast replicative lifespan, likely through a mechanism related to glucose restriction and metabolic reprogramming. This finding highlights the potential of targeting glucose metabolism as a strategy to delay aging and age-related diseases.

Gene Mutant
ALA1-DAmP-BC
YDR341C-DAmP-BC
DED81-DAmP-BC
DPS1-DAmP-BC
YNL247W-DAmP-BC
GUS1-DAmP-BC
GLN4-DAmP-BC
GRS1-DAmP-BC
ILS1-DAmP-BC
CDC60-DAmP-BC
KRS1-DAmP-BC
MES1-DAmP-BC
FRS2-DAmP-BC
FRS1-DAmP-BC
YHR020W-DAmP-BC
SES1-DAmP-BC
THS1-DAmP-BC
WRS1-DAmP-BC
HTS1-DAmP-BC
TYS1-DAmP-BC
VAS1-DAmP-BC
ALG7-DAmP-BC
CCT4-DAmP-BC
CDC42-DAmP-BC
CEG1-DAmP-BC
GAA1-DAmP-BC
GPI15-DAmP-BC
GUK1-DAmP-BC
HAS1-DAmP-BC
JAC1-DAmP-BC
LAS1-DAmP-BC
RPB11-DAmP-BC
RPC11-DAmP-BC
RPC17-DAmP-BC
RPC19-DAmP-BC
RPC25-DAmP-BC
RPS31-DAmP-BC
SIK1-DAmP-BC
SRB7-DAmP-BC
SUI1-DAmP-BC
TEN1-DAmP-BC
TIF11-DAmP-BC

Gene Mutant
TIF35-DAmP-BC
TOM40-DAmP-BC
AVO1-DAmP-BC
PGI1-DAmP-BC
NOP1-DAmP-BC
GCR1-Damp-BC
PGK1-DaMP-BC
FBA1-DaMP-BC
UBP8
TAT2
MRP20
FOB1
RRD2
REI1
TIS11
MRT4
RPL21B
RPL19B
RPL29
RPL34B
BST1
SIS2
YKR073C
TKL1
MUB1
SPT4
ATO2
MFA2
MSL1
SAP190
RPL19A
GPR1
RPL13A
ATG1
MSW1
MDL2
YLR124W
RAD52
RTT109
RAX1
HST3

Gene Mutant
SKN7
STE20
HOM2
RTG2
YGR012W
RAD6
PHB2
SPS19
YDR269C
SMF1
THR1
RIC1

Figure 2.1 List of Genetic Mutants

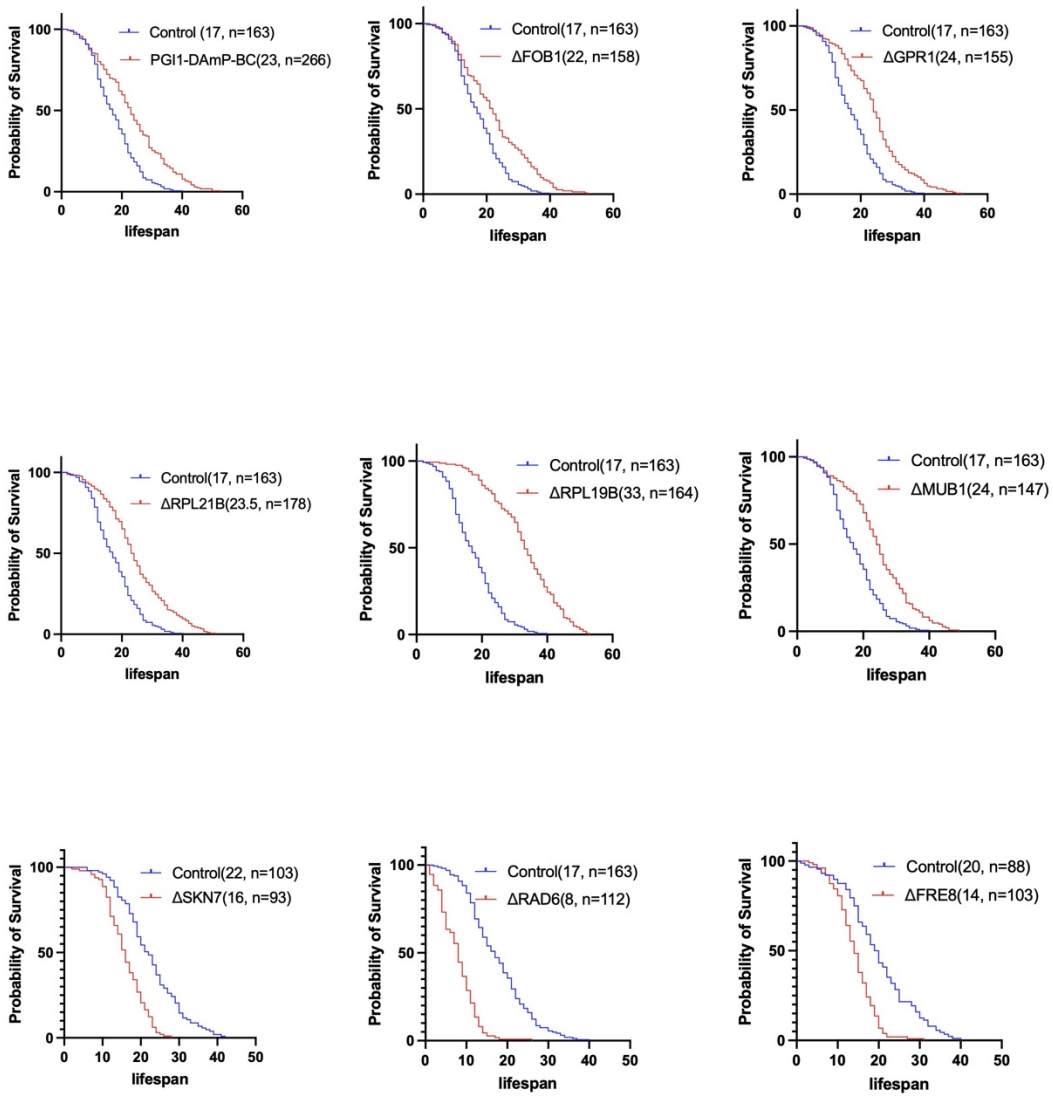


Figure 2.2 Genetic Mutants Lifespan Curve Examples Survival curves comparing the replicative lifespan of various yeast strains with genetic modifications versus wild type as control, including PGI1-DAmP-BC, fob1, gpr1, rpl21b, rpl19b, mub1, skn7, rad6, and fre8. Median values and mother cell number are shown. *P <0.05, **P <0.01 and ****P <0.001, by two-sided Wilcoxon rank-sum test.

CHAPTER 3 COMPOUND SCREENING

Introduction

This chapter outlines the methodology and results of screening both FDA-approved and bioactive compound libraries to identify potential anti-aging agents in *Saccharomyces cerevisiae*. The identification of novel compounds with anti-aging properties is crucial for understanding the molecular mechanisms underlying aging and for developing targeted interventions to promote healthy aging [Longo et al., 2015].

Screening of FDA-Approved and Bioactive Compound Libraries

We utilized comprehensive libraries of FDA-approved and bioactive compounds to accelerate the discovery of agents with potential anti-aging properties. These libraries were chosen for their diverse chemical compositions and known bioactivities, providing a broad basis for identifying novel interventions in aging. FDA-approved compounds are particularly attractive as they have already undergone rigorous safety testing and have well-characterized pharmacological properties, potentially expediting their translation into clinical applications [Snell et al., 2012]. Bioactive compound libraries, on the other hand, contain a wide range of natural products and synthetic molecules with diverse biological activities. These compounds have been shown to modulate various cellular pathways and processes, making them valuable tools for exploring the complex mechanisms of aging. By screening these libraries, we aimed to identify compounds that could extend yeast lifespan and provide insights into the underlying molecular pathways that regulate aging.

Systematic Screening Strategies

Initial Screening - The initial screening aimed to identify compounds that showed a promising increase in the lifespan of yeast. This stage utilized a fluorescence-based assay to rapidly evaluate the effects of each compound on yeast longevity. The assay employed genetically engineered yeast strains expressing fluorescent markers that allow for the quantification of lifespan extension based on the intensity and duration of the fluorescent signal. Compounds demonstrating significant lifespan extension compared to untreated controls were selected for further analysis. The initial screening provided a high-throughput method to assess a large number of compounds and identify those with the most promising anti-aging potential. This approach allowed for the efficient prioritization of compounds for subsequent follow-up studies.

First Follow-Up - The first follow-up phase involved a more detailed assessment of candidate compounds that showed the most promise in the initial screenings. These compounds were subjected to dose-response analysis to determine the optimal concentrations for lifespan extension. Dose-response testing is critical for establishing the efficacy and potential toxicity thresholds of each compound [Blagosklonny, 2009]. Yeast cells were treated with varying concentrations of each compound, and their lifespan was measured using the fluorescence-based assay. The dose-response curves generated from these experiments provided valuable information on the concentration-dependent effects of each compound on lifespan extension. Compounds exhibiting a clear dose-response relationship and significant lifespan extension at non-toxic concentrations were prioritized for further evaluation.

Second Follow-Up – The second follow-up focused on a narrower set of hits, selected based on their performance in previous tests. This stage employed a microfluidics-based assay, which allowed for precise manipulation and observation of yeast cells at a single-cell level, providing

detailed insights into the compounds' effects on cell division and longevity. The microfluidics platform enabled the tracking of individual yeast cells over their entire lifespan, allowing for the accurate determination of replicative lifespan. By monitoring cell division events and morphological changes, we could assess the impact of each compound on the aging process at a high resolution. This approach provided a more comprehensive understanding of the compounds' anti-aging effects and helped to validate the findings from the initial screening and first follow-up studies.

Focused Screening on mTOR Inhibitors and Rapalogs

In addition to the broad screening of FDA-approved and bioactive compounds, we conducted a focused screening of rapalogs, which are specific inhibitors of the mTOR pathway and derivatives of rapamycin. The mTOR pathway is a central regulator of cell growth, metabolism, and aging, and its inhibition has been shown to extend lifespan in various model organisms [Johnson et al., 2013]. Rapalogs were evaluated for their capacity to increase the replicative lifespan of yeast using the microfluidics-based assay. Yeast cells were treated with different concentrations of rapalogs, and their lifespan was monitored at the single-cell level. The results of this focused screening provided valuable insights into the potential of mTOR inhibition as an anti-aging strategy in yeast. The identification of specific rapalogs that significantly extend yeast lifespan could pave the way for the development of targeted anti-aging interventions. These compounds could serve as valuable tools for further dissecting the molecular mechanisms underlying mTOR-mediated lifespan extension and for exploring the conservation of these pathways across different species.

In conclusion, the systematic screening of FDA-approved and bioactive compound libraries, along with the focused screening of mTOR inhibitors and rapalogs, has provided a comprehensive approach to identifying potential anti-aging agents in yeast. The multi-stage screening process,

involving initial high-throughput assays and subsequent follow-up studies, has enabled the identification of compounds with robust lifespan-extending effects.

Results

To identify small molecule compounds with potential anti-aging effects, we conducted a comprehensive library screening using our advanced fluorescence-based assay. Our screening strategy involved testing compounds from the FDA-approved Drug Library and the Bioactive Compound Library, which together contain over 4,000 molecules (**Figure 3.1**). These libraries were selected for their diverse chemical structures and known biological activities, providing a rich source of potential anti-aging compounds. For the initial screening, we utilized a high-throughput fluorescence-based assay to measure the replicative lifespan of yeast cells treated with individual compounds at three serial concentrations (0.1 uM, 1 uM, and 10 uM). This as-say allows for the rapid and efficient evaluation of lifespan-extending effects by quantifying the fluorescence intensity of genetically engineered yeast strains expressing age-dependent fluorescent markers. A representative scatter plot from one of the screening plates demonstrates the distribution of lifespan effects, with each point corresponding to a single well containing a unique compound (**Figure 3.1**). Compounds exhibiting a greater than 30 % increase in mean replicative lifespan compared to wildtype controls were considered as hits and selected for further validation.

From the primary screen, we identified 30 compounds that significantly and consistently extended yeast replicative lifespan (**Figure 3.2**). These top hits included several drugs with diverse mechanisms of action, such as TOR inhibitors, GSK-3 inhibitors, and topoisomerase inhibitors. The identification of TOR inhibitors, such as rapamycin and its analogs, is consistent with previous studies demonstrating their lifespan-extending effects in various model organisms, including yeast, worms, flies, and mice [Johnson et al., 2013, Bitto et al., 2016]. Similarly, GSK-3 inhibitors have been shown to extend lifespan in C. elegans and Drosophila, potentially through the modulation of stress response pathways and the regulation of protein homeostasis [McColl et al., 2008,

Castillo-Quan et al., 2016]. The presence of these known lifespan-extending compounds among our top hits supports the translatability of our yeast-based screening platform and validates its ability to identify novel anti-aging compounds. To further characterize the dose-response relationships of the most promising hits, we performed extensive experiments at a broad range of concentrations for each compound. Replicative lifespan analysis revealed that the lifespan extension effects were dose-dependent, with optimal concentrations varying among the different drugs (**Figure 3.3**).

This observation highlights the importance of determining the appropriate dosage for each compound to maximize its anti-aging benefits while minimizing potential adverse effects. The dose-response data also provide valuable insights into the therapeutic windows of these compounds, informing future studies aimed at optimizing their efficacy and safety profiles. In addition to the primary hits, our screening approach also identified several novel compounds that have not been previously reported to extend lifespan in any model organism. These compounds represent exciting new leads for further investigation into their mechanisms of action and potential as anti-aging interventions. By leveraging the power of yeast genetics and molecular biology, we can rapidly elucidate the cellular pathways and molecular targets through which these com- pounds exert their lifespan-extending effects. Moreover, the identification of novel anti-aging compounds underscores the value of unbiased, high-throughput screening approaches in uncovering new therapeutic strategies for promoting healthy aging.

To further investigate the anti-aging potential of mTOR inhibitors, we conducted a focused screening of rapalogs, a class of rapamycin analogs that share the same molecular scaffold but possess different physicochemical properties. Our screening campaign included some established rapalogs, such as Ridaforolimus and Temsirolimus, and novel compounds like Rapalink(**Figure 3.4**).We evaluated the dose-response relationships of selected rapalogs in our yeast replicative lifespan assay. All rapalogs showed significant positive lifespan extension effects at certain

concentration range. These results are consistent with the reported potency of these compounds in mammalian systems and highlight the evolutionary conservation of mTOR signaling in the regulation of aging. Particularly Rapalink at the concentration range 5uM-10uM demonstrated most potent effects. These findings suggest that structural modifications of the rapamycin scaffold can enhance the anti-aging activity of mTOR inhibitors. Collectively, our focused screening of rapalogs provides compelling evidence for the anti-aging potential of this class of mTOR inhibitors. The identification of novel rapalogs with enhanced lifespan-extending activity and distinct mechanistic profiles opens new avenues for the development of optimized anti-aging therapeutics targeting the mTOR pathway. Further investigation of the molecular and physiological basis of rapalog-mediated lifespan extension in yeast may inform the design of next-generation mTOR inhibitors with improved efficacy for clinical translation.

In summary, our yeast-based screening approach successfully identified several compounds that extend replicative lifespan, including both known lifespan-extending drugs and novel compounds. These findings provide a foundation for future mechanistic studies to elucidate the underlying pathways and targets responsible for the observed anti-aging effects. Moreover, the most promising compounds warrant further investigation in mammalian models to assess their translational potential as interventions to promote healthy aging in humans. By combining the power of high-throughput screening with the versatility of yeast as a model organism, our study demonstrates the potential for accelerating the discovery of new anti-aging compounds and advancing our understanding of the fundamental mechanisms of aging.

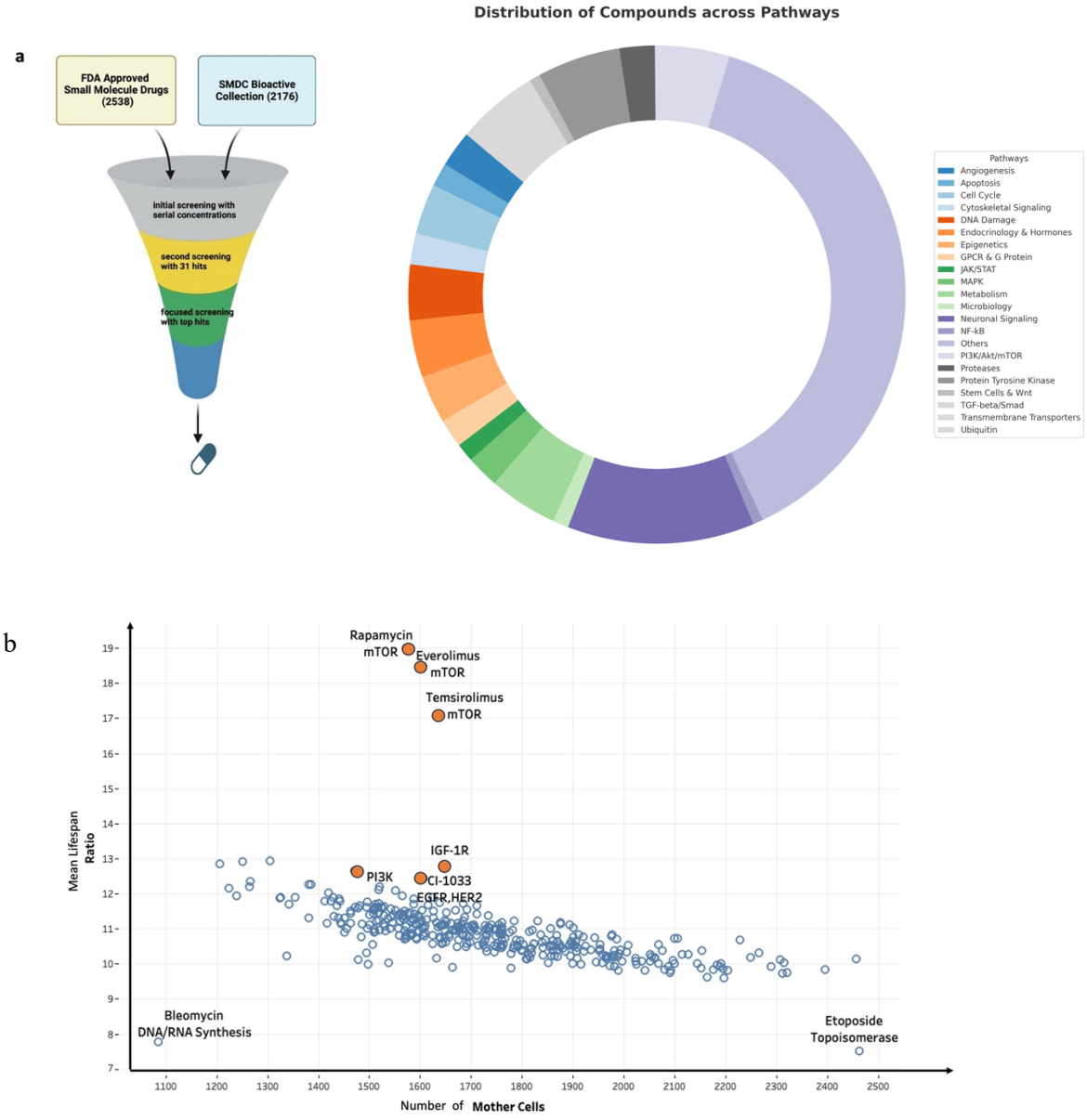


Figure 3.1 Compound screening workflow and initial screening results. (A) Schematic overview of the compound screening roadmap employed in this study. The process begins with comprehensive FDA-approved drug libraries and bioactive compound libraries, which collectively contain over 4,000 small molecules with diverse chemical structures and known bioactivities. An initial high-throughput screening is performed using a fluorescence-based assay to measure the replicative lifespan of yeast cells treated with individual compounds at multiple concentrations. Compounds exhibiting significant lifespan extension compared to wildtype controls are identified as hits and selected for further validation and characterization. (B) Representative scatter plot from the initial screening of one 384-well plate. Each data point corresponds to a single well containing a unique compound treatment. The x-axis represents the compound identifier, and the y-axis depicts the measured lifespan ratio.

Name	Targets
Naratriptan	5-HT Receptor
Pyronaridine Tetraphosphate	anti-malaria
Artesunate	
Sulfadoxine	
Gefitinib	EGFR
CI-1033	
Stigmasterol	multiple cell growth pathways
GSK-3 inhibitor	GSK-3
IGF-1R inhibitor	IGF-1R
proteasome inhibitor	inhibit protein synthesis
Kanamycin	
Cycloheximide	
Emetine, Emetine Dihydrochloride	
Dioscin	mTOR
Ridaforolimus	
Temsirolimus	
mTOR inhibitor	
mTOR/PI3K dual inhibitor	
Tepoxalin	NSAID
Salicyl Alcohol	
Rofecoxib	
Salsalate	
Mexiletine	sodium channel
Sparteine Sulfate	
Dyclonine	
Irinotecan	Topoisomerase
Pirarubicin	
Etoposide	
LE-SN38	
Epirubicin	

Figure 3.2 Summary of top hits from initial compound screening and their potential mechanisms of action as anti-aging interventions.

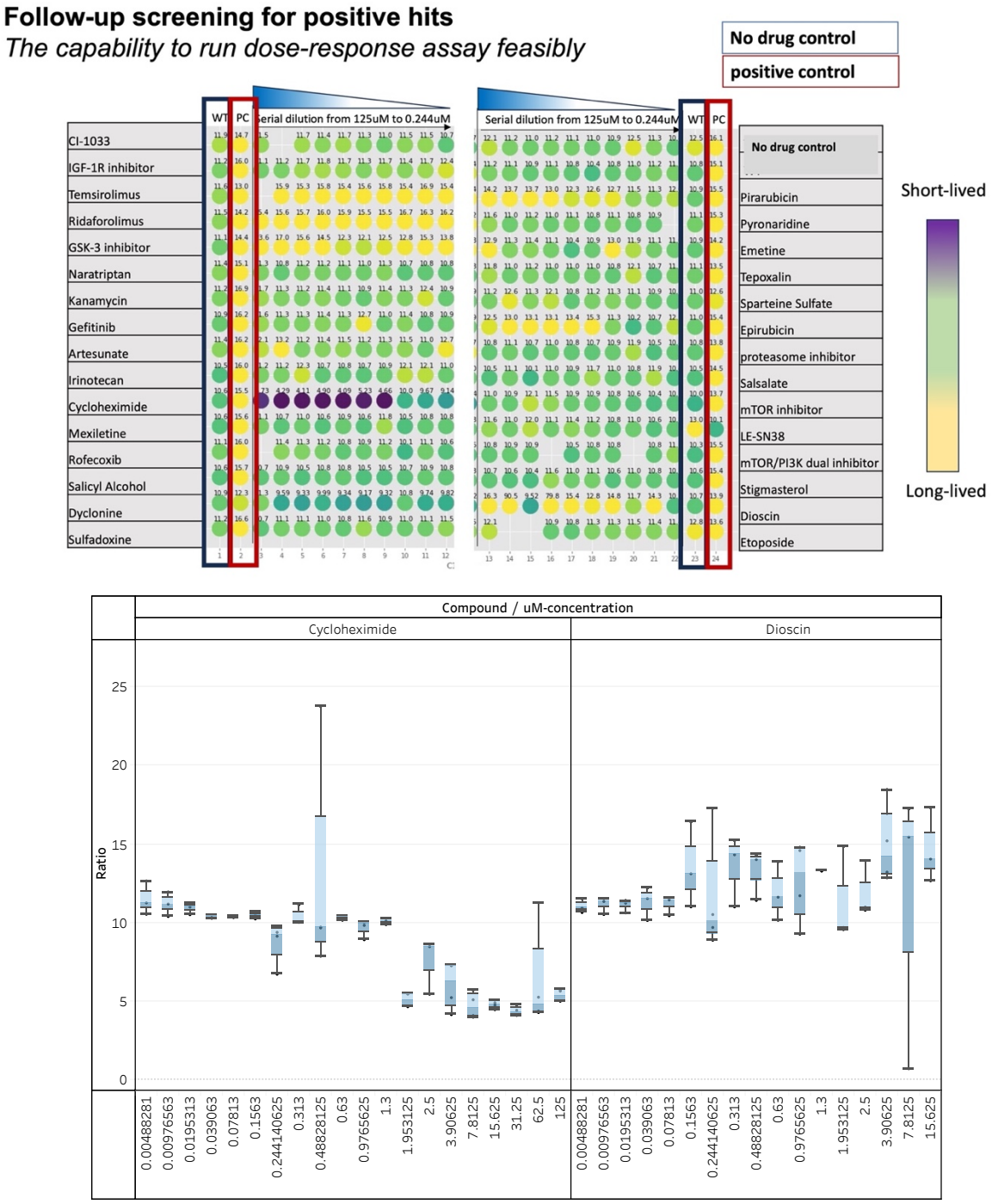


Figure 3.3 Dose-response analysis of selected compounds from the initial high-throughput screen.

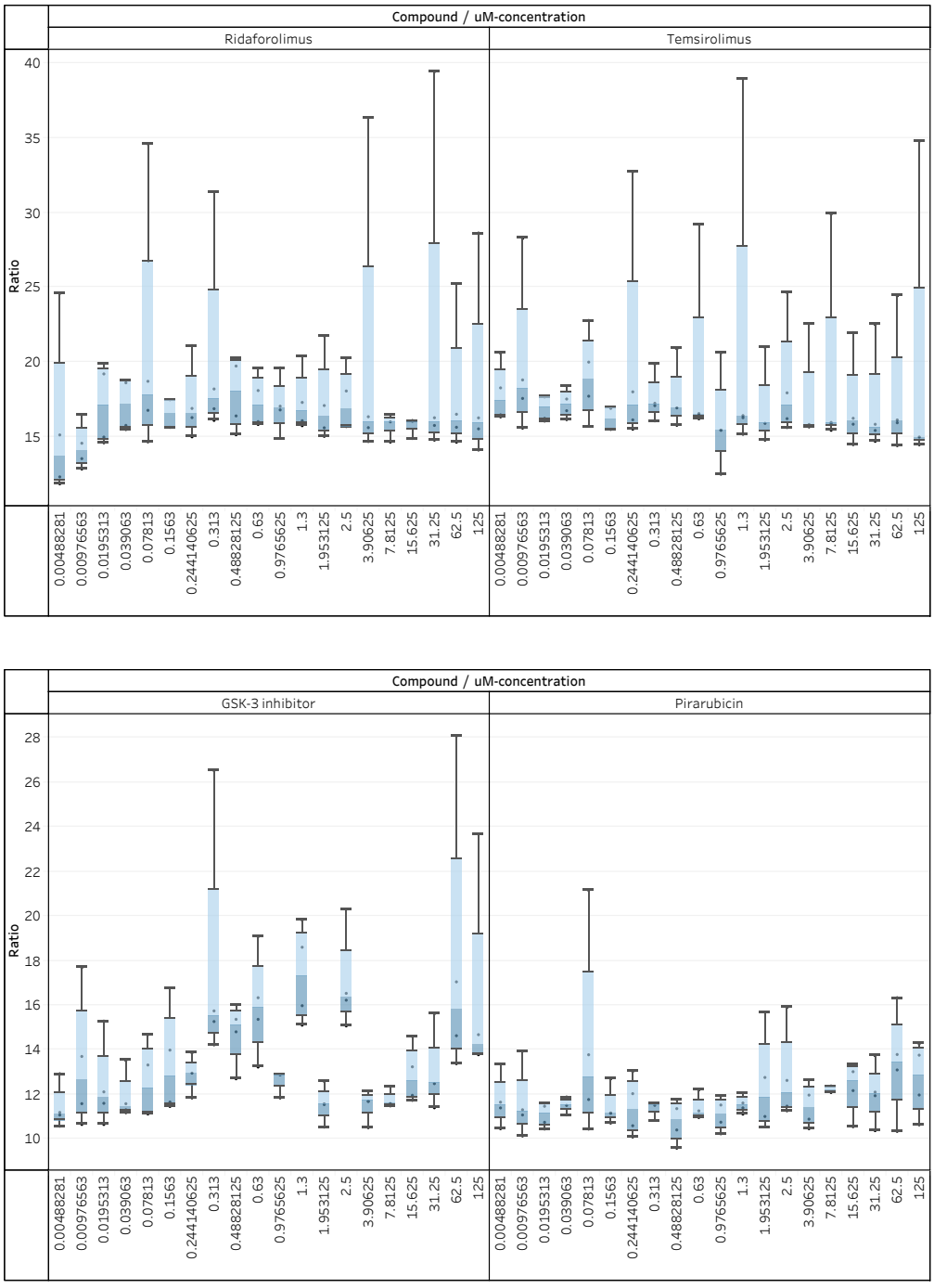


Figure 3.3(continued) Dose-response analysis of selected compounds from the initial high-throughput screen.

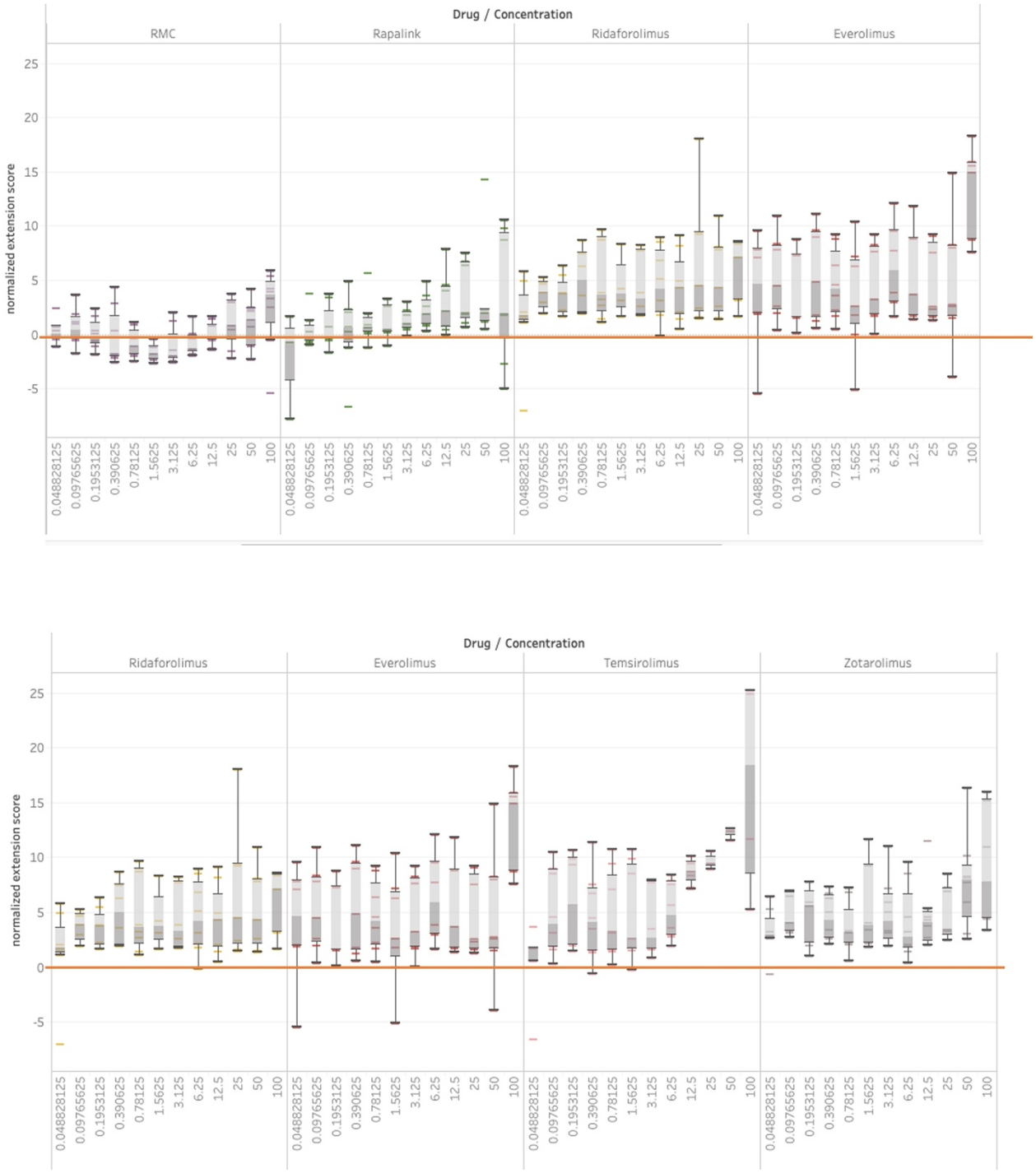


Figure 3.4. Dose-response analysis of rapalogs on yeast replicative lifespan. A series of panels illustrating the effects of various rapalog compounds, specific inhibitors of the mTOR pathway, on replicative lifespan in the yeast model system.

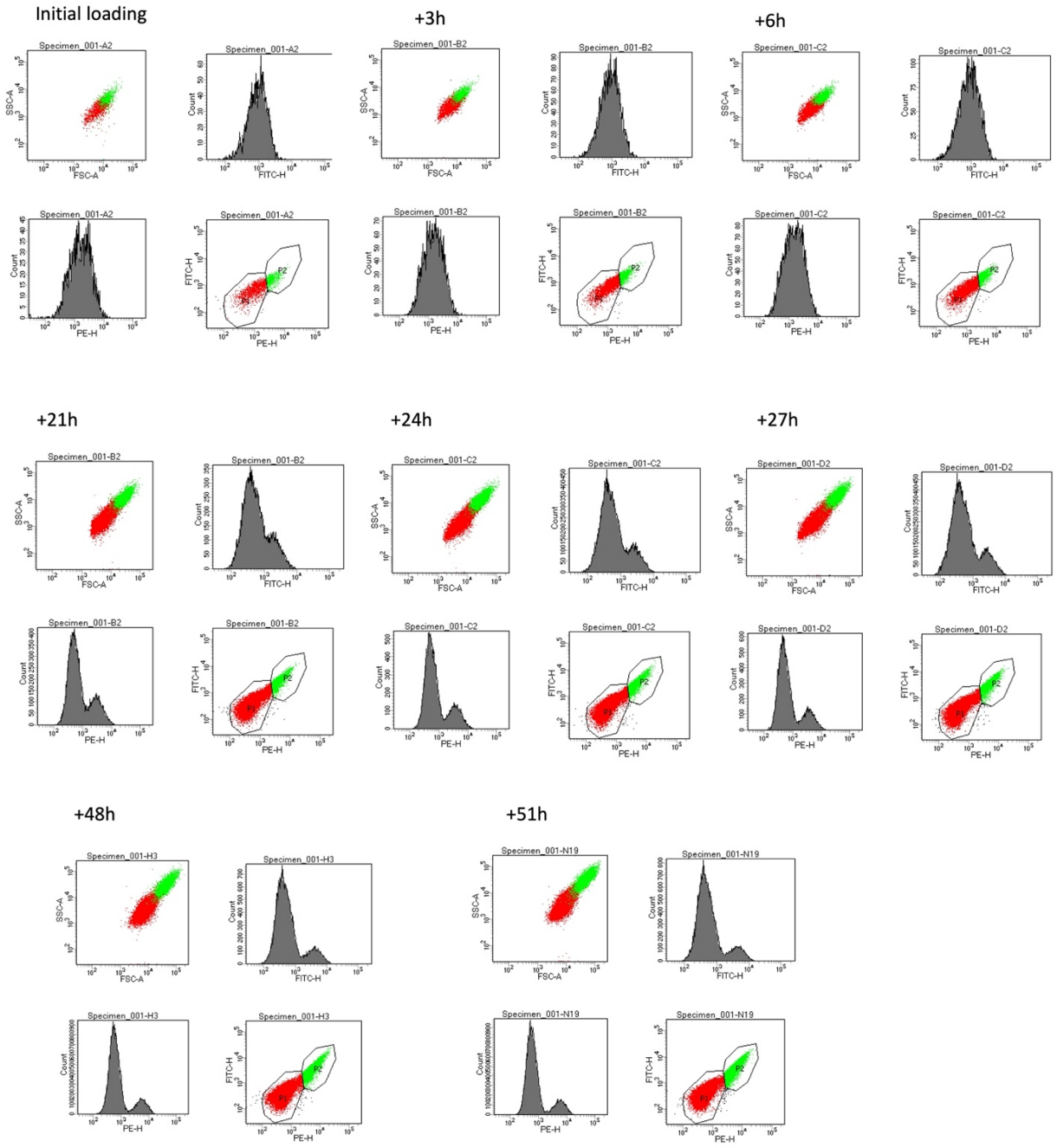
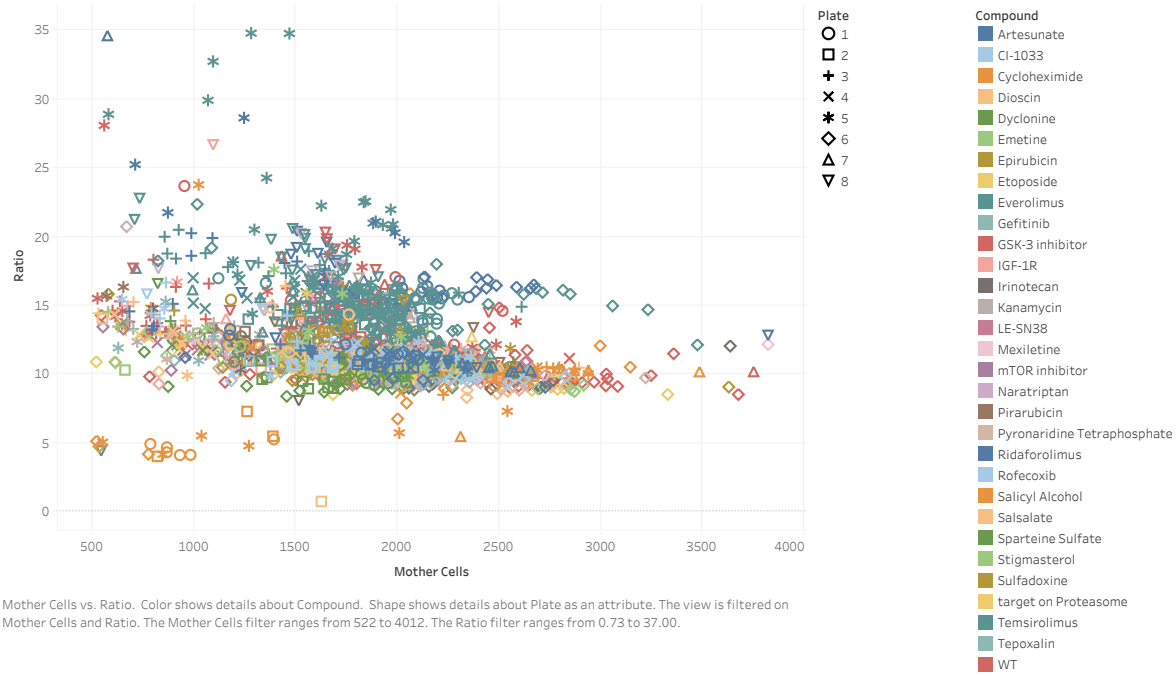


Figure s1. Temporal dynamics of GFP and RFP fluorescence in the Daughter Arrest Program (DAP) with histone marker yeast strain measured by flow cytometry. Samples were collected at the indicated time points and analyzed on a BD FACS Celesta flow cytometer equipped with 488 nm and 561 nm lasers for GFP and RFP excitation, respectively.



Mother Cells vs. Ratio. Color shows details about Compound. Shape shows details about Plate as an attribute. The view is filtered on Mother Cells and Ratio. The Mother Cells filter ranges from 522 to 4012. The Ratio filter ranges from 0.73 to 37.00.

Figure s2. Dose response assay summarized data.

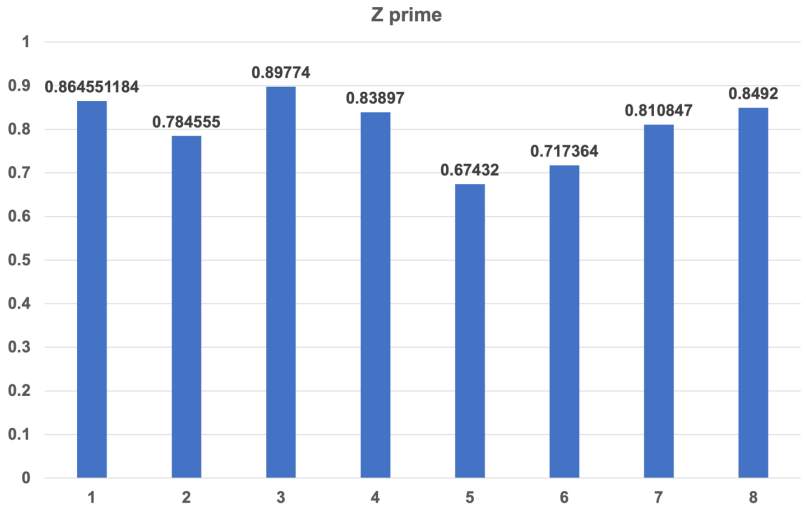


Figure s3. Z-prime analysis for phenotypic assay validation. The graph depicts the Z- prime values calculated across 8 different measurements or conditions for a phenotypic assay. The Z-prime is a statistical metric used to assess the robustness and quality of an assay, with values above 0.5 generally considered acceptable for high-throughput screening (HTS) assays. Each data point represents the Z-prime value obtained for the corresponding measurement or condition on the x-axis.

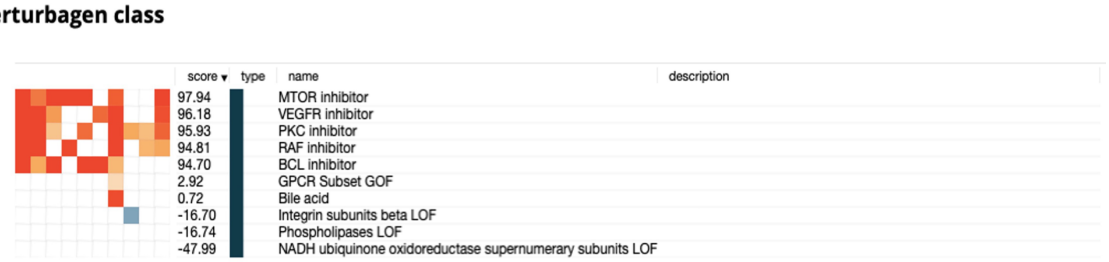


Figure s4. Connectivity Map (CMap) analysis of potential drug targets and mechanisms of action. The CMap analysis pipeline utilizes a gene expression signature derived from the positive hit compounds identified in the initial screening assay. This query signature is compared against a reference database of gene expression profiles induced by various perturbagens (com- pounds and genetic modifications) using a pattern-matching algorithm. Results from the CMap analysis, represented as a heatmap, reveal potential drug targets and mechanisms of action associated with the positive hit compounds. Each row corresponds to a perturbagen class, with columns indicating the connectivity score and a brief description. Positive connectivity scores suggest that the perturbagen induces a similar gene expression pattern as the query signature, while negative scores indicate an opposing pattern. The top-scoring perturbagen classes include mTOR inhibitors, VEGFR inhibitors, PKC inhibitors, RAF inhibitors, and BCL inhibitors, among others. These results implicate the mTOR, VEGF, PKC, RAF, and BCL pathways as potential targets or mechanisms underlying the anti-aging effects of the positive hit compounds. The CMap analysis provides a computational framework for generating hypotheses and prioritizing follow-up experiments.

DISCUSSION

The development of high-throughput screening platforms for measuring yeast replicative lifespan and identifying potential anti-aging interventions represents a significant advancement in the field of aging research. The novelty and potential impact of this work lie in the innovative technologies that accelerate the discovery process, the comprehensive screening of diverse compound libraries, and the insights gained into the genetic and molecular mechanisms of aging.

One of the key strengths of this research is the ability to rapidly screen a vast array of compounds and genetic modifications using the Daughter Arrest Program (DAP) strain [Hendrickson et al., 2018, Lindstrom & Gottschling, 2009]. This technological breakthrough enables the identification of novel interventions at an unprecedented pace, potentially revolutionizing our understanding of the aging process and facilitating the development of targeted therapies. The fluorescence-based assay further enhances the high-throughput capabilities, allowing for the efficient analysis of hundreds of drug treatment conditions and thousands of yeast cells [Hendrickson et al., 2018, Zimmermann et al., 2018].

The comprehensive screening of FDA-approved and bioactive compound libraries is another significant aspect of this research. By leveraging these diverse chemical libraries, the scope of potential anti-aging interventions is greatly expanded, moving beyond traditional candidates [Snell et al., 2012]. The identification of unexpected compounds that extend yeast lifespan, such as mTOR inhibitors and rapalogs, highlights the power of unbiased screening approaches and provides valuable insights into conserved pathways that regulate aging across species [Johnson et al., 2013, McCormick et al., 2015, Wang et al., 2021].

However, it is important to acknowledge the limitations and challenges in translating findings from yeast to humans. While yeast serves as a valuable model organism for studying the fundamental

mechanisms of aging, the complexity of human biology and the influence of environmental and lifestyle factors cannot be fully captured in a single-celled organism [Longo et al., 2012, Kaeberlein, 2010]. Therefore, the anti-aging effects of compounds identified in yeast will need to be validated in more complex animal models and eventually in human clinical trials. Additionally, the long-term safety and efficacy of these interventions will require careful evaluation, considering the potential for unintended consequences and the need for personalized approaches.

The genetic screening aspect of this research, utilizing non-essential gene deletion mutants and the DAmP library, sheds light on the complex interplay between genetics, environment, and aging. The identification of specific genes and pathways that modulate lifespan, such as the mTOR pathway, provides a foundation for exploring the conservation of these mechanisms in higher organisms [McCormick et al., 2015, Wang et al., 2021, Matecic et al., 2010]. These findings underscore the multifactorial nature of aging and the importance of considering both genetic and environmental factors in developing targeted interventions.

Looking forward, several exciting avenues for future research emerge from this work. The integration of high-throughput screening data with other omics technologies, such as transcriptomics, proteomics, and metabolomics, could provide a more comprehensive understanding of the molecular networks and pathways that govern aging. This systems-level approach may facilitate the identification of novel biomarkers of aging, the development of more targeted interventions, and the prediction of individual responses to anti-aging therapies [Longo et al., 2012, Kaeberlein, 2010].

Furthermore, the high-throughput nature of these screening platforms enables the generation of large datasets that can be mined using advanced computational tools, such as machine learning and artificial intelligence [Gallagher et al., 2020, Kaeberlein, 2010]. These approaches can uncover

hidden patterns and relationships in the data, leading to the discovery of new mechanisms and pathways involved in aging. The integration of computational biology with experimental research will be crucial for accelerating the pace of discovery and translating basic science findings into clinical applications.

Another promising direction is the exploration of combinatorial interventions that target multiple aging pathways simultaneously. By leveraging the knowledge gained from high-throughput screens, researchers can design rational combinations of compounds or genetic modifications that synergistically extend lifespan and healthspan. This approach may overcome the limitations of single interventions and provide more robust and effective anti-aging strategies.

Moreover, the insights gained from yeast aging research can guide the development of novel therapeutic strategies for age-related diseases in humans. Many of the pathways and processes implicated in yeast aging, such as mTOR signaling, autophagy, and mitochondrial function, are conserved in higher organisms and have been linked to various age-related pathologies [Longo et al., 2012, Kaeberlein, 2010]. By targeting these fundamental mechanisms, it may be possible to delay the onset or progression of multiple age-related diseases simultaneously, thereby extending healthspan and improving quality of life in the aging population.

In conclusion, the development of high-throughput screening platforms for measuring yeast replicative lifespan and identifying potential anti-aging interventions represents a significant milestone in aging research. The innovative technologies, comprehensive screening approaches, and insights into the genetic and molecular mechanisms of aging provide a solid foundation for future discoveries. As these findings are translated into more complex systems and eventually into clinical applications, they hold immense promise for promoting healthy aging, alleviating the burden of age-related diseases, and transforming the way we approach aging as a society.

Continued collaboration across disciplines, careful consideration of translational challenges, and the integration of computational approaches will be essential for realizing the full potential of this research and advancing the field of geroscience.

METHODS

Fabrication of the Microfluidic Device

(*protocol cited from related patent document by Dr.Changhui Deng)

To fabricate the microchip, we design two photomasks for two-layer UV exposure. The first layer is for the microstructure with 3.5 μm of the floor-to-top height and the second layer is for deeper flow channel with 30 μm of the floor-to-top height surrounding the island of the microstructures. Each channel span 3 wells with the left well for cell loading and media flowing in, the right well for media flowing out and the middle well for microscopic observation and taking photos or movie.

3D Printing of 32-Channel Cover

To load and wash cells and fill the microfluidic chips with water or cell culture media (YEPD or YEPD + drug), we design a 32-channel cover which has three channels: one vacuum sealing channel, one cell loading channel (with vent holes in wells in columns 1, 4, 7, 10), one flow-out vent channel (with vent holes in wells in columns 3, 6, 9, 12). The 3D structure of the cover is designed with AutoCAD and fabricated by 3D printing.

The side with channel vent holes is covered by a soft transparent PDMS gel with holes at appropriate positions, which can help seal the cover and the 96-well plate by applying vacuum.

Fabrication of the mode

Bake the wafer at 200 ℃ for 5 minutes to evaporate water vapor, followed by cooling down at room temperature for 5 minutes. For the first layer, dispense 4 ml of SU-8 3005 on 4-inch silicon wafer (for 16-channel) or 5 ml of SU-8 3005 on 5-inch wafer (for 32-channel), spin at 500 rpm for 10 seconds with acceleration of 100 rpm/second, spin at 5000 rpm for 30 seconds with acceleration of 300 rpm/second. Bake the coated wafer at 60 ℃ for 3 minutes, followed by baking at 95 ℃ for 3 minutes, then cool down at room temperature for 5 minutes.

Expose the SU-8 3005 photoresist under 12.7 mW/cm2 365 nm UV for 2 seconds, followed by baking at 60 ℃ for 3 minutes and 95 ℃ for 3 minutes, cool down at room temperature for 5 minutes.

For the second layer, dispense 4 ml of SU-8 2015 on 4-inch silicon wafer (for 16-channel) or 5 ml of SU-8 2015 on 5-inch wafer (for 32-channel), spin at 500 rpm for 10 seconds with acceleration of 100 rpm/second, spin at 1500 rpm for 30 seconds with acceleration of 300 rpm/second. Bake the coated wafer at 60 ℃ for 5 minutes, followed by baking at 95 ℃ for 5 minutes, then cool down at room temperature for 5 minutes.

Align the second layer photomask with the first layer alignment marks, expose the photoresist under 12.7 mW/cm2 365 nm UV for 12 seconds, followed by baking at 60 ℃ for 5 minutes and 95 ℃ for 5 minutes, cool down at room temperature for 5 minutes.

Develop the UV exposed photoresist in SU-8 developer with gentle shaking for 5 minutes, spray/wash the developed image with fresh developer solution for approximately 3x10 seconds, followed by a second spray/wash with Isopropyl Alcohol (IPA) for another 3x10 seconds. Air dry with filtered, pressurized air or nitrogen. Bake the imaged resist at 200 ℃ for 30 minutes, cool down at room temperature for 5 minutes.

Fabrication of the microfluidic chips

Immobilize the silicon wafer mold to a 15 cm-diameter plastic Petri dish using scotch tape, with the pattern side facing up. Each mold can be re-used many times to fabricate microfluidic chips.

Place a clean weighing boat on a balance and tare the balance. Pour 50 g of PDMS base into the weigh boat. Add 5 g of PDMS curing agent to the weigh boat (w/w ratio of 1:10 to the PDMS base). This volume is based on a 15 cm-diameter Petri dish with the mold. Adjust the amount of reagent if using a Petri dish of a different size.

Stir the PDMS base and the curing agent with a disposable pipette. Start from the edge of the weighing boat and slowly move inwards. Stir thoroughly for several minutes until small bubbles form throughout the mixture; thorough mixing is essential for PDMS polymerization.

Pour the mixture slowly into the Petri dish; the mixture must completely cover the silicon wafer mold. Place the Petri dish in a vacuum for 30 minutes to remove all the air bubbles from the PDMS mixture. If bubbles remain on the surface of the mixture, use a pipette to blow them out.

Incubate the silicon wafer mold filled with PDMS in an oven at 70 °C for about 2 hours. Cool down at room temperature for 30 minutes, then cut the PDMS directly from the silicon wafer mold with a minimum 5-mm margin around the pattern using a single-edge industrial razor blade, and gently peel the PDMS layer off the wafer mold. Be careful to avoid any damage to the construction of the wafer mold.

Place the PDMS layer on the cutting pad with the pattern side facing up, use a punch pen (1.2 mm I.D.) to punch holes straight down through the inlet and outlet circles on each side of the channels. These holes create the pathway for the flow of medium. Therefore, it is crucial to go through the circles and punch all the way through the PDMS layer. Make sure to remove the PDMS columns from the hole.

Check every punched hole by inserting the punch pen needle again into the hole. Make sure the needle can come out from the other side, indicating that there is no blockage. Apply tape to the pattern surface, then gently peel off the tape to clean dust particles. Repeat this step at least three times. Leave a clean piece of scotch tape on the PDMS to maintain sterilization. Repeat this procedure on the opposite side of the PDMS and leave the last piece of tape on as well.

Prepare a 50 x 75mm (for 16-channel) or 105 x 75 mm (for 32-channel) cover glass with a thickness of 0.13-0.17 mm. Spray 70% ethanol on the glass and dry with dust remover to clean the

surface; additionally, the glass can be washed with autoclaved water and dried with dust remover. Transfer the cover glass and PDMS to a plastic plate. Remove the scotch tape from the PDMS and place the pattern side facing up. Avoid any contact with the pattern surface during the transfer.

Place the plastic plate in the plasma machine. Apply oxygen plasma treatment to the PDMS and the cover glass to render the surfaces hydrophilic with the following operation parameters: exposure, 90 seconds; gas stabilization, 20 cc/min; pressure, 200; and power, 100 W.

Carefully align and place the PDMS onto the cover glass, connecting both hydrophilic surfaces (the surfaces that faced up during the plasma treatment). Make sure that there are no air bubbles between the PDMS and the cover glass. Incubate the PDMS chip in an oven at 70 °C for at least 2 h. Check the bond between the PDMS and the cover glass by slightly lifting the PDMS from the edges with tweezers, a successful bond wouldn't separate the PDMS from the cover glass by this lifting.

Assemble of Microfluidic Plates

Drill holes with a 2 mm diameter drill bit at columns 1, 3, 4, 6, 7, 9, 10, 12 in a 96-well flat bottom polystyrene microplate. Wash the plate with double distilled water to remove the plastic debris and dry it at 70 °C for 30 minutes.

Clean once the bottom surface of the plate and the PDMS surface opposite to the cover glass on the chip with scotch tape. Apply oxygen plasma treatment to the PDMS and the bottom surface of the plate to render the surfaces hydrophilic with the following operation parameters: exposure, 90 seconds; gas stabilization, 20 cc/min; pressure, 200; and power, 100 W.

Place two layers of kimwipes tissue on the plate bottom surface. Evenly add 3 ml 1% APTMS [(3-Aminopropyl)trimethoxysilane] aqueous solution on the tissue and remove the air bubble between the surface and the tissue. Incubate at room temperate for 30 minutes.

Evenly add 3 ml 1% GOTMS [(3-Glycidyloxypropyl)trimethoxysilane] aqueous solution on the PDMS surface and incubate at room temperate for 30 minutes.

Remove the excessive APTMS or GOTMS aqueous solution, then wash the plate bottom and the PDMS surface with double distilled water, remove the water remains with the dust remover, dry them at 37 ℃ for 30 minutes.

Align and attach the PDMS surface on the plate bottom, gently press the PDMS around the edge of the bottom to form a sealed environment. Seal the plate with the 32-channel cover and apply vacuum to remove the air between the PDMS and the surface of the plate bottom, keep the bonding at room temperature for 30 minutes, which will attach the microfluidic chip to the bottom of the 96-well plate very tightly. Release the vacuum.

Add 300 µl autoclaved double distilled water to the wells in columns 1, 4, 7, 10, seal the plate with the 32-channel cover by applying vacuum to the sealing channel, fill the microfluidic channels with water by applying 2 psi air pressure to the loading channel for 240 seconds, add 200 µl autoclaved double distilled water to the wells in columns 3, 6, 9, 12, cover with the lid and leave the microfluidic plate at room temperature for overnight, now the plate is ready for next-step experiment. Keep the chips filled with water will help prevent the channels from collapsing.

High throughput measurements of replicative lifespan

Lifespan measurement using the microfluidic device and microscope

Culture the IDS2GH or IDS2RH yeast cells in YEPG [For 1 L medium: Bacto yeast extract (Difco 0127-17) (1%) 10g, Bacto peptone (Difco 0118-17) (2%) 20g, Galactose (2%) 20g] at 30 ℃ for 15 hours, dilute the cell with the ratio of 1:20 in fresh YEPG and grow for another 3 hours, spin down the cells and wash 3 times with YEP [For 1 L medium: Bacto yeast extract (Difco 0127-17) (1%) 10g, Bacto peptone (Difco 0118-17) (2%) 20g], suspend the cells in equal volume of YEP,

then dilute the cells with 1:5 ratio in fresh YEP and incubate at 30 ℃ for another 3 hours, the last step is important to separate the aggregated cells.

Discard the water in the wells, add 100 µl of cells (~1x106 cells/ml) in cell loading wells in columns 1, 4, 7, 10. Seal the plate with the 32-channel cover by applying vacuum to the sealing channel, load cells by applying 4 psi air pressure to the loading channel for 10 seconds. Discard the remained cells in the wells, add 300 µl autoclaved ddH2O, wash the cells by applying 25 psi air pressure to the loading channel for 10 seconds. Discard the remained water in the wells, add 300 µl YEPD [For 1 L medium: Bacto yeast extract (Difco 0127-17) (1%) 10g, Bacto peptone (Difco 0118-17) (2%) 20g, Dextrose (2%) 20g], wash the cells with by applying 2 psi air pressure to the loading channel for 240 seconds. Discard the YEPD remains in the wells and add 300 µl fresh YEPD in each well in columns 1, 3, 4, 6, 7, 9, 10, 12. For drug screen, wash and culture cells by adding YEPD with appropriate concentration of drug. Incubate the plate with loaded cells at 30 ℃ for overnight.

Take the images after the cells are incubated for 84 hours. Upload the images to the server for next-step analysis. The dTomato signals are used for counting initial mother cells and the bright-field signals are used for counting total cell number. To calculate the replicative life span (RLS), discard the cells with multiple mother cells in one circle, therefore, for one selected yeast mother cell, its RLS = total cell number in the circle – 1.

Lifespan Measurement Using the Fluorescence-Based Assay

To prepare IDS2GH or IDS2RH yeast cells similarly to the DAP induction, culture them in YEPG [For 1 L medium: Bacto yeast extract (Difco 0127-17) (1%) 10g, Bacto peptone (Difco 0118-17) (2%) 20g, Galactose (2%) 20g] at 30°C for 15 hours. Subsequently, dilute the cells with a ratio of 1:20 in fresh YEPG and allow growth for an additional 3 hours. Centrifuge the cells, perform three

washes with YEP [For 1 L medium: Bacto yeast extract (Difco 0127-17) (1%) 10g, Bacto peptone (Difco 0118-17) (2%) 20g], resuspend in an equivalent volume of YEP, and dilute further at a 1:5 ratio in fresh YEP, incubating at 30°C for another 3 hours. This final step aids in dispersing cell aggregates.

Following aggregation separation, cells are cultured in YEPD with a drug solution diluted to 1:100. Plate the cells in either 96-well or 384-well plates and incubate at 30°C for three days. Analysis is performed using a flow cytometer (models BD Celesta and Symphony, equipped with a high-throughput plate loading station) using BD FacsDIVA software. The pipeline for data analysis includes(developed by Dr.Jiashun Zheng):

1. Classification of Cells: Utilize the RFP channel to classify events into single daughter cells and mother cells, with mother cells typically representing a smaller subset. This classification can be achieved by fitting a mixture model or using k-means clustering for two distinct classes.
2. Counting Mother Cells: Quantify the number of mother cells (M).
3. Counting Free Daughter Cells: Optionally count free daughter cells (D1).
4. Average Daughter Cell Fluorescence: Calculate the mean fluorescence intensity of GFP in single daughter cells (FITC-H).
5. Total Daughter Cell Count: Determine the total number of daughter cells by dividing the total GFP fluorescence by the mean fluorescence of single daughter cells (assuming most GFP contribution is from daughter cells), denoted as D.
6. Generation Calculation: Estimate the number of generations as D/M - 1, assuming that mother cells exhibit the same level of GFP as single daughter cells.

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