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The genetic architecture of lifespan extension by Dietary Restriction in the Budding Yeast

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ABSTRACT

Dietary restriction is probably the most promising non-pharmacological intervention to extend human life and health span. Yet, only a handful of the genetic regulators that mediate the cellular response to dietary restriction are known. To grant a much needed comprehensive view of how longevity by dietary restriction is elicited, we measured at the genome-wide level the chronological lifespan of *Saccharomyces cerevisiae* gene-deletion strains under two contrasting nitrogen-source regimens, glutamine (non-restricted) and γ-aminobutyric acid (restricted). We identified 472 mutants with diminished or enhanced extension of lifespan under limited nitrogen conditions. Functional analysis of such dietary-restriction genes allowed us to re-discover the cellular mechanisms of aging that have been previously associated with dietary restriction. Moreover, our analysis revealed novel processes underlying longevity elicited by nitrogen limitation. The list of genes that displayed important phenotypes for dietary restriction response allowed us to generate a prioritized catalogue of transcription factors orchestrating the dietary-restriction response, which underscored the relevance of cell cycle control as a mechanism of chronological longevity in yeast. Importantly, deletion of transcription factors Msn2, Msn4, Snf6, Tec1, and Ste12 resulted in defects in the cell cycle control upon nitrogen or carbon starvation. Flow cytometry experiments with rapamycin suggest that Ste12-dependent cell cycle arrest is not under the control of the TOR pathway, showing that the pheromone/invasive growth pathway might represent an alternative mechanism of regulation of cell survivorship in stationary phase in response to nutrients. Our global picture of the genetic players of longevity by dietary restriction highlights intricate regulatory cross-talks in aging cells.

RESUMEN

La restricción dietética es probablemente la intervención farmacológica más prominente para extender la esperanza de vida y de salud humanas. Sin embargo, se conocen tan solo un puñado de los reguladores genéticos que median la respuesta a la restricción dietética. Para adquirir una visión a nivel genómico de la longevidad por restricción dietética, medimos la esperanza de vida cronológica de mutantes sencillas de *Saccharomyces cerevisiae* bajo dos condiciones contrastantes de fuente de nitrógeno, glutamina como fuente rica de nitrógeno y ácido ɣ-aminobutírico como restricción de nitrógeno. Identificamos 472 mutantes con esperanza de vida disminuida o extendida bajo limitación de nitrógeno. El análisis funcional de estos genes permitió redescubrir los mecanismos celulares de envejecimiento que han sido asociados previamente a la restricción dietética. Más aún, este análisis reveló procesos nuevos que determinan la longevidad por limitación de nitrógeno. La lista de mutantes con fenotipos relevantes en respuesta al cambio de nutrientes permitió generar un catálogo de factores de transcripción que regulan la respuesta a la restricción dietética. Así mismo, la identidad de estos factores indica la relevancia del control de ciclo celular como mecanismo de control de la esperanza de vida cronológica. De forma importante, la mutación de los factores de transcripción Msn2, Msn4, Snf6, Tec1, y Ste12 resultó en defectos importantes en el arresto del ciclo celular en respuesta a la falta de nitrógeno y carbono. La dinámica del ciclo celular con rapamicina sugiere que el arresto del ciclo mediado por Ste12 en respuesta a la carencia de nutrientes no está bajo el control de la vía TOR. Esto último, sugiere que la vía de respuesta a feromona y el crecimiento filamentoso representan un mecanismo alternativo de regulación de la supervivencia en fase estacionaria en respuesta a nutrientes. La visión sistemática que aquí se presenta de los reguladores genéticos de la longevidad por restricción dietética realza la intricada disposición de la regulación del envejecimiento celular.

1. INTRODUCTION

Aging, defined as a decline in function throughout the lifespan of an organism, is considered the greatest risk factor in a plethora of chronic human diseases, including diabetes, cancer, and Alzheimer-disease (Lopez-Otin et al. 2013). Notably, recent improvements in health conditions have highlighted the role of age-related disease in human health. On top of that, the decrease in fertility observed in developed countries have resulted in aged human populations in need of specific treatments for aging-related conditions (Vaupel 2010; Lee 2011). Thus, understanding the molecular mechanisms that contribute to lifespan will lead to improved treatments for these diseases (Christensen et al. 2006). In this regard, several genetic, pharmaceutical, and nutrimental interventions that promote longevity in a diverse set of model organisms have been researched in an effort to translate this treatments into human life and health span extension (Humfrey 1998; Wanke et al. 2008; Morselli et al. 2009; Fontana & Partridge 2015).

Dietary restriction is the only intervention that has effectively been proven to prevent a wide range of age-associated changes. Importantly, the sole restriction of calories or other specific nutrients from the diet has shown to extend lifespan in nearly every organism studied in the laboratory (Masoro 2005). This intervention, known as dietary restriction, protects against age-associated disease in mammalian models, including neurodegenerative disorders (Zhu et al. 1999) and cancer (Yamaza et al. 2010; Martín-Montalvo et al. 2011), promoting not only a longer lifespan but also healthier aging (Fontana & Partridge 2015). Furthermore, dietary restriction delays the onset of aging-related physiological changes in humans (Holloszy & Fontana 2007; Omodei & Fontana 2011), making dietary restriction the most promising intervention targeted to extend human lifespan.

The great number of factors that shape lifespan makes it a particularly complex phenotype, where the study of the interactions between its components is much needed in order to gain a systems view that allows a deeper understanding on its mechanisms (Kirkwood 2008).In recent years, research on aging has taken advantage of genome-wide approaches, enabling a comprehensive description of

genes involved in lifespan regulation. Calorie restriction-genes have been targeted in the past with the aid of high-throughput screens methodologies, however few candidates have been put forward (Matecic et al. 2010). In addition, computational methods have predicted many more genes are involved in lifespan extension, exhibiting our lack of experimental information (Wuttke et al. 2012).

In this thesis, we focused on the phenotypic contribution to lifespan of aging-related genes and dietary restriction in single-gene deletions of *S. cerevisiae*, through the application of yeast large-scale phenotyping methods (Garay et al. 2014; DeLuna et al. 2008) that allow for precise identification of gene-diet interactions. Using this approach we have uncovered a great number of genes that modulate the response to dietary restriction. The results of this thesis provide the means for a systems view of lifespan extension by dietary restriction.

2. BACKGROUND

2.1 Dietary restriction

Over 80 years ago McCay *et al*. discovered that restricting the daily calorie intake lead to lifespan extension in rats (Mccay, C. M. 1935). Lifespan extension by calorie restriction is usually achieved by limiting the source of carbon without limiting essential nutrients to avoid malnutrition (Koubova & Guarente 2003). However, others forms of dietary restriction have been shown to have a beneficial effect in lifespan as well, these include the reduction in the nitrogen content (Jiang et al. 2000; Mirzaei et al. 2014) or restriction of specific amino acids from the diet (Powers et al. 2006; Ruckenstuhl et al. 2014). As lifespan extension can be elicited using different dietary protocols, the more general term, dietary restriction (DR), has been adopted to describe several forms of lifespan extension by nutrimental limitation (Mair & Dillin 2008).

Ever since its discovery, dietary restriction has proven to be effective in plenty of species, including yeast, nematodes, flies, rodents, dogs and primates (Masoro 2005), delaying the appearance of several age-related physiological markers in mice such as high blood pressure (Mager 2006), accumulation of oxidative damage in skeletal muscle (Lass et al. 1998) and accumulation of triglycerides (Wang et al. 2006). Moreover, dietary restriction also offers protection against age-associated disease in mice, including neurodegenerative disorders (Zhu et al. 1999) and neoplastic growth (Yamaza et al. 2010), increasing health span along with half-life and maximum lifespan (Weindruch et al. 1986). Importantly, this intervention reduces the mortality rate in non-human primates by delaying the onset of several diseases (Colman et al. 2014). Moreover, a couple of studies in humans that voluntarily consumed 30% fewer calories than age-matched control groups in the USA showed that dietary restriction provides a strong protection against type-2 diabetes, inflammation and cardiac dysfunction (Omodei & Fontana 2011). In addition older cohorts in human populations that have been historically subjected to DR show less signs of neurodegeneration and cancer (Holloszy & Fontana 2007; Fontana & Partridge 2015). DR is the only intervention that produces a robust result

in health span and lifespan in a variety of organisms, hence deciphering its molecular mechanisms promise to yield effective treatments for age-related disease.

2.2 Dietary restriction, an evolutionarily conserved mechanism of longevity

Senescence or aging is a byproduct of expression changes throughout the development of the organisms, some to optimize reproduction and fitness, but in the long run, these changes likely result in pleiotropic effects that ultimately bring inadequacy (Williams 1957). Due to the differences in life histories across multiple taxa, the final effectors that bring about developmental changes in different species might impact aging through specific or 'private' mechanisms (Martin et al. 1996). Notably, others mechanisms of aging have been proven to be 'public' or conserved. For instance, since the discovery of the first long-lived mutant in *C. elegans* (Kenyon et al. 1993), that carried a mutation in *DAF*-2 insulin receptor, mutations in other components of the conserved IIS/IGF/TOR nutrient sensing pathway have been shown to extend lifespan in distantly related organisms (Kaeberlein et al. 2005; Holzenberger et al. 2004; Fabrizio et al. 2001; Bartke 2008). These discoveries highlight the role of nutrimental status in lifespan regulation across taxa.

The evolutionary basis of the effects of dietary restriction has been proposed to trace back to a trade-off between reproduction and lifespan (Holliday 1989). The most cited explanation for the effect of dietary restriction is that organisms with genotypes able to delay reproduction during a temporal lack of resources might increase their fitness due to greater investment in maintenance processes, thereby maximizing their probabilities for reproduction later (Masoro & Austad 1996). In this regard, there is evidence that dietary restriction results in a steep decline in fecundity or delay in reproduction in *Drosophila*, *C*. *elegans* and mice (Partridge et al. 2005); moreover, ablation of the germline in nematodes lead to lifespan extension, further reinforcing the idea of a trade-off (Hsin & Kenyon 1999). In this same vein, Ratcliff et al. proposed a mathematical model that predicts the increase in the frequency of genotypes that facultatively delay reproduction in response to temporal resources shortages even without affecting individual fitness in several resources availability scenarios (Ratcliff et al. 2009). Assuming food shortages are ubiquitous for many

species, then increasing lifespan in response to specific environmental cues to enhance reproductive success might represent a direct evolutionary adaptation (Kirkwood & Shanley 2005).

Importantly, a conserved feature of dietarily restricted organisms is higher resistance to several types of stress (Fontana & Partridge 2015). For instance, dietary restriction in juvenile nematodes results in the formation of a dauer, which has enhanced resistance to oxidative and heat stress (Fielenbach & Antebi 2008). Likewise, several animal models subjected to dietary restriction show enhanced resistance to several types of stress through induction of autophagy, superoxide dismutase and heat-shock protein (Fontana & Partridge 2015). The hormesis theory of aging proposes that lifespan can be extended through exposition to mild stressors; in this sense, lifespan extension is a side-effect of the anti-stress response to dietary restriction (Masoro 1998). In support of this theory several stressors enhance longevity in *C*. *elegans* (Cypser & Johnson 2002), small doses of ionizing radiation and periodic immersion in cold water result in lifespan extension in rodents (Cameron 2005; Holloszy & Smith 1986). An extension of this theory states that the anti-stress pathways could be promoted through environmental cues that announce harsh conditions in advance (Sinclair 2005). This is intriguing given that resveratrol, curcumin and other plant compounds extend lifespan in animals by modulating the same pathways as dietary restriction (Howitz & Sinclair 2008). The study of these dietary restriction mimetics, is paving the way for pharmacological treatment of aging, hence the relevance of understanding the pathways that lead to longevity by dietary restriction.

Whether the response to dietary restriction has been conserved or it is the product of evolutionary convergence, the study of dietary restriction on model organisms has proven that aging is remarkably similar in distantly related phyla (Holzenberger et al. 2004; Smith et al. 2008; Gems & Partridge 2013; Fontana & Partridge 2015). In a sense, dietary restriction represents a true “public” mechanism of longevity, this is of particular importance since the study of its mechanisms in

laboratory models are susceptible of being translated into treatments for aging-related diseases in humans (Partridge & Gems 2002).

2.3 The genetics of longevity by dietary restriction

Despite the effects of DR were discovered a long time ago its mechanisms of lifespan extension have remained largely unknown, in part because most DR research focused on mice, where genetic techniques were not available. However, the introduction of model organisms in the aging field such as *Caenorhabditis elegans*, *Drosophila melanogaster* and *Saccharomyces cerevisiae*, has allowed better strategies to dissect lifespan extension by DR (Gems & Partridge 2013). Normally DR-related genetic interventions are identified comparing its effect with and without DR. This is, a genetic intervention that results in increased lifespan must decrease its effect as nutrients are limited. While interventions necessary for DR show the largest effect during full DR. Utilizing this comparisons several DR-related genes have been reported in laboratory models were the genetic tools are available (Mair & Dillin 2008).

The insulin/IGF-1 pathway was associated to aging and dietary restriction by the finding of a long-lived *C. elegans* strain that carried a mutation in the insulin receptor *daf-2*, in addition, lifespan extension required the activity of the FOXO transcription factor *daf-16* (Kenyon et al. 1993). These mutations were later linked to DR, as lifespan extension in nematodes requires *daf-16* (Kenyon 2011). Later it was shown that mutations in genes of the insulin/IGF-1 pathway result in lifespan extension in *D. melanogaster*, mammals (van Heemst 2010) and even in *S. cerevisiae* (Wei et al. 2008). Insulin/IGF-1 is a highly conserved pathway that signals the availability of nutrients through insulin receptor and FOXO transcription factors to regulate growth, reproduction and maintenance (Gems & Partridge 2013). In Ames dwarf mice, a mutation in Pit impairs their production of growth hormone, this hormone is the main activator of the IGF-1 pathway, thus resulting in low IGF-1 signaling (Bartke et al. 1998). Curiously, these mice are longer-lived compared to mice with normal levels of growth hormone (Brown-Borg et al. 1996). In addition, these same hormonal profiles have been observed in dietary restricted animals, suggesting that the IGF-1

pathway regulation is part of the DR mechanisms of lifespan extension (Koubova & Guarente 2003).

The TOR pathway is also a nutrient sensing pathway that has been implicated in lifespan extension, its master regulator, the target of rapamycin (mTOR), regulates protein and lipid synthesis, lysosome biogenesis, energy metabolism and autophagy (Laplante & Sabatini 2012). Interestingly, mTOR activity has also been related to type-2 diabetes and cancer progression, given that the highest risk for both diseases is age, diminished activity of mTOR is expected to produce health benefits (Laplante & Sabatini 2012). Concomitantly, genetic intervention of mTOR or inhibition by rapamycin results in lifespan extension in yeast, flies and nematodes and decreases the additional lifespan extension expected from DR (Johnson et al. 2013). In addition, inhibition of TOR decreases translation, enhances autophagy and increases respiration in all model organisms, this same profile is observed in organisms subjected to DR, linking the TOR pathway to lifespan extension by nutrient restriction (Kenyon 2010).

Several cellular processes are necessary for lifespan extension by DR, among them perhaps the most studied are autophagy, translation and mitochondrial respiration. In the case of autophagy, it presumably increases lifespan by degradation of aged cellular components maintaining cellular homeostasis (Johnson et al. 2013). In *C*. *elegans*, the disruption of genes necessary for autophagosome formation shortens the lifespan of *eat2* mutants, *eat2* mutants regularly display long lifespan due to low food intake, relating it indirectly with DR (Hansen et al. 2008). In yeast, deletion of autophagy genes *ATG1* and *ATG7*, decreases lifespan in rich medium; moreover, amino acid addition-related lifespan extension is suppressed in *atg1*Δ and *atg7*Δ mutant strains (Alvers et al. 2009). Recently, it was shown that lack of autophagy is a trigger of senescence in mammalian cells, moreover, that stem cells maintain their status by activating autophagy (García-Prat et al. 2016). However, it is not yet clear through which mechanisms is autophagy being deregulated in aging cells and how exactly autophagy maintains youthfulness.

Inhibition of translation results in lifespan extension in yeast and nematodes (Hansen et al. 2007; Steffen et al. 2008). Translation is activated by mTOR when nutrients are available (Johnson et al. 2013) dietary restriction leads to inhibition of translation in yeast (Schleit et al. 2013). The rationale for this relation has been explained through a possibility of damage infliction by translation (Hansen et al. 2007). Alternatively, the diversion of resources from this costly process to cellular maintenance can extend lifespan (Pan et al. 2007). Although it seems that reduction of translation is a step needed for lifespan extension by DR the genes that regulate this shift are unknown.

DR induces mitochondrial respiration in yeast and nematodes (Lin et al. 2002; Schulz et al. 2007), also deletion of raptor, a member of mTOR complex, in mice show increase mitochondrial respiration and protection against type-2 diabetes.. In agreement with increase respiratory rates during DR, it appears that the effect of DR on translation are selective, as mitochondrial translation remains the same upon treatment with DR (Miller et al. 2012). The mitochondria theory of aging states that reactive oxygen species (ROS) produced in the mitochondria are at least in part responsible for aging (Harman 1956). Concomitantly, it was discovered recently that deletion of elements of the electron transport chain decreases the effect of DR while increasing levels of ROS (Kwon et al. 2017). Although the reduction in ROS levels seems a plausible mechanism of lifespan extension by DR, the genetic manipulation of antioxidant enzyme SOD has given rise to conflicting evidence, as deletion of superoxide dismutase (SOD) does not shorten lifespan in all models, while in *C. elegans*, the deletion of *sod2* results in lifespan extension (Gems & Partridge 2013). A more detailed study of the interactions of mitochondrial genes with the environment could grant a deeper understanding of the relation between respiration and DR.

2.4 The budding yeast as an aging model

Since the first studies in *Saccharomyces cerevisiae* in which yeast cells were shown to have a finite number of replications (Mortimer & Johnston 1959), the budding yeast has continued to be one of the most relevant organisms where aging is studied

(Kaeberlein et al. 2007). The relative ease with which yeast can be manipulated in the laboratory and its short lifespan have kept yeast in the spotlight of discovery for new aging factors and dietary restriction factors (Kaeberlein 2010). There are at least two well-established models to study aging in the budding yeast. The first model to be explored was replicative lifespan, which refers to the number of daughters yeast cells are able to produce mitotically; in this vein, there is an asymmetric distribution of cellular damage resulting in a limited number of replications a single cell can undergo, the same has been observed for certain human cell lineages (Denoth Lippuner et al. 2014; Vaziri & Benchimol 1998). Chronological lifespan (CLS) is defined as the time a yeast population remains viable in stationary phase and is a better model of aging for post-mitotic cells (Longo et al. 2012). Noteworthy, dietary restriction affects both aging paradigms making them pivotal in aging research (Kaeberlein 2010).

One of the earliest contributions in the molecular mechanisms of aging resulted from a genetic screening in yeast, where a long-lived strain turned out to have a mutation in *SIR4*, an NAD+-dependent deacetylase. *SIR4* along with SIR2 and SIR3 participate in the assembly of silencing domains at telomeres, mutation of *SIR4* results in the formation of more silencing complexes at the rDNA, halting the formation of deleterious extrachromosomal circles with each replication (Kennedy & Guarente 1996). Although extrachromosomal circles are not produced in other model organisms during aging, the levels of expression of *SIR2* correlate with lifespan in nematodes and flies and its over expression leads to lifespan extension in mice (Finkel et al. 2009). Interestingly, sirtuins have been proposed to modulate lifespan in response to dietary restriction in yeast, nematodes and mice through the regulation of NAD+ levels (Guarente & Picard 2005). The latter suggests that while some specific-mechanisms of aging are not present in all taxa, the role of sirtuins in lifespan regulation is conserved, opening the possibility that other shared aging-factors have kept their ancestral roles in lifespan (Kaeberlein 2010).

Lifespan extension by dietary restriction is commonly achieved in yeast by reducing glucose concentration from 2% to 0.5% or lower, this has shown to extend

both chronological and replicative lifespan, indicating overlapping pathways between the two models (Kaeberlein 2010). Noteworthy, other dietary protocols result in lifespan extension in yeast, for instance Jiang et al. reduced the amino acid concentration in the medium, increased CLS was assumed to be due to lower caloric content (Jiang et al. 2000). However, the substitution of preferred amino acids also extend CLS without necessarily reducing the total amount of calories in the medium (Powers et al. 2006), also the replacement of glucose exhausted medium for water in stationary phase cultures can increase CLS (Fabrizio & Longo 2003). However, the fact that very different dietary protocols can enhance lifespan in yeast suggests that there is an important overlap between the mechanisms of longevity by multiple forms of dietary restrictions (Kennedy et al. 2007).

In yeast, dietary restriction regulates lifespan at least through the modulation of the conserved TOR and Ras/cAMP/PKA pathways, which regulate cellular growth and maintenance in response to nutrient availability (Kaeberlein et al. 2005). Moreover, deletion of some key the genes within this nutrient-sensing pathway *TOR1*, *SCH9* and *RAS2* results in long-lived strain (Kaeberlein et al. 2005; Powers et al. 2006). In the same way, low nutrient conditions inhibit TORC1 activity leading to activation of the serine/threonine kinase Rim15, which in turns phosphorylates transcription factors Msn2, Msn4, or Gis1, activating an anti-aging response (Fabrizio et al. 2004). However, the *msn2*Δ *msn4*Δ *gis1*Δ triple mutant still shows lifespan extension by DR (Wei et al. 2008). This observation suggests that there is an unknown number of longevity regulators that are yet to be identified.

The study of both aging models in yeast has already proven fruitful in the discovery of possible anti-aging therapies. For instance, the lifespan extension properties of the drug rapamycin were exposed using the CLS model in yeast (Powers et al. 2006; Kaeberlein et al. 2005). Rapamycin exerts its effect through inhibition of the conserved TOR complex, it has also been shown to extend lifespan in mice (Harrison et al. 2009) and is one of the few molecules being tested to treat aging in humans (Kraig et al. 2018). In addition, the lifespan effect of several genetic interventions within the TOR pathway has been revealed in yeast before uncovering

this effect in other organisms (Fabrizio et al. 2001). Likewise, spermidine, a naturally occurring polyamine found in ribosomes, was first reported to increase CLS in yeast by inducing autophagy (Eisenberg et al. 2009) and was later found to have cardiac protection and lifespan extension in mice (Eisenberg et al. 2016). Importantly, both of these molecules are considered dietary restriction mimetics, hence the study of dietary restriction in yeast will surely keep revealing future treatments for aging diseases.

There have been concerns about the conservation of yeast CLS mechanisms in other species, in particular given that during yeast cells excrete acetic acid, acidifying the medium and shortening lifespan, this trait has been called a private mechanism of yeast (Burtner et al. 2009). However, there are similarities between stationary phase yeast cells and mammals. For example, acetic acid signal inhibits Sod2 expression in yeast, in mammalian cells growth signaling leads to MnSod suppression and inhibition of G1 arrest through the activity of Sch9 homologue AKT (Burhans & Weinberger 2009). The power of the CLS model in yeast can be better illustrated with a particular case in humans, where a rare genetic polymorphism that causes a deficiency in growth hormone receptor, results in downregulation of IGF-1, *mTOR*, *PKA* (homologue of yeast *SCH9*) and *RAS*; this mutation renders them resistant to age-associated diseases (Guevara-Aguirre et al. 2011). The CLS of a yeast triple knockout mutant (*tor1*Δ *sch9*Δ *ras*Δ) was increased, suggesting that the health benefits observed in growth hormone receptor-deficient humans can translate into the CLS model of yeast.

In general, yeast aging models offer a reasonable explanation of how lifespan is determined, especially concerning the response to cellular damage, which appears as widely conserved across species. In addition, several biological functions including nutrient sensing, mitochondrial metabolism and mRNA translation are relevant for lifespan across taxa (Smith et al. 2008). This suggests both aging models in yeast will most likely continue to shed light on the mechanisms that underlie aging in other organisms.

2.5 Genome-wide screens for longevity factors in yeast

In recent years, aging research in yeast has taken advantage of genome-wide approaches and high-throughput screenings enabling a comprehensive description of genes involved in lifespan regulation. For instance, a recent systematic study of replicative lifespan of most viable deletion strains revealed that translation, the SAGA complex, and the TCA cycle mediate longevity (McCormick et al. 2015). Several studies have relied in a pooling strategy to estimate survival of single-gene deletion mutants in parallel through microarray profiling or bar-code sequencing (Matecic et al. 2010; Gresham et al. 2011; Fabrizio et al. 2010). A different approach is the direct phenotyping of each single deletion strain aged in co-culture with a wild type reference (Garay et al. 2014). These strategies have allowed the discovery of new aging-related processes such as fatty acid transport, tRNA methylation, cytoskeleton function, purine biosynthesis pathway and the Swr1 complex.

Importantly some of the mentioned studies aimed at finding nutrient-response genes, specifically screening for gene deletion strains under leucine and phosphate limitation (Gresham et al. 2011) and by switching stationary-phase cells to water (Fabrizio et al. 2010). Still, these lacked a nutrient-rich condition comparison, forbidding the appointment of specific dietary restriction genes. On the other hand, the work targeted by Matecic *et al*. calorie restriction-unresponsive strains, yet only two candidates were put forward. Noteworthy, an RLS screen of 166 gene-deletion mutants revealed that vacuolar pH, mitochondria Sod1 and mitochondrial proteostasis are required for lifespan extension by DR (Schleit et al. 2013).

Other strategies have increase our knowledge of dietary restriction factors in the CLS model. For instance, metabolomics profiling of single-deletion mutants under rich conditions revealed similarities of long-lived strains *hxk2*Δ, *gpa2*Δ and *tor1*Δ with WT cells aged under DR (Yoshida et al. 2010). Non phenotyping strategies have also been used to enquire dietary restriction response, in silico prediction based in a guilt-by-association network successfully predicted eight vacuolar genes necessary for lifespan extension by dietary restriction and recapitulated most processes previously related with dietary restriction (Wuttke et al. 2012). In addition,

a differential gene-expression analysis also revealed several genes to be up- and down-regulated in response to DR (Choi et al. 2013) , most of which lack direct phenotypic confirmation. Although lifespan extension by DR has been explored through genome-wide approaches, it appears that we are still missing a global picture of the genetic architecture of such a response, which is needed to grant a deeper understanding of the genotype-phenotype relationship of aging and longevity (Schleit et al. 2013).

3. HYPOTHESIS

Dietary restriction increases the survival of wild-type yeast populations in stationary phase. In addition, stationary-phase survival is a highly quantitative trait and varies greatly with the environment. Given those assumptions, we hypothesize that a large number of gene-deletions strains will impact the magnitude and direction of the effect of DR and these changes will be coherent with defined biological modules.

4. AIMS

The general goal of this work is to elucidate the genotype-phenotype relation of chronological lifespan extension by dietary restriction at a genome-wide level in the budding yeast *Saccharomyces cerevisiae*.

To accomplish the general goal of this work, we proposed the next set of specific aims:

1) To characterize an adequate dietary intervention to use in our large-scale phenotyping method.
2) To screen most viable deletion mutants in our collection under nutrient-rich and dietary restriction conditions. This aim requires to develop a custom statistical approach to detect genes that quantitatively change the effect of dietary restriction.
3) To analyze the transcriptional regulation of the newly-discovered set of DR-genes to find DR-related transcription factors.
4) To analyze the possible mechanisms of lifespan extension of master regulators of DR response.

5. METHODS

Strains and media.

Fluorescent single-gene deletion strains for the genome-wide screen are prototrophic haploids (*MAT*a *xxx∆::kanMX4 PDC1-mcherry-CaURA3MX4 can1∆:STE2pr-SpHIS5 lyp1∆ his3∆1 ura3∆0 LEU2*) derived from crossing the *MAT*α YEG01-RFP SGA-starter with 4,340 viable deletion strains from the *MAT*a BY4741 collection from the Saccharomyces Genome Deletion Project (Garay et al. 2014). All *de novo* single-gene deletions were generated in the YEG01-RFP parental strain (*MAT*α *PDC1-mcherry-CaURA3MX4 can1∆:STE2pr-SpHIS5 lyp1∆ his3∆1 ura3∆0 LEU2*) by direct gene replacement with the *natMX4* module conferring resistance to clonNAT. The Ste12 overexpression strain was generated by inserting the *CUP1* promoter and GFP-fusion construct from plasmid pYM-N4 in the 5' region of *STE12* ORF (Janke et al. 2004).

Non-restricted (NR) aging medium contained 0.17% yeast nitrogen base (YNB) without amino acids and ammonium sulfate, 2% glucose, 0.07% amino acid supplement mix (see **Appendix 1** for media summary). Dietary-restricted (DR) aging medium was prepared substituting glutamine with 25 mM of GABA. All other nitrogen sources tested (ammonium sulfate, methionine, asparagine, phenylalanine, leucine, isoleucine, and valine) were also supplemented at 25 mM. SC medium used for glucose restriction was 0.17% yeast nitrogen base (YNB) without amino acids, 0.2% amino acid supplement mix, and 0.5% or 2% glucose. Outgrowth cultures for all CLS experiments were performed in low-fluorescence medium (**Appendix 2**).

Nitrogen-starvation medium for cell-cycle progression experiments was 2% glucose and 0.17% yeast nitrogen base without amino acids and ammonium sulfate. Glucose starvation medium contained no glucose and 0.67% yeast nitrogen base without amino acids and 0.2% amino acid supplement mix.

Methods were taken from: Campos SE, et al., (2018) Genome-wide mechanisms of chronological longevity by dietary restriction in budding yeast. *In press*.

Automated competition-based CLS screens and data analysis

Fresh cultures of 4,340 tagged gene-deletion strains were replicated in 96-well plates (Corning 3585) with 150 μl of NR or DR aging medium. Deletion strains with slow growth and/or low fluorescence signal were discarded, only 4,050 strains passed this filter. In addition, several strains were lost during the subsequent steps of the automated experimental setup, resulting in the recovery of only 3,718 deletion strains that were tested under both glutamine and GABA. Saturated cultures were mixed with a CFP-labeled WT reference strain in a 2RFP:1CFP ratio for all mutants and WT controls to increase the dynamic range of mutant measurements, as many displayed low fluorescence. Mixed cultures were then replicated by pinning into 96-deep well plates (Nunc 260251), containing 700 μl of NR or DR aging medium, and grown at 30°C and 70% relative humidity, without shaking, in an automated system (Tecan Freedom EVO200) integrated to a multi-label plate reader (Tecan M1000). Four days after inoculation into deep-well plates, cultures were fully re-suspended and 5 μl outgrowth cultures were inoculated every other day into 150 μl of fresh low-fluorescence medium (**Appendix 2**) with the aid of an automated robotic arm. Absorbance at 600nm (OD_{600}) and fluorescence (*RFP* and *CFP*) measurements were taken every 150 min throughout 14 hrs with a Tecan multi-plate reader. An apparent survival coefficient, *s*, and its standard error, σ_s, were obtained from the slope of the linear regression (Robustfit, Matlab) of the log ratio of *RFP* to *CFP* signal at a fixed interpolation time point in the outgrowth culture (10 hrs), throughout 21 days in stationary phase.

Scoring CLS phenotypes and lifespan extension coefficients

Short- and long-lived knockouts under NR or DR were determined by assigning a *Z*-score ($Z = \frac{s_{mut} - \mu_{wt}}{\sigma_{wt}}$) to each mutant's *s* coefficient; the distribution's mean (μ) and standard deviation (σ) of the population were taken from the measurement of 264 WT_{RFP}/WT_{CFP} independent co-cultures under either condition, assuming equal distribution of errors in the mutants and the WT. Two-tailed *p*-values were obtained

from each *Z*-score to compute a false-discovery rate (FDR); we assigned significant phenotypes using a 5% FDR. While the mutants were not replicated under the same condition, the correlation of technical replicates for this assay has been measured previously (*r*=0.88, Pearson) (Garay et al. 2014).

The effect of dietary restriction on the gene-deletion strains relative to the WT was evaluated by calculating their relative lifespan extension defined as $LE = \frac{s_{DR}+1}{s_{NR}+1}$, where s_{NR} and s_{DR} are the *s* coefficients of a given deletion strain obtained from the screen under NR and DR, respectively. A *Z*-score was assigned to the LE of each mutant compared to the distribution of LE values of 264 independent WT reference experiments; significant LE<1 and LE>1 values were assigned at a 5% FDR.

Small-scale CLS assay measured by outgrowth kinetics

Selected strains were grown individually in the indicated non-restricted or dietary restriction media for 48 hours at 30°C 200 rpm in aerated tubes, then transferred to 96-well plates. In this step, each strain tested was inoculated several times in parallel from a single culture stock, the same setup was used throughout all our single-culture assays performed in deep well plates. These plates were replicated onto 96 deep-well plates containing 700 µl of NR or DR medium and left for the entire experiment at 30°C and 70% relative humidity without shaking. From here on, all experimental steps were performed in an automated robotic station (Tecan Freedom EVO200). After 4 days, 10 µl aliquots were taken with an automated 96-channel pipetting arm to inoculate 96-well plates containing 150 µl of low fluorescence medium. OD_{600} was obtained in a plate reader (Tecan M1000) every 1.5 hours until saturation was reached; this first outgrowth-kinetics curve was regarded as the first time point (T_0, age = 0 days). Sampling was repeated every 2-3 days for 21-28 days. From these outgrowth kinetics, we extracted the doubling time and the time shift to reach mid-exponential phase (OD_{600}=0.3) that occurred between the first day of measurements (T_0) and each day in stationary phase (T_n). Relative cell viability was calculated from these data, as reported by Murakami *et al* (Murakami et al. 2008).

Viability data points relative to T_0 were used to plot a survival curve, which was fitted (all data points) to an exponential decay model ($N(T) = N_0 e^{-rT}$) where N_0 is the percentage of viability at T_0, T is time in days, and r is the rate of death. For validation of CLS effects, mutants were taken from the RFP-tagged deletion collection (or generated *de novo*, when indicated) and viability was assayed to calculate death rates in at least 7 experimental replicates which were compared to replicates of a WT strain; significant CLS effects were considered using a $p<0.05$ cutoff (*T*-test).

CLS assay in standard aeration conditions

Standard culture conditions were used, as described (Hu et al. 2013). In brief, pre-inoculums from three different colonies of each strain were set in 5 mL SC medium for 24 hours, these were diluted 1:100 v/v in 10 mL SC with 2% glucose or 0.5% glucose aging medium in 50 mL tubes for 48 hours with shaking (200 rpm) at 30 °C. Viability at each measuring point was obtained by monitoring the change in outgrowth-kinetics parameters with time in stationary phase using three technical replicates for each colony tested, as described above.

Visualization of functional clusters

Gene Ontology (GO) associations and phenotype terms were downloaded from the *Saccharomyces* Genome Database (SGD, last updated December 2016) to build two *m* by *n* matrixes, where *m* is the number of DR genes (219 and 253 for $LE<1$ and $LE<1$, respectively) and *n* is the number of GO and phenotypic terms (1,748). Each term was used to evaluate the overall agreement between gene-pairs to calculate Cohen's *kappa* ($kappa = \frac{\mathrm{Pra}(a)-\mathrm{Pr}(e)}{1-\mathrm{Pr}(e)}$). Where *Pra*(*a*) is the number of associated-terms and not associated- terms that each gene-pair shares, divided by the total number of terms downloaded from SGD in the matrix (denominator is the same for any gene pair), and *Pr*(*e*) is the hypothetical probability for each member of the gene-pair to be associated by chance.

A matrix representing the agreement between each gene-pair was built with the *kappa* values. Gene-pairs that showed *kappa*>0.35 were regarded as likely similar, according to previous reported thresholds for large datasets (Huang et al. 2009). Gene-pairs formed in the first step were used as cluster seeds to form larger groups of genes; *ie*. groups of genes that shared at least 50% of their members merged in subsequent iterative steps, thus creating larger groups in each iteration until only groups with dissimilar members remained. Clusters with at least four elements were manually named by inspection in the SGD and GO enrichment. We performed all procedures related to Kappa calculation and analysis on Matlab Network representation was created using Cytoscape; edges between nodes represent kappa agreement above the established threshold (*kappa*>0.35).

TFRank analysis

TFRank was used at http://www.yeastract.com/ formrankbytf.php using the *TF Rank* algorithm option (Gonçalves et al. 2011), with a heat diffusion coefficient of 0.25. All DR-genes were introduced as Target ORF/Genes, with all transcription factors selected (*n*=197). A weight is assigned to each TF according to the presence or absence of target genes and their regulators in the regulatory network of *S. cerevisiae*. The weight of the transcription factor increases according to the number of direct and indirect targets to rank the transcription factors; this weight is re-evaluated through a diffusion coefficient, which takes into account the number of layers of the transcription factors hierarchy to obtain a list of prioritized regulatory players.

Alive/dead staining assay

We used the same scheme of 96 deep-well plates (one plate per replicate) to age cells in SC with 0.5% or 2% glucose. Each day, a single well of each strain was collected. Cells were centrifuged, washed, and dyed with LIVE/DEAD® FungaLigth™ Yeast Viability Kit, following manufacturer's instructions. Propidium iodide (IP) and Syto®9 fluorescence were measured by cell cytometry (LSRFortessa™, Becton Dickinson) at early stationary phase (4 days after

inoculation) and at different time-points until 21 days in stationary phase. IP was excited with a 591-nm laser, fluorescence was collected through a 586/15 band-pass filter; Syto9 was excited with a 488-nm laser, and fluorescence was collected through 525/50 band-pass and 505LP emission filters. Cell-viability percentage was obtained by staining dead and alive cells with SYTO9 and subtracting the number of dead-cell events only as stained by propidium iodide.

Cell-cycle assays

WT and mutant strains were grown in flasks containing 50 ml of NR aging medium at 30 °C and shaken at 200 rpm until mid-logarithmic phase ($OD_{600} \cong 0.5$). Cells were centrifuged, washed twice with sterilized water, and transferred to nitrogen or glucose starvation medium. For rapamycin-induced arrest, 10 nM rapamycin was directly added to mid-log phase cell cultures. Samples were taken at the moment of either transfer to starvation medium or rapamycin addition (time 0) and 1, 2 and 4 hours after that time point. Fixation and dying with SYTOX™ Green were performed as described elsewhere. Cells were analyzed by flow cytometry (LSRFortessa™, Becton Dickinson); SYTOX Green was excited with a 488-nm laser, and fluorescence was collected through a 525/50 band-pass filter.

RNA-seq analysis

Three different colonies of the WT and *ste12*Δ strains for each condition were cultured overnight at 30°C, 200 rpm in 7 ml tubes SC medium. OD was diluted to 0.1 in 5 ml SC in separated 25 ml plastic tubes for each colony. Cultures were grown until OD was 0.8 at that moment rich medium samples were frozen in liquid nitrogen. Nitrogen depletion samples were centrifuged, washed once with deionized water and then re-suspended in 5 ml SC 2% glucose medium without ammonium sulfate, samples were frozen after 4 hours. Samples were thaw and RNA extraction was performed with the RiboPure kit (Applied Biosystems, AM1926). mRNA enrichment was performed with the Qiagen Oligotex mRNA Mini Kit (Cat. # 70022) and then samples were concentrated with the Zymo RNA Clean & Concentrator Kit 5 (Cat. #R1015). cDNA libraries were prepared with the NEBNext Directional RNA-Seq kit

(Cat. # E7420). Library quantification was done with Qubit and quality was checked on Bioanalyzer. cDNA libraries were sequenced with HiSeq4000 at the Center for Advance Technology at UCSF, San Francisco. Sequencing reads were mapped to Saccharomyces cerevisiae genome using *kallisto* (Bray et al. 2016) and transcripts abundances were analyzed using sleuth (Pimentel et al. 2017).

6. RESULTS

6.1 Identification of lifespan extension by nitrogen source substitution

To describe the genetic architecture of lifespan extension by DR, we set out to establish an adequate mode of dietary restriction for high-throughput phenotyping. We selected 9 nitrogen sources based in a classification reported by Godard *et al*., where amino acids were classified in three distinct groups, namely preferred, intermediate and poor nitrogen sources according to the gene-expression profiles these induced in yeast (Godard et al. 2007). We measured the lifespan of a wild-type (WT) strain aged under the different nitrogen sources by adapting an established method for quantitative analysis of yeast CLS based on outgrowth kinetics (Murakami et al. 2008) to a high-throughput robotic platform (see Methods). The half-life of the WT strain varied substantially, from 5.6 days in the rich nitrogen source ammonium to 26.5 days in GABA (**Table 1**; **Figure 1A**). The use of GABA as the sole nitrogen source resulted in extended lifespan without major effects on the growth kinetics (**Table 1**; **Figure 1B**); therefore, we selected GABA as our dietary-restricted condition. The non-restricted medium chosen was glutamine as the sole source of nitrogen, as it resulted in one of the shortest half-lives measured, it provided growth at a rate similar to GABA, and it has been classified as a preferred nitrogen source (Godard et al. 2007). The choice of a non-preferred nitrogen source for DR instead of limited glucose concentrations overcomes the dramatic metabolic changes due to glucose repression in yeast (Kresnowati et al. 2006), while facilitating the parallel characterization of stationary-phase cultures in low volumes, given that cell yields are similar under non-restricted and dietary-restriction conditions. Ammonium sulfate was not used because of its toxicity in stationary phase (Santos et al. 2015). Further testing of our selected conditions, showed a robust lifespan difference as nitrogen-based dietary-restriction model resulted in a 91% extension of the CLS, from a WT half-life of 14.7 days in glutamine to 28.1 days in GABA (**Figure 1C**).

Results sections 6.1 to 6.7, Table 1 and Figures 1 to 13 were modified from: Campos SE, et al., (2018) Genome-wide mechanisms of chronological longevity by dietary restriction in budding yeast. *In press*. Supplementary data might be found at http://www.langebio.cinvestav.mx/deluna/CamposThesis/

Table 1. Chronological lifespan of the WT strain aged under different nitrogen sources

Nitrogen source	Death rate, *r* [a]	Half-life, days [a]	Doubling time, hours [b]
Ammonium	-0.137 ± 0.010	5.6 ± 0.4	2.70 ± 0.11
Methionine	-0.061 ± 0.005	11.4 ± 0.9	3.59 ± 0.09
Glutamine	-0.057 ± 0.004	13.1 ± 1.0	2.64 ± 0.09
Asparagine	-0.049 ± 0.004	15.3 ± 1.0	2.69 ± 0.09
Phenylalanine	-0.046 ± 0.006	15.9 ± 1.8	2.91 ± 0.12
Leucine	-0.041 ± 0.003	18.0 ± 1.3	4.31 ± 0.02
Isoleucine	-0.041 ± 0.004	18.3 ± 2.2	3.05 ± 0.12
Valine	-0.039 ± 0.004	18.9 ± 2.1	2.76 ± 0.07
GABA	-0.029 ± 0.005	26.5 ± 4.4	2.82 ± 0.07

a. Death rate and half-life are obtained from fitting survival curves to an exponential decay model; values are the average ± S.E.M. from at least 5 experiments.

b. Obtained from the rate during the exponential phase of growth.

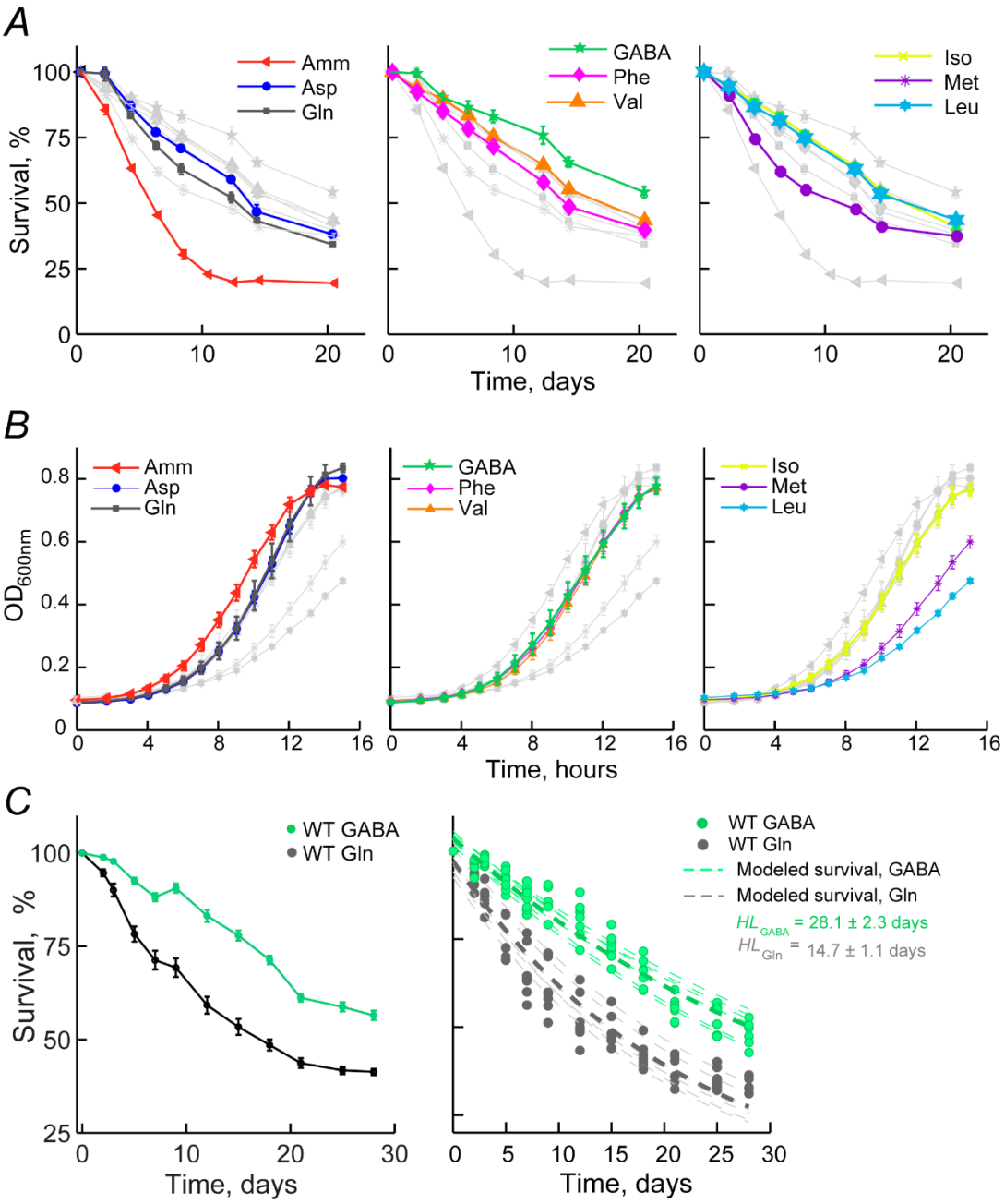


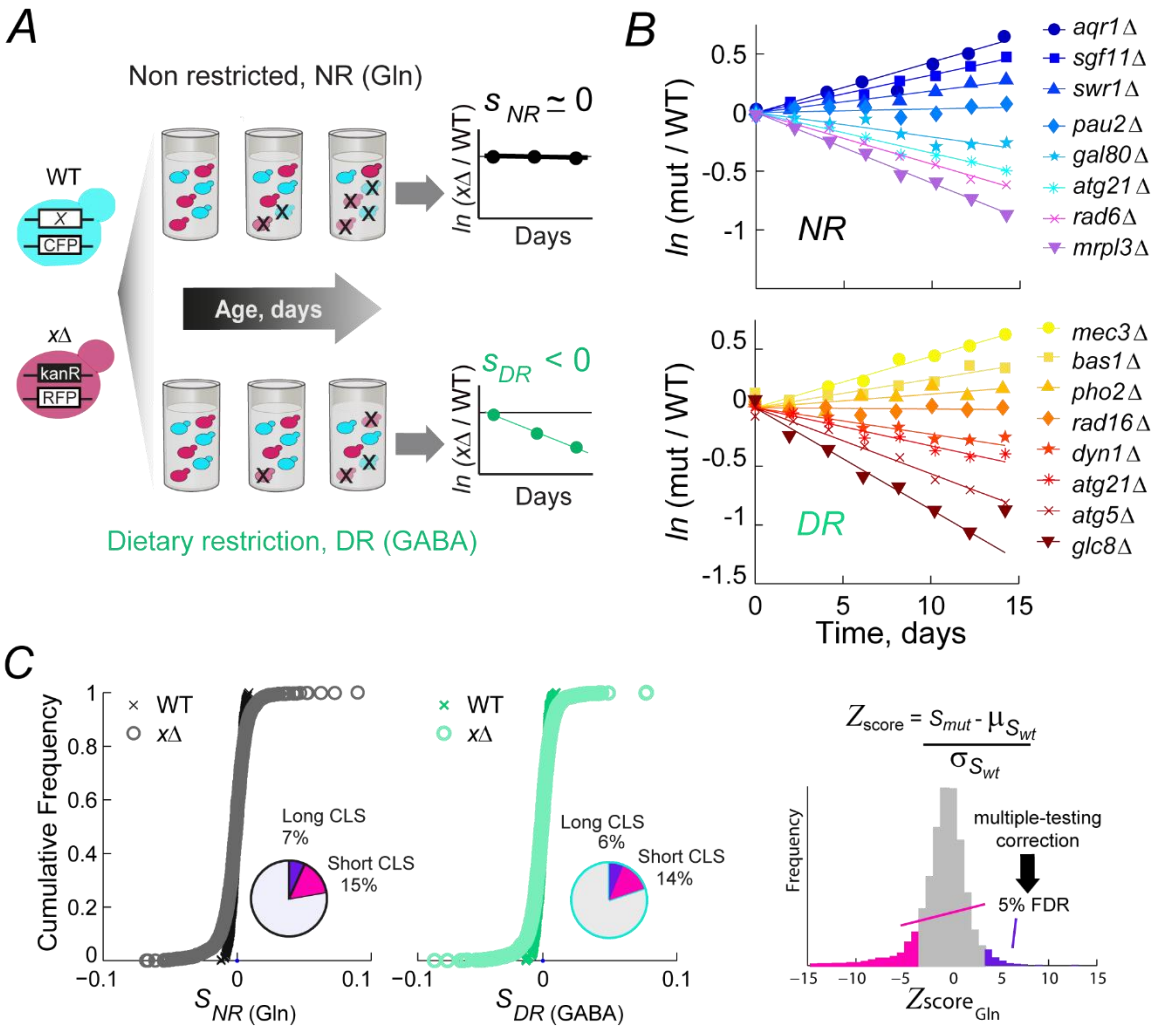
Figure 1.

The CLS of yeast is affected by the quality of the nitrogen source in the aging medium. (**A**) Survival curves of the WT strain obtained with a CLS method based on outgrowth kinetics (adapted from Murakami et al. 2008); survival curves on different nitrogen sources are shown according to transcriptomic response to supplementation with these amino acids (Godard et al. 2007): rich (left), intermediate (middle) and poor nitrogen sources (right). All of these conditions were tested in a single 96-deepwell plate, to avoid experimental batch variation, at least 6 replicates were aged and measured for each condition. (**B**) Growth kinetics of the WT strain under the different nitrogen sources. (**C**) An independent experiment for survival of the WT strain aged under Glutamine and GABA (left); error bars are the S.E.M. (n=6). Data of each survival curve was adjusted to an exponential decay model to obtain the decay rate and half life under each condition (HL, right).

Supplementary data might be found at http://www.langebio.cinvestav.mx/deluna/CamposThesis/

6.2 Genome-wide profiling of gene-deletion strains under two conditions

To identify the genes that determine lifespan under dietary restriction at the genome-wide level, we measured the CLS of 3,718 gene-knockout strains under GABA (dietary-restricted) and glutamine (non-restricted) media. We used an assay developed at our laboratory, based on the measurement of a relative survival coefficient (*s*) of each knockout strain aged in co-culture with the WT strain in rich or restricted medium (**Figure 2A**). Our model of stationary-phase survivorship assumes exponential death, thus the natural logarithm of the ratio of each mutant strain and the WT reference was adjusted to a linear fit; short-lived mutants display negative slopes (survival coefficient, *s*) while long-lived strains show positive slopes (see examples under the two conditions in **Figure 2B**). In addition, we obtained the *s* of 264 WT *versus* WT competitions under both nutrimental conditions and used the mean and standard deviation of this population to calculate a Z-score for each mutant, assuming that the variance in the mutants was the same as in the WT. Finally, we used the Benjamini-Hochberg false-discovery rate (FDR) to correct for multiple hypothesis testing (**Figure 2C**). Using a FDR threshold of 5%, we scored 573 significantly short-lived and 254 long-lived single knockout strains in the non-restricted medium, while dietary restriction resulted in 510 short-lived and 228 long-lived mutants (**Data-S1;**). A considerable fraction of the strains tested showed condition-specific CLS effects (**Figure 2D**). Importantly, the error in the fitted model of the mutants was not correlated to the magnitude of the CLS effect (**Figure 3A-B**).



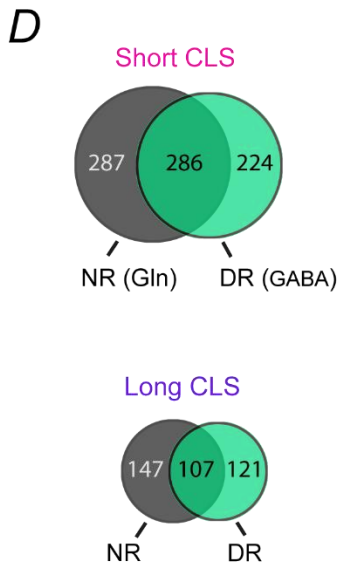


Figure 2.

Genome-wide profiling of chronological-lifespan under two dietary regimens. (**A**) Schematic representation of the screening strategy; an RFP-labeled deletion strain (xΔ) is aged in co-culture with a CFP-labeled WT strain; a survival coefficient (s) relative to WT is obtained for each mutant under each aging condition. (**B**) Examples of the survival coefficients of selected long-lived and short-lived mutants under glutamine (non restricted, upper panel) and GABA (dietary restriction, lower panel) are shown. For comparison purposes, initial offsets were corrected to start at zero. (**C**) Cumulative distributions of s values for the 3,718 single deletion strains under glutamine (grey circles) and GABA media (green circles), 264 WT replicas were included (black and green crosses). Right, Z-scores were obtained (only glutamine is shown) by comparing the mutants' survival coefficient (s) to the s distribution of WT-reference experiments; multiple-testing correction was used to define hits at a 5% FDR. (**D**) Venn diagrams show the overlapping short- and long-lived mutant strains scored under glutamine (NR, dark grey) and GABA (DR, green).

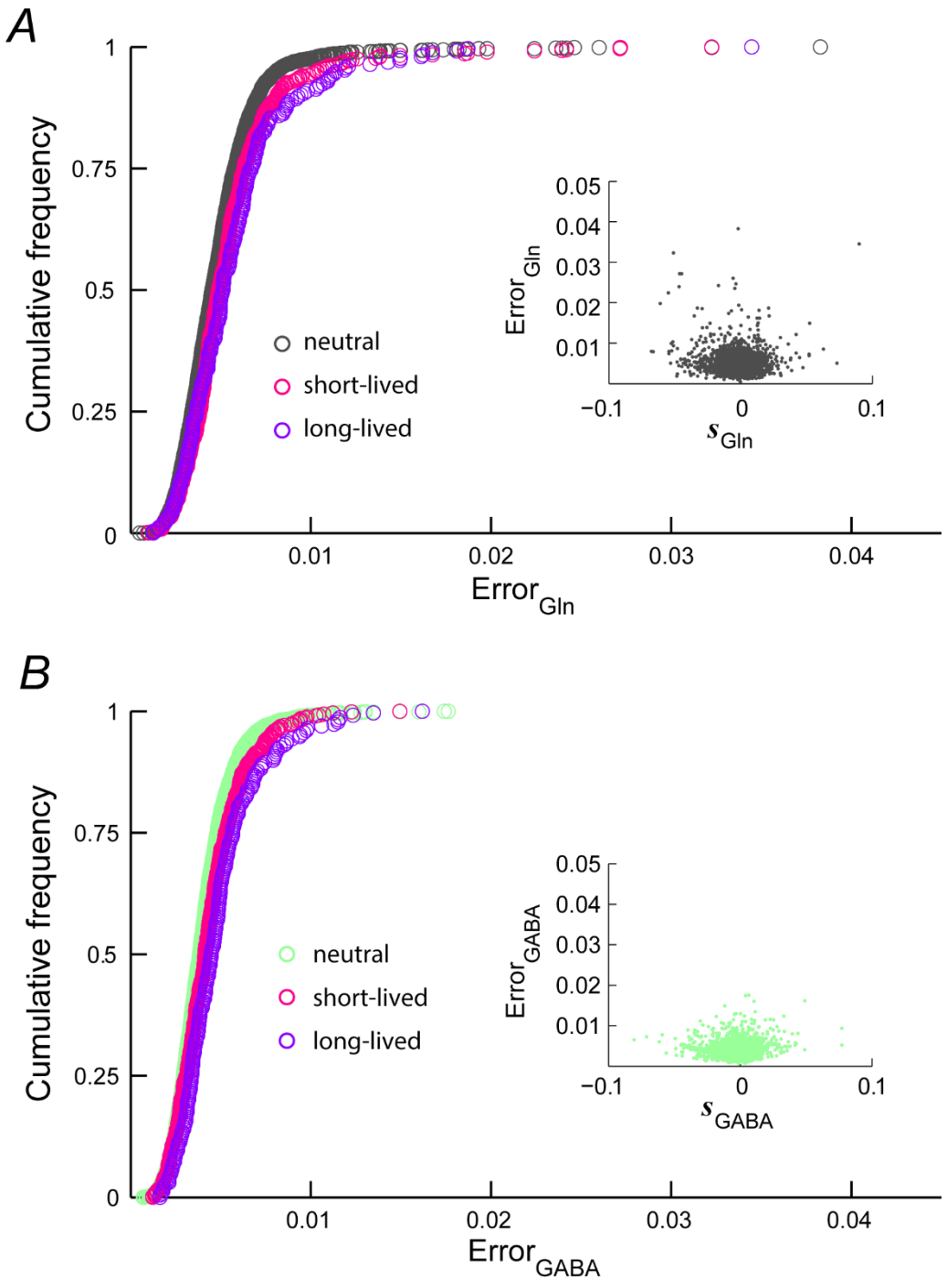


Figure 3

The error in the fitted model shows no correlation with lifespan phenotypes scored nor estimated survival coefficients. Cumulative frequencies of the errors in the survival coefficients measured under (**A**) glutamine or (**B**) GABA for neutral, short-lived, and long-lived knockout strains. The error is from the coefficients of the linear regression equation calculated by robust fit (Matlab). Insets show the error in the fit as a function of the survival coefficient, s.

To validate the results of our genome-wide screens, we turned again to the small-scale outgrowth-kinetics CLS approach (Murakami et al. 2008) and measured in single-strain cultures the lifespan of a set of randomly-selected knockouts with a CLS effect in the genome-wide screen (5% FDR). First off, we calculated survival as a function of days under each of the two nutrient conditions and then obtained a rate of death by assuming exponential death, mutant death rates were compared to those of WT replicates (see **Methods**). Twelve out of 16 (75%) strains that were re-tested under non-restricted glutamine medium recapitulated the CLS effects observed in the genome-wide screen (**Figure 4A**; $p<0.05$, *T*-test), while 11 out of 17 (65%) strains were consistent with the results under GABA dietary restriction (**Figure 4B**). Notably, the rate of confirmed hits is considerably higher compared to screening assays that have used pooled yeast deletion strains (6–31% hits validated) (Fabrizio et al. 2010; Matecic et al. 2010). To directly compare the results obtained with the conventional single-culture CLS assay and with our high-throughput screen, we modeled a survival curve from the survival coefficients of the corresponding knockout strains (**Figure 4A-B**). Together, the results of these validation experiments indicate that our profiling approach provides accurate and quantitative CLS scores under different conditions, allowing the description of gene-diet interactions underlying this phenotype.

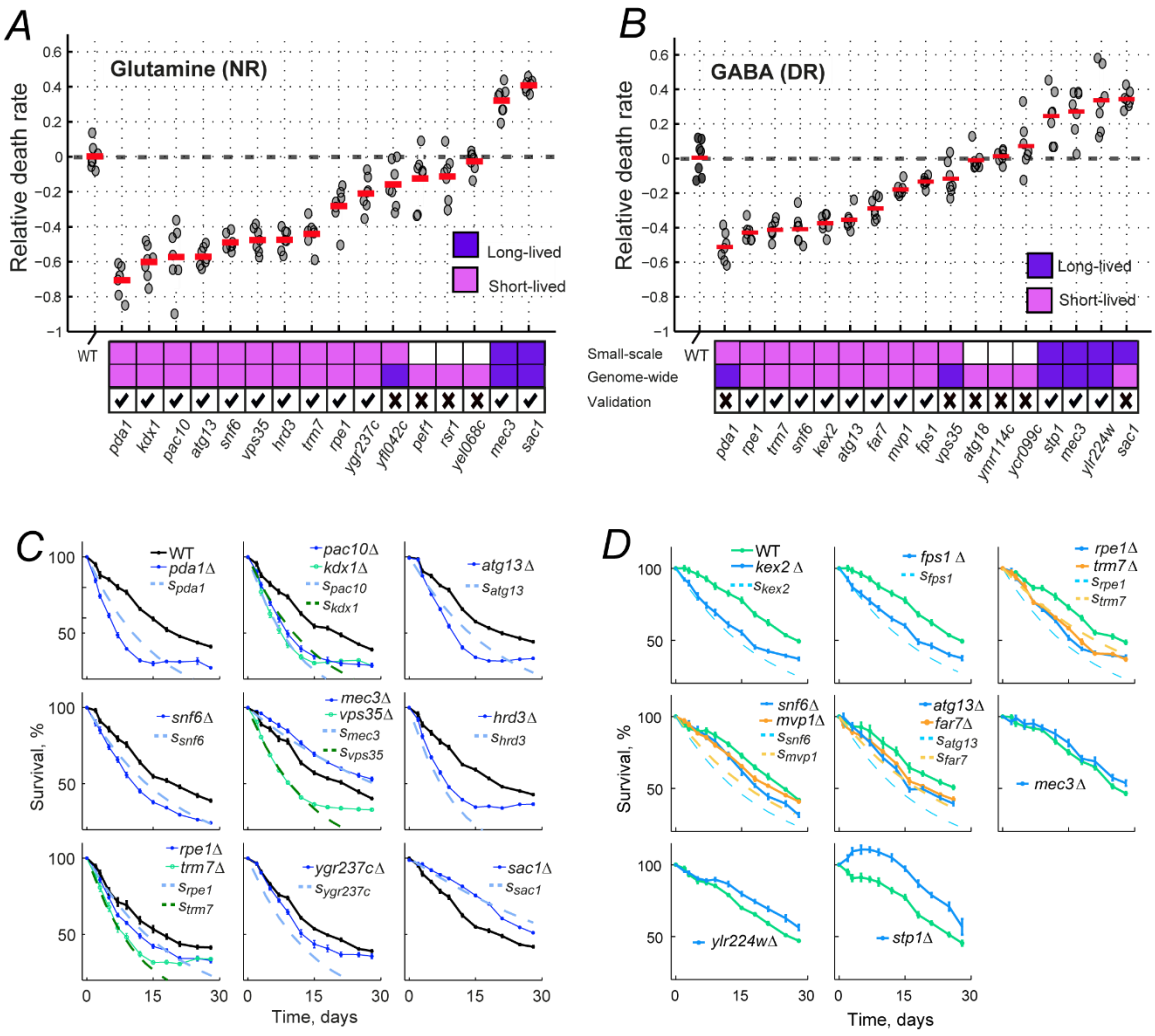


Figure 4

Validation of CLS hits from the genome-wide screen. Top plot shows the relative rates of death of WT and mutant strains, -1*ln(rx / rwt), aged under (**A**) glutamine and (**B**) GABA medium, measured by outgrowth kinetics. Death rates were obtained by fitting the survival curves to an exponential decay model, as shown in Figure 1C.. Hits from the genome-wide screen were considered valid when the death rate of the corresponding mutant was consistent (short-lived or long-lived) and statistically different from that of the WT ($p<0.05$, T-test). Bottom plots show the survival of single cultures (solid lines) in (**C**) glutamine or (**D**) GABA ; only validated strains are shown, along with the specific WT of each experimental batch. Error bars are the S.E.M. (n=7). In addition, survival coefficients (s) from the genome-wide screen where used to model a survival curve for the corresponding knockout strain (dashed lines). Given the slow death rate of yeast cells in GABA, positive survival coefficients $s>0.02$ cannot be used to model survival curves, and are therefore not show for the long-lived *mec3*Δ, *ylr224w*Δ, and *stp1*Δ knockouts in GABA.

6.3 Systematic identification of dietary-restriction genes in yeast

To gain insight into the genes that mediate the lifespan-extending effects of dietary restriction, we searched for deletion strains that showed important differences when

comparing the relative CLS under the two nitrogen sources tested. Specifically, we sought to identify strains that showed a shorter survival coefficient under DR compared to the non-restricted glutamine medium (diminished lifespan extension); likewise, we scored cases where the survival coefficient was relatively larger under DR (enhanced lifespan extension) (see examples in **Figure 4**).

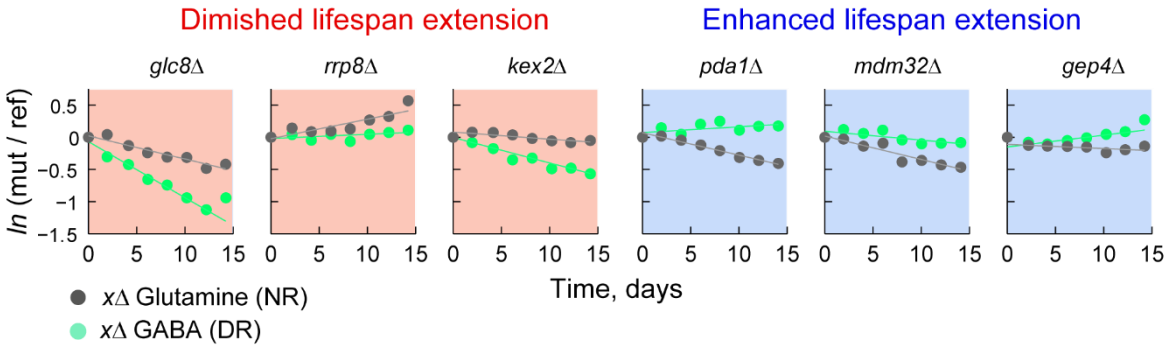


Figure 5

Dietary restriction-genes show differential CLS under DR. Selected knockout strains with differences in their selection coefficients obtained under glutamine (non restricted) and GABA

We quantitatively defined the relative lifespan extension of each knockout as $LE = \frac{s_{DR}+1}{s_{NR}+1}$ (see Methods). Next, search for genes with an LE statistically different from that of WT, to do this we used the LE of 264 independent WT replicates to obtain a *Z*-score (**Figure 6A**). After correcting for multiple tests we initially scored 583 mutant strains with a significant LE; for higher stringency, we filtered out strains that did not show a significant CLS effect in either condition. The final list included 472 gene-knockouts with altered dietary-restriction response (5% FDR; **Figure 6B**; **DATA-S2**). This comprehensive set, which we termed DR-genes, includes 219 knockouts with diminished longevity (LE<1) and 253 gene-knockouts that displayed enhanced lifespan extension (LE>1). These results indicate that many gene-environment interactions underlie longevity by GABA dietary restriction in yeast.

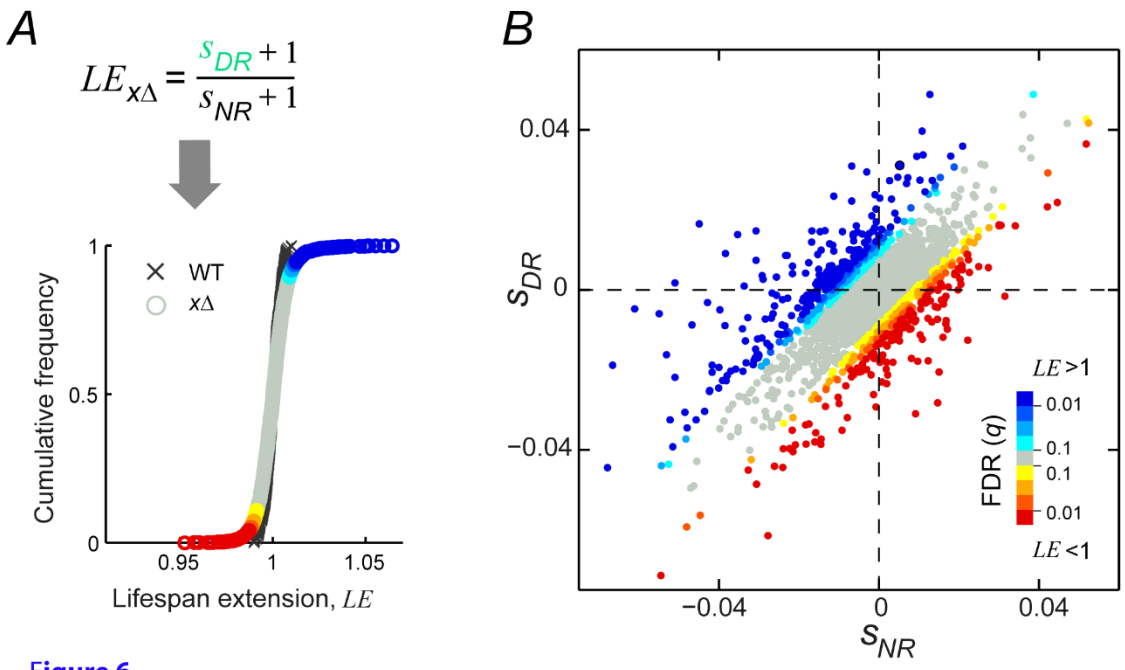


Figure 6

A comprehensive compendium of dietary-restriction genes. (**A**) A relative lifespan extension, LE, was calculated for each mutant strain (circles) and compared to that of the WT replicates (crosses) to score statistical significance. (**B**) Scatter plot of the sSurvival coefficients of 3,718 deletion strains under non-restricted medium (horizontal axis) and dietary restriction (vertical axis); data points above or below the diagonal are colored according to the lifespan extension (LE) significance (q).

6.4 Functional classification of dietary-restriction genes

To describe the cellular functions related to lifespan extension by DR, we sought to classify the 472 DR-genes according to their annotated functional features. We used a kappa statistic approach (Huang et al. 2009) to cluster genes by shared GO terms and mutant phenotypes—as reported in the Saccharomyces Genome Database—, providing functional associations between the gene deletions. Gene-pairs were deemed as similar when Kappa value was above 0.35, in accordance to previously reported thresholds for large gene databases (Huang et al. 2009). With this approach, genes were clustered into discrete groups, allowing a non-supervised identification of the cellular processes underlying DR. The analysis was performed separately for genes with diminished (LE<1) or enhanced (LE>1) lifespan extension; moreover, kappa values above the threshold were used to create a network of

functional gene relationships (**Figure 7A-B; Data-S3**). Some clusters recapitulated cellular functions previously related to lifespan regulation, such as autophagy (Meléndez et al. 2003), mitochondrial function (Aerts et al. 2009), and cytosolic translation (Pan et al. 2007; Hansen et al. 2007). Importantly, this classification also revealed novel processes such as maintenance of cell-cycle arrest mediated by pheromone and establishment of nucleus localization in the cell. Our clustering approach recapitulated functions previously associated to dietary restriction and allowed the appointment of novel biological processes.

Clusters with diminished lifespan extension included mutant strains that displayed exacerbated deleterious effects under DR and gene-deletions where beneficial CLS effects on rich-medium were concealed under DR. For instance, deletion of genes necessary for the maintenance of cell-cycle arrest *FAR7*, *FAR8* and *FAR11* and INO80 chromatin remodeling protein *IES5* resulted in diminished lifespan extension, suggesting a functional association between INO80 complex, cell cycle control and longevity. Moreover, novel regulators of longevity were found in gene-deletions required for processing and correct localization of ribonucleoprotein complexes, which resulted in a strongly diminished lifespan extension. Likewise, deletion of *DYN1-3*, *JNM1*, *NUM1*, and *PAC1*, all involved in nuclear movement along microtubules, resulted in diminished lifespan extension. The way in which these processes regulate lifespan are yet to be explored.

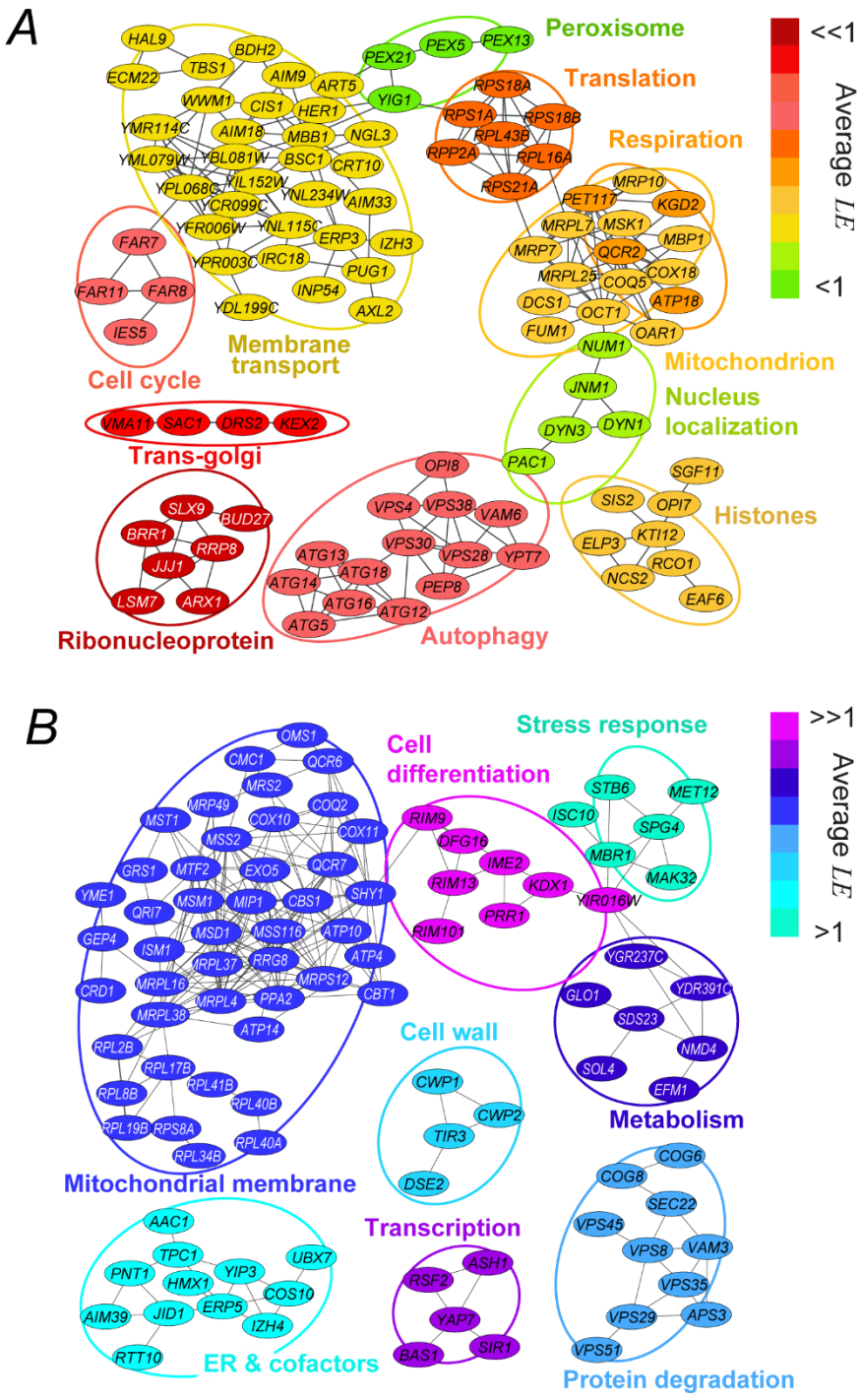


Figure 7

Functional clustering of dietary-restriction genes. Network representation of functional clusters of genes with (**A**) diminished (LE<1) and (**B**) enhanced (LE>1) lifespan extension. Edges denote agreement between pair of genes (kappa>0.35) denoting functional association., which suggests functional association between the genes; clusters were formed between at least four associated genes. Node color code indicates decreasing or increasing ranks of mean LE for each cluster.

Clusters with enhanced lifespan extension included mostly gene-deletions with deleterious effects that were buffered or alleviated under DR. A great number of mitochondrial genes (Mitochondrial membrane) fell under this category, although specifically deletion of respiratory genes were among the strains with diminished lifespan extension (**Figure 7A-B**). Most of the genes within this category of lifespan extension present buffering mechanisms for CLS deleterious effects. On the other hand, no single cluster arose with only gene-deletion with beneficial effects under NR and no significant Gene Ontology enrichment was found for genes with this specific phenotypes either. Surprisingly, several gene-deletions within the Protein degradation cluster showed buffered lifespan extension which was not expected from a set of genes related to autophagy. Importantly, genes appointed in the Stress response and Cell wall clusters have not been linked to longevity before. Interestingly, the network representation shows that in some cases clusters are not completely discrete entities, but rather they are linked to other clusters possibly due to real functional associations between genes. Taken together, these findings suggest that lifespan extension in response to dietary restriction is a complex phenotype resulting from the interplay of many downstream cellular processes.

6.5 Lifespan extension by dietary restriction is regulated by a specific set of transcription factors

Lifespan extension by DR is regulated by at least several signal pathways that activate *de novo* transcriptional responses (Mair & Dillin 2008). To determine the transcription factors (TF) that regulate our set of DR-genes we recurred to TFRank (Gonçalves et al. 2011), a graph-based approach that takes advantage of all known regulatory paths in *S. cerevisiae.* A weight is assigned to each TF according to the presence or absence of target genes and their regulators in the regulatory network of *S. cervevisiae*. The weight of the transcription factor increases according to the number of direct and indirect targets to rank the transcription factors; this weight is then re-assigned through the diffusion coefficient, which takes into account the number of layers of the transcription factors hierarchy to obtain a list of prioritized regulatory players. Based on this analysis, we established a possible role in lifespan

extension to the top-ranked transcription factors (**Table 2**); the analysis also revealed a number of possible regulators that were not included in our gene-knockout screen due to gene essentiality or sterility. Transcription factors within the top 5% ranked DR-regulators included Msn2 and Msn4 which are well-known regulators of lifespan (Fabrizio et al. 2004). Intriguingly, most top-ranked transcription factors had not been previously associated to longevity in yeast. Noteworthy, the top hits (Ace2, Ash1, Tec1, Spf1, and Ste12) regulate different aspects of cell-cycle progression.

Table 2. A catalogue of transcription factors regulating dietary-restriction genes in yeast (DR-regulators, top 5% rank)

Rank	TF	Weight[a]	% Regulated[b]	Description
1	Ace2	3.15	75.5	Involved in G1/S transition of the mitotic cell cycle; activates cytokinetic cell separation
2	Ash1	2.94	50.4	Negatively regulates G1/S transition of mitotic cell cycle; activates pseudohyphal growth
3	Tec1	2.81	63.0	Transcription factor targeting pseudohyphal-growth genes and Ty1 expression
4	Sfp1	2.55	66.3	Regulates ribosomal-protein genes, response to nutrients and stress, and G2/M transitions of the mitotic cell cycle
5	Ste12	2.45	58.2	Activates genes involved in mating or pseudohyphal-growth pathways
6	Bas1	2.36	44.9	Involved in regulating the expression of genes of purine and histidine biosynthesis
7	Snf6	2.19	37.4	Subunit of the SWI/SNF chromatin remodeling complex
8	Msn2	2.10	54.3	Regulation of transcription in response to a wide variety of stresses
9	Yrm1	1.79	44.5	Transcription factor involved in multidrug resistance
10	Gcn4	1.76	46.7	Activator of amino acid biosynthetic genes; responds to amino acid starvation
11	Ixr1	1.73	26.4	Transcriptional repressor that regulates hypoxic genes during normoxia
12	Abf1	1.723	45.3	DNA binding protein with possible chromatin-reorganizing activity
13	Msn4	1.65	44.0	Regulation of transcription in response to a wide variety of stresses
14	Rap1	1.53	41.4	Essential DNA-binding transcription regulator; role in chromatin silencing and telomere length

a. TFRank weight (Gonçalves et al. 2011).

b. Percentage of dietary-restriction genes regulated by the transcription factor.

To evaluate the validity of the TFRank algorithm to uncover transcription factors specific of DR response, we compared the ranks of the transcription factors that showed gene-deletion lifespan phenotypes either in glutamine or GABA media (CLS transcription factors) with that of transcription factors showing no lifespan effect (non-CLS transcription factors). DR-regulators with a deletion phenotype were typically ranked higher by the TFRank algorithm than regulators with no CLS effect ($p<10^{-5}$, Wilcoxon ranked-sum test) (**Figure 8**). The average ranks for transcription factors were 63.5 and 112.9 for regulators with and without a CLS gene-deletion phenotype, respectively. This indicates that the downstream DR-genes and cellular pathways identified in our genome-wide screen are linked to a coherent set of transcription factors that influence the CLS of yeast.

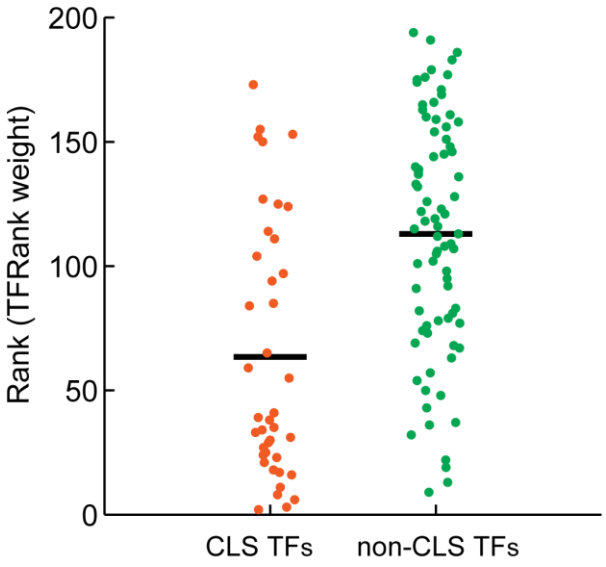


Figure 8

Regulatory ranks accurately predicts lifespan phenotypes of transcription factors. Plots indicate the rank of transcription factors from TFRank-analysis of DR-genes (n=197). Transcription factors with a gene-deletion effect are shown in red (CLS TFs, n=40) and those without an effect are shown in green (non-CLS, n=79 out of 119). Only 119 transcription factors measured under both dietary conditions are considered. Solid black lines indicate the average rank of each group.

The set of DR-genes was defined from our genome screens under a particular dietary-restriction regime based on nitrogen limitation. To test the generality of DR transcriptional regulation regardless of the mode of DR, we evaluated the CLS those DR-regulators in lifespan extension by calorie restriction, a classic form of DR in yeast and other organisms (Koubova & Guarente 2003). To do this we generated *de*

novo deletion mutants for eight top-ranked transcription factors and measured their CLS in 2% and 0.5% glucose media using the small-scale outgrowth-kinetics approach. Strikingly, the deletion of all of these transcription factors resulted in altered CLS under 0.5% glucose: six strains were short-lived, while the remainder strains (*ace2*Δ and *bas1*Δ) were long-lived under the glucose-restricted condition (**Figure 9**). As expected, the same mutants showed altered CLS when characterized under glutamine or GABA as nitrogen source (**Figure 10**). Although the observed lifespan effects under glucose- or nitrogen-restricted conditions were moderate, this is in agreement with the fact that transcription factors in yeast typically act upon overlapping targets, providing functional compensation to one another (Zheng et al. 2010). Together, these results show that top TFRank hits are general regulators of lifespan extension by dietary restriction in yeast.

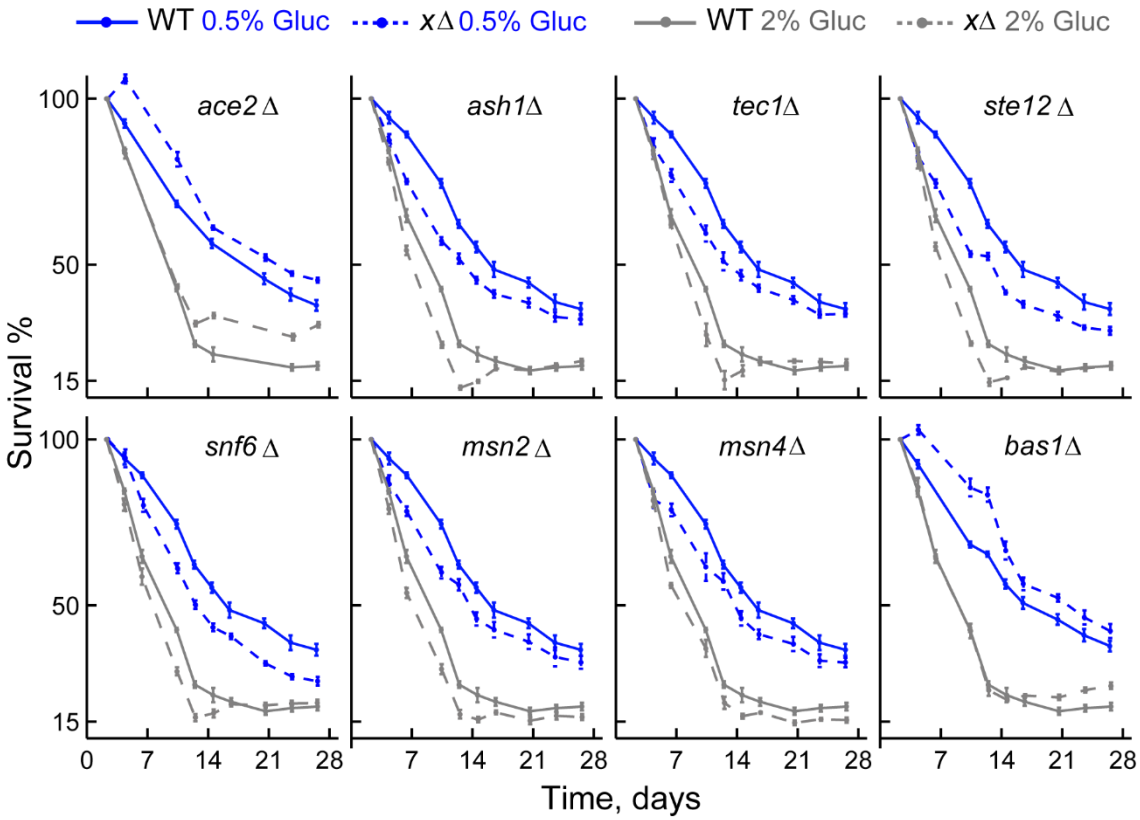


Figure 9

Altered chronological lifespan in strains deleted for dietary-restriction regulators. Survival curves of WT and gene-deletion strains aged in SC medium with 2% glucose (non-restricted, grey lines) or 0.5% glucose (dietary restriction, blue lines). Transcription factor Ddeletion strains are shown in discontinuous lines (discontinuous lines) are for genes coding for transcription factors Ace2, Ash1, Tec1, Ste12, Snf6, Msn2,v Msn4, and Bas1. Error bars are the S.E.M. (n=5).

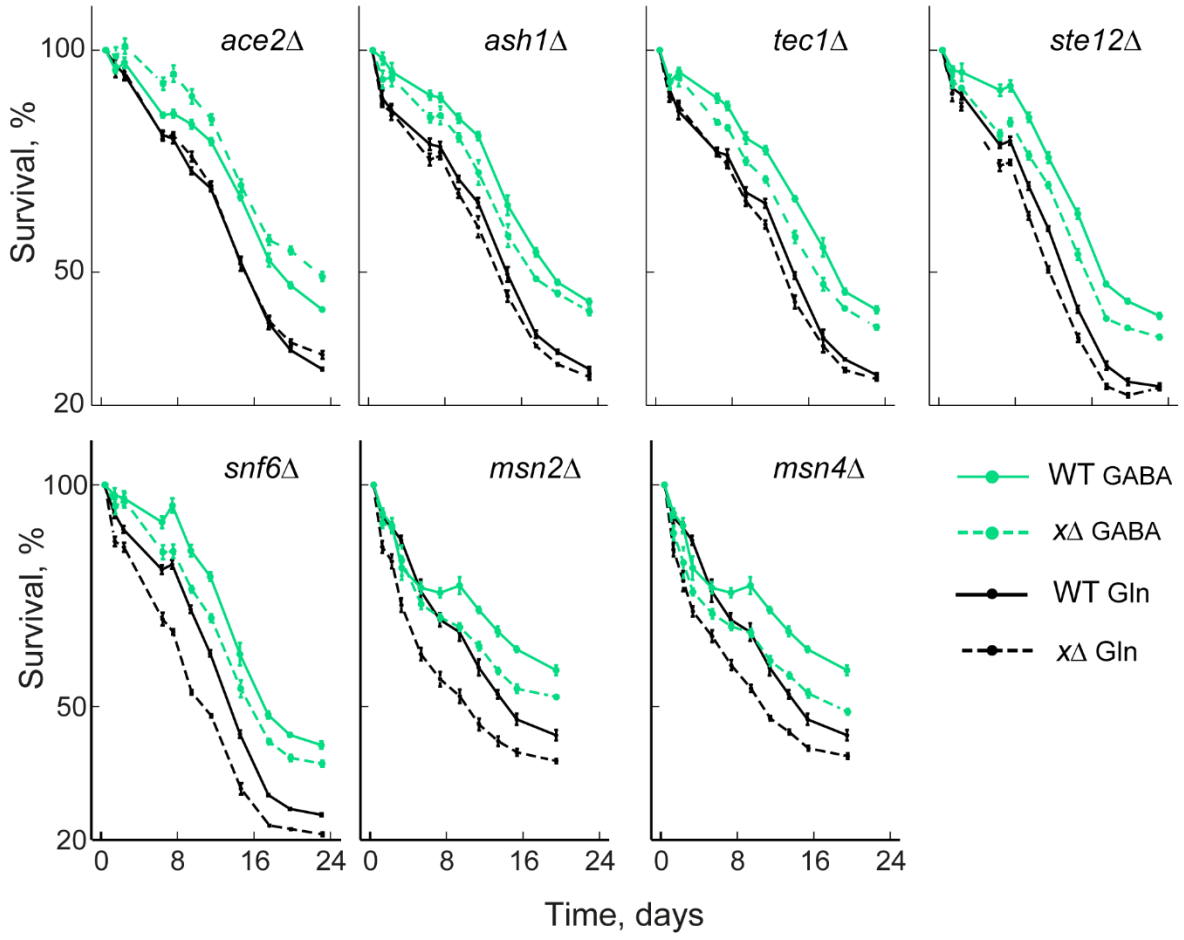


Figure 10

CLS of gene deletions of transcription factors that regulate nitrogen-based dietary restriction. Survival curves of WT and gene-deletion strains aged in SC medium with 2% glucose and glutamine (non-restricted, black lines) or GABA nitrogen source (dietary restriction, green lines). Deletion strains (discontinuous lines) are for genes coding for transcription factors Ace2, Ash1, Tec1, Ste12, Snf6, Msn2, and Msn4. These strains are the exact same strains shown in Fig4B; the experimental batch shown here did not include the bas1Δ strain. The *msn2*Δand *msn4*Δ strains were characterized in a separate batch along with the WT shown in these two panels. At least seven replicates were aged in parallel from a single stock for each deletion strain (a single colony verified from one transformation event) in the same deep-well plate. Error bars are the S.E.M. (n=7).

6.6 Transcription factors involved in lifespan regulation mediate cell-cycle arrest in response to nitrogen or carbon starvation

Our functional classification of DR-genes the transcription factors that regulate them underscore the contribution of cell-cycle control as a mechanism of lifespan extension. In an attempt to query the cell cycle dynamics upon DR, we measured the DNA content of growing cells starved for nitrogen or carbon by flow cytometry. Interestingly, most mutants (Tec1, Ste12, Snf6, Msn2, and Msn4) failed to arrest G1 at a WT rate after nitrogen or carbon depletion (**Figure 11A-B**). The long-lived *bas1*Δ cells arrested faster compared to the WT, but only under carbon starvation conditions (**Figure 11B**). The *ace2*Δ strain showed aberrant DNA content; this mutation affects mother-daughter cell separation, which explains the large fraction of events with >2n DNA content. These findings suggest that many of the transcription factors that act as DR-regulators are involved in the control cell-cycle arrest in response to nutrient limitation.

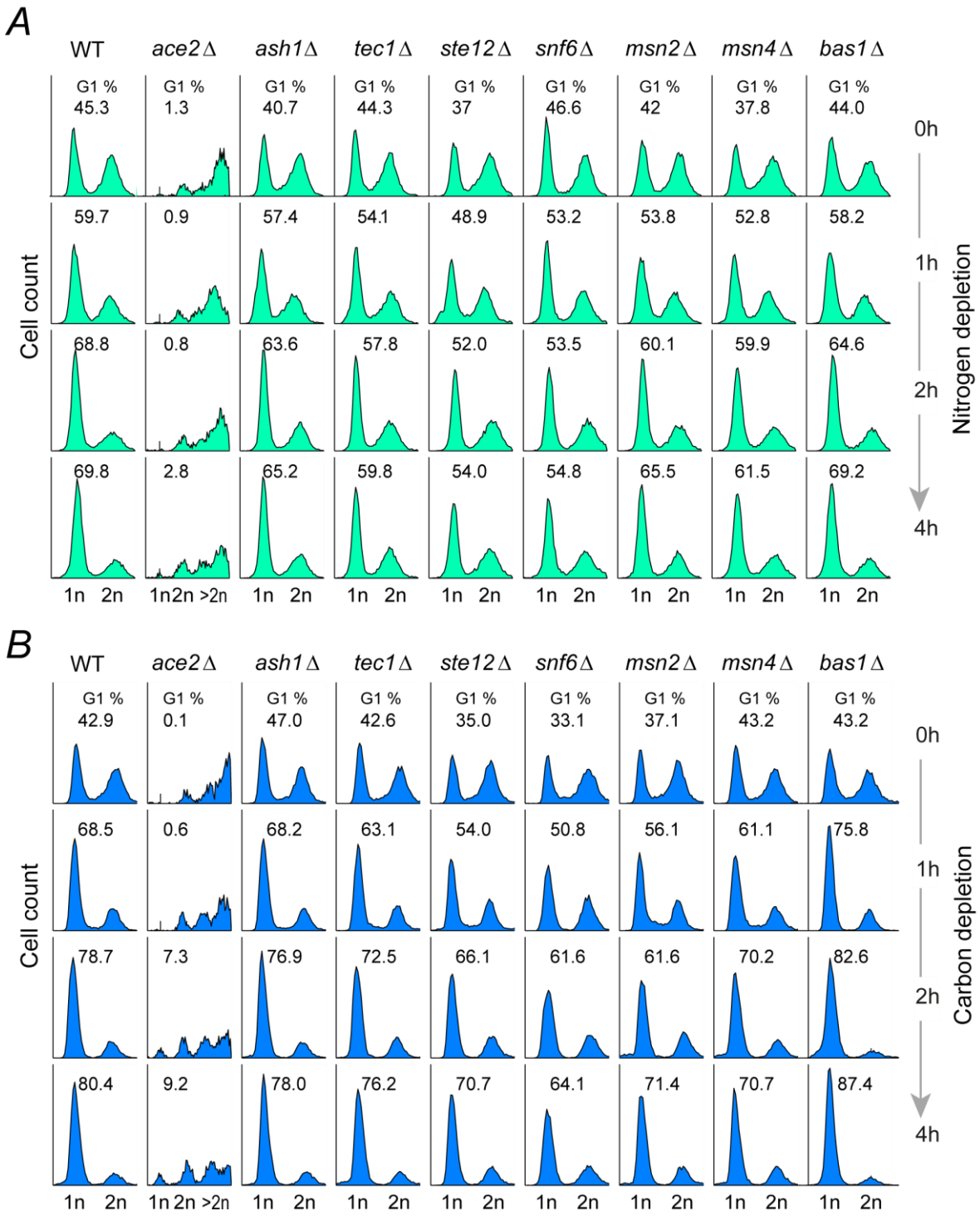


Figure 11

Several dietary-restriction regulators mediate cell-cycle arrest in response to nutrient limitation. Histograms show the DNA content of populations of WT and transcription-factor mutant cells at 0, 1, 2, and 4 hours after nitrogen (**A**) or carbon (**B**) depletion. Cellular DNA content was measured by SYTOX-Green staining followed by flow cytometry. Numbers indicate the percentage of cells at G1 in each population.

6.7 Ste12 is a positive regulator of longevity by dietary restriction and cell-cycle arrest in response to nutrients

Among the top hits of our DR-regulators was Ste12 and it showed one the lowest G1 arrest rates under starvation. Ste12 activates downstream genes involved in mating or pseudohyphal growth (Dolan et al. 1989; Roberts & Fink 1994) and it is an important regulatory hub during stationary phase (Wanichthanarak et al. 2015). However its role in stationary-phase survival in response to nutrients has not been confirmed, as opposed to other hits such as Msn2 and Msn4 (Fabrizio et al. 2004). To confirm its role in lifespan extension, we evaluated cell survival of *ste12*Δ under standard conditions of full aeration (Hu et al. 2013). While the lifespan of the *ste12*Δ strain was not affected under non-restricted 2% glucose, this strain showed diminished longevity in the 0.5% glucose dietary-restriction medium (**Figure 12A**). In addition, given that our method for measuring CLS in yeast relies in the ability of stationary-phase cells to re-enter the cell cycle, and that Ste12 is itself involved in cell-cycle arrest, we ruled out technical artifacts by using an alternative cell-viability assay. Direct staining of dead and alive cells in stationary phase showed that the alive *ste12*Δ population died faster than the WT under limited glucose and, to a lesser extent, under non-restricted conditions (**Figure 12 B**). Together, these results confirm that the lifespan effects of the *STE12* deletion are maintained regardless of the experimental conditions and methodology used to infer population survivorship in stationary phase.

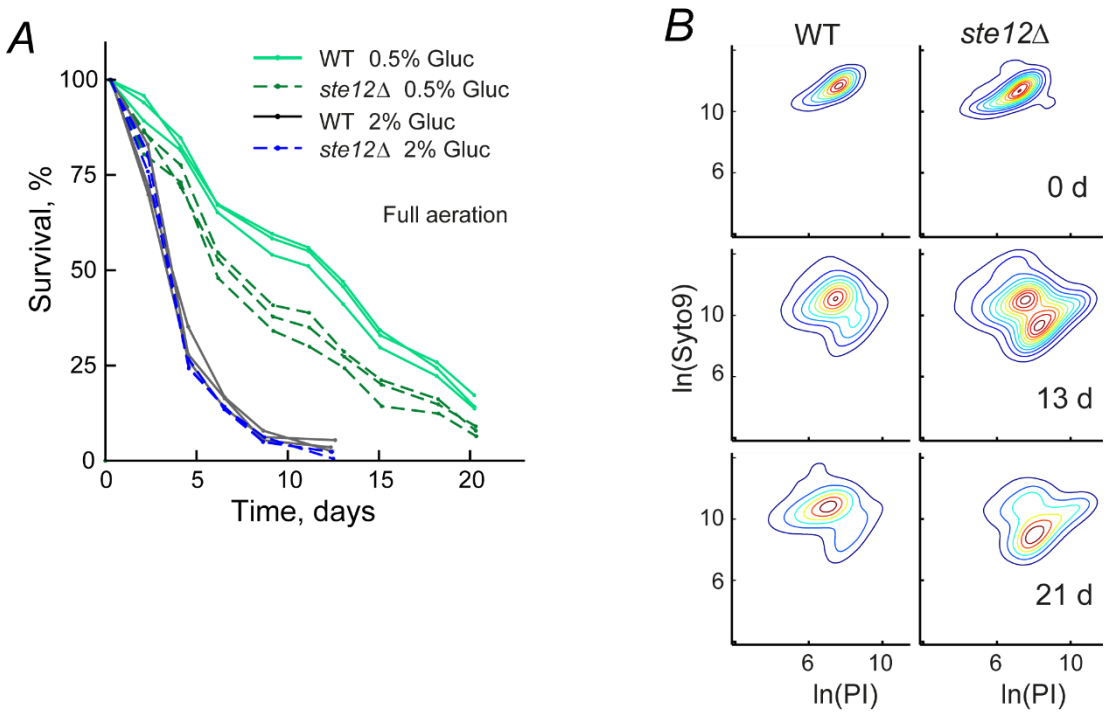


Figure 12

Lifespan extension deffects of *ste12Δ* is detectable by several CLS assays. (**A**) Survival curves of WT and *ste12Δ* strains aged in fully-aerated tubes with 10 mL SC containing 0.5% or 2% glucose. Lines shown in the same color are colonies from three independent transformation events. (**B**) Contour plots showing WT and *ste12Δ* populations of dead and alive cells under SC 0.5% glucose; fluorescence of Syto9 (alive cells) and propidium iodide (PI, dead cells) is shown in the vertical and horizontal axes, respectively, across time in stationary phase.

High expression of a bona fide positive regulator of lifespan is expected to cause increased lifespan. To test Ste12 for this trait, we generated a copper-inducible strain with a GFP fusion to track Ste12 protein levels; the WT and *pCUP1-STE12* strains were aged under varying concentrations of copper sulfate in 2% glucose SC medium. We found that the CLS of a *STE12*-overexpression population increased readily as a function of copper concentration even under non-restricted conditions (**Figure 13A**). Importantly, GFP signal in the nucleus increased as a function of copper concentration (**Figure 13B**), confirming a link between lifespan and increased levels of Ste12. Increased copper concentrations had no effect on the CLS nor growth of the WT strain (**Figure 13C-D**). Together, these results unambiguously confirm that Ste12 is a novel positive transcriptional regulator of lifespan in yeast.

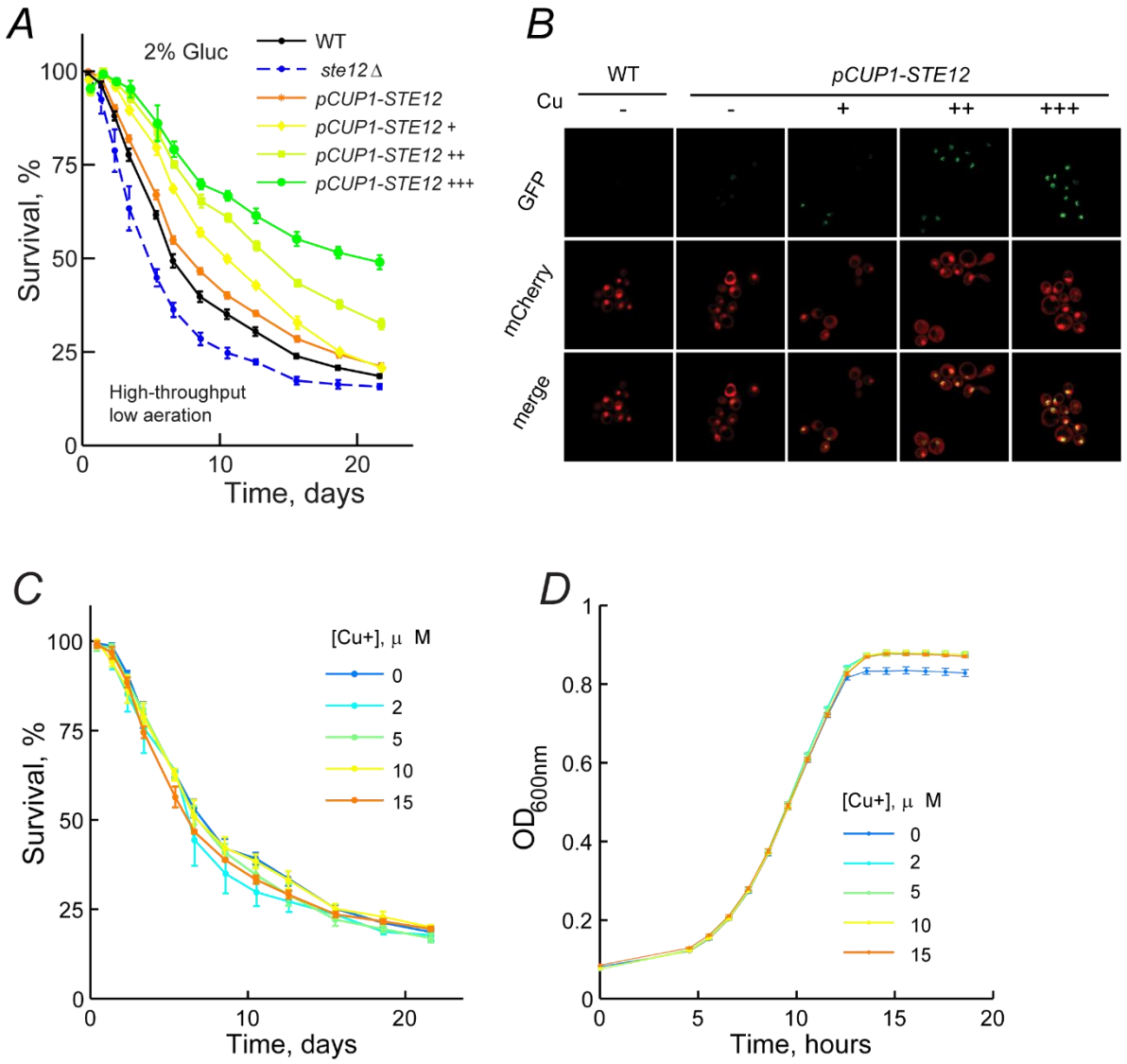


Figure13

Ste12 is a positive regulator of lifespan. (**A**) Survival curves of the WT, *ste12*Δ, and pCUP1::GFP-STE12 overexpression strains aged in SC 2% glucose in non-induced conditions (orange) or induced with 2 μM (+), 5 μM (++), or 15 μM (+++) copper sulfate. Error bars are the S.E.M. (n=7). (**B**) Micrographs of WT and pCUP1::GFP-STE12 strains with GFP, mCherry (constitutive), and merge filters, showing untreated (-) or induced cells with 2 μM (+), 5 μM (++), or 15 μM (+++) copper sulfate.(**C**) Survival curves of a WT strain treated with increasing concentrations of copper sulfate. Different concentrations of copper in the WT were aged in a single deepwell plate with no aeration (n=5) (**D**) Plot shows growth curves of WT strain treated with increasing concentrations of copper sulfate. Error bars are the S.E.M. (n=8).

6.8 Ste12 role in lifespan regulation is linked to the pheromone pathway

To gain further insight on the cellular pathways of lifespan extension by DR, we asked whether the lifespan effect of *STE12* was linked to Tec1, a transcription factor of the invasive-growth pathway that forms a heterodimer with Ste12 activating filamentous-growth genes (Madhani et al. 1999). Moreover, Tec1 has also been associated to lifespan regulation in response to rapamycin (Brückner et al. 2011). The effect of the deletion of *TEC1* alone displays a smaller effect and the CLS phenotype of a *ste12*Δ*tec1*Δ double deletion was not the additive (**Figure 14A**). Seemingly the effect of Tec1 is a subset of the effect of Ste12 suggesting that Tec1 depends upon Ste12 to promote longevity by dietary restriction. We also asked whether the effect of Ste12 is associated to other elements of the pheromone-responsive MAPK pathway. Deletion of the pheromone-receptor genes *STE2*, *STE3* and *KSS1* did not show a lifespan-extension defect in response to glucose restriction (**Appendix 7**). Importantly, deletion of *FUS3*, a MAPK involved in mating, increase CL under DR; moreover the CLS of a *fus3*Δ*ste12*Δ double mutant shows that the beneficial effect of deleting FUS3 is dependent upon STE12 (**Figure 14B**). Altogether, these results indicated that Ste12 regulates lifespan extension in response to nutrients through functional association to Tec1 and the invasive-growth pathway, but not under the control of upstream pheromone-pathway components.

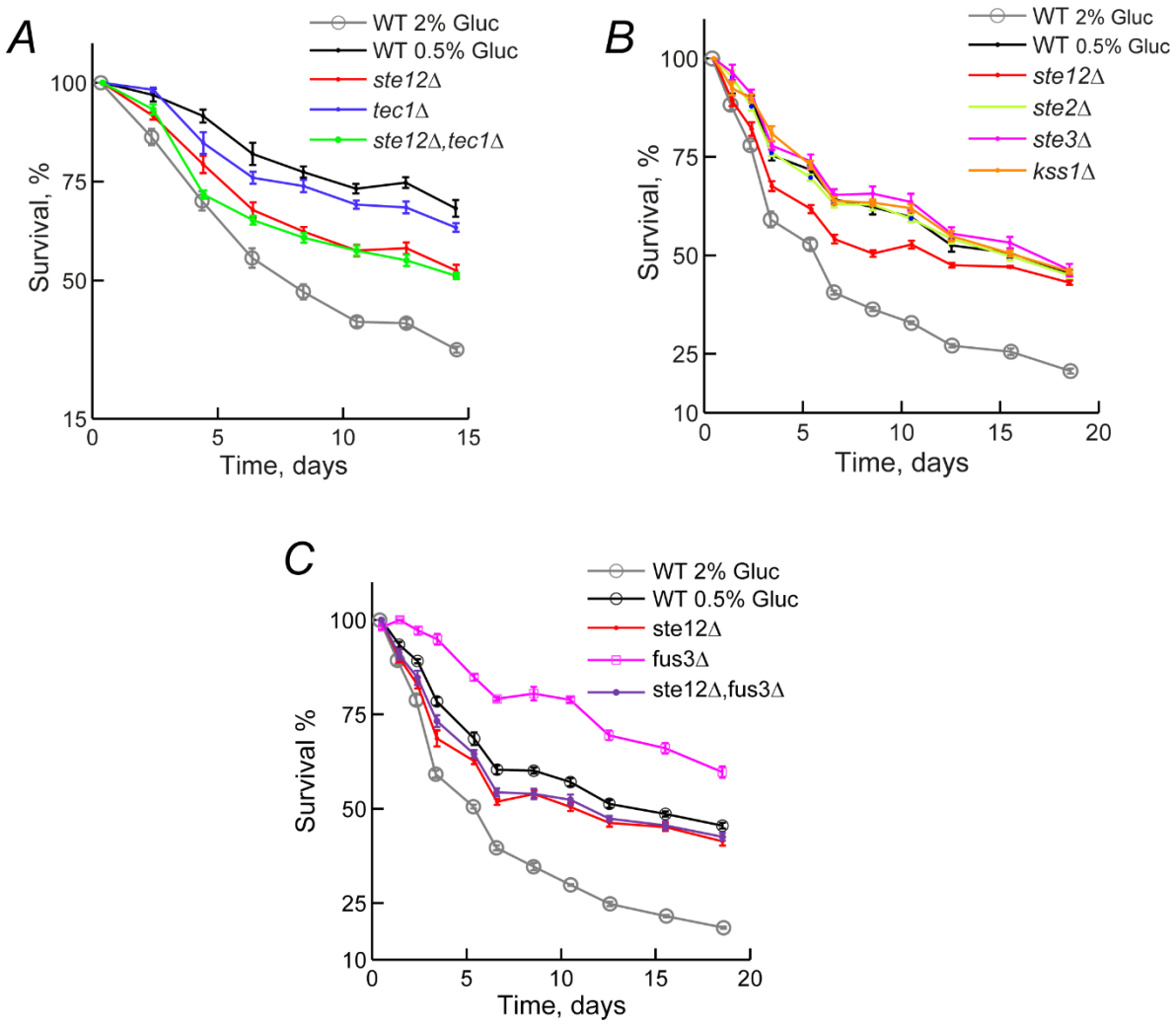


Figure 14

Ste12 promotes longevity in association to mating and invasive growth genes. (**A**) Survival curves of *ste12*Δ and *tec1*Δ single and double knockout strains under dietary restriction (0.5% glucose), WT survival curve under 2% glucose is shown for comparison purposes (**B**) Survival curves of WT, *ste12*Δ, *ste2*Δ, *ste3*Δ and *kss1*Δare shown under dietary restriction (0.5% glucose). (**C**) Survival curves of *ste12*Δ and *fus3*Δ single and *ste12*Δ *fus3*Δ double knockout strains are shown under dietary restriction (0.5% glucose).

The TOR pathway along with *RAS2*, are masters regulators of cell proliferation and differentiation programs in response to nutrient availability, both converging at Rim15 to regulate cellular maintenance (Longo et al. 2012). To investigate the relationship between Ste12 role in lifespan and the TOR pathway we built de novo deletion strains *rim15*Δ, *ras2*Δ, *tor1*Δ. In addition, we also enquired the

link between Far7 and Far8 and the TOR pathway, in our screen both deletion strains showed diminished lifespan extension and these are part of the cell cycle arrest mechanism in response to pheromone (Kemp & Sprague 2003). Deletion of *RIM15*, resulted in severe shortening of CLS even under GABA as a nitrogen source, however, deletion of *STE12*, *FAR7* and *FAR8* in a *rim15*Δ background resulted in additional shortening of CLS (**Figure 15A-C**). The phenotype of the double knockout strains was not completely epistatic, suggesting partial overlap in the effects of all mutants tested. On the other hand, *ras2*Δ strain is longer-lived than the WT strain under dietary restriction (**Figure 15D-F**), showing additional effects from those elicited by DR. Deletion of *STE12* in this background showed complete epistasis, while the effect of depleting Far7 and Far8 in this background had little to no effect, suggesting deletion of *RAS2* overrides the defects caused by deletion of *FAR7* and *FAR8*. Lastly, *tor1*Δ strain display a small long-lived effect under DR, as in *ras2*Δ strain deletion of *TOR1* shows further increase in lifespan even in after dietary restriction (**Figure 15G-I**). Depletion of Ste12, Far7 and Far8 in *tor1*Δ strain resulted in almost the same CLS as in the single deleterious mutants, suggesting that the effects of deleting *TOR1* partially overlap with the role of these three genes from the pheromone pathway. Together, these results suggest that Ste12, Far7 and Far8 seem to be linked to the TOR pathway at least partially. However part of the life-extending mechanisms of Ste12 when nutrients are limited might be independent of the downstream response of TOR.

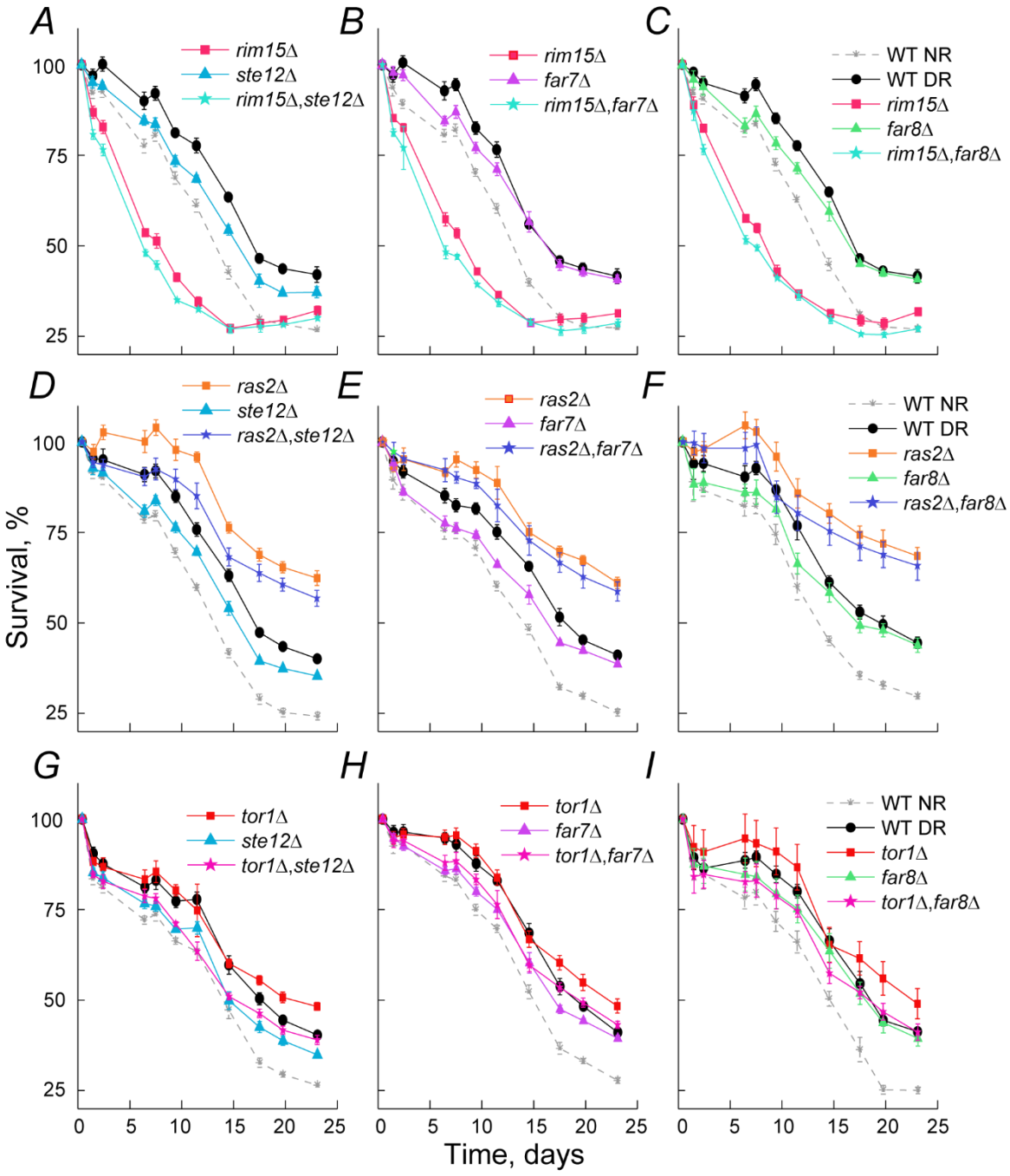


Figure 15

The role of Ste12 pathway in lifespan is partially independent from TOR and RAS pathways. Survival curves of *ste12*Δ, *far7*Δ and *far8*Δ strains are shown to compare their CLS to that of *rim15*Δ (**A-C**), *ras2*Δ (**D-F**) and *tor1*Δ (**G-I**) strains. Survival curves for double mutants *xxx*Δ-*rim15*Δ, *xxx*Δ-*ras2*Δ and *xxx*Δ-*tor1*Δ (xxx = ste12, far7 or far8).

The TOR pathway regulates cell cycle transitions upon nutrient depletion through Rim15 activity (Pedruzzi et al. 2003). It was thus tempting to ask whether Ste12 mediates cell-cycle arrest in response to nutrient limitation in concert with the TOR pathway. To this end, we used flow cytometry to monitor the cell-cycle dynamics following treatment with the TOR-inhibiting drug rapamycin, which is enough to trigger cell cycle arrest even in rich nutrient conditions. We confirmed that the *ste12*Δ mutant was impaired in cell-cycle arrest upon nitrogen depletion to compare the effect to that of rapamycin. However, mutant cells treated with rapamycin were unaffected in their cell-cycle dynamics when compared to the WT (**Figure 11C**). This result indicates that TOR-mediated cell-cycle arrest does not require activity of Ste12, suggesting that this transcription factor integrates nutrient signals leading to cell cycle arrest independently of TOR signaling.

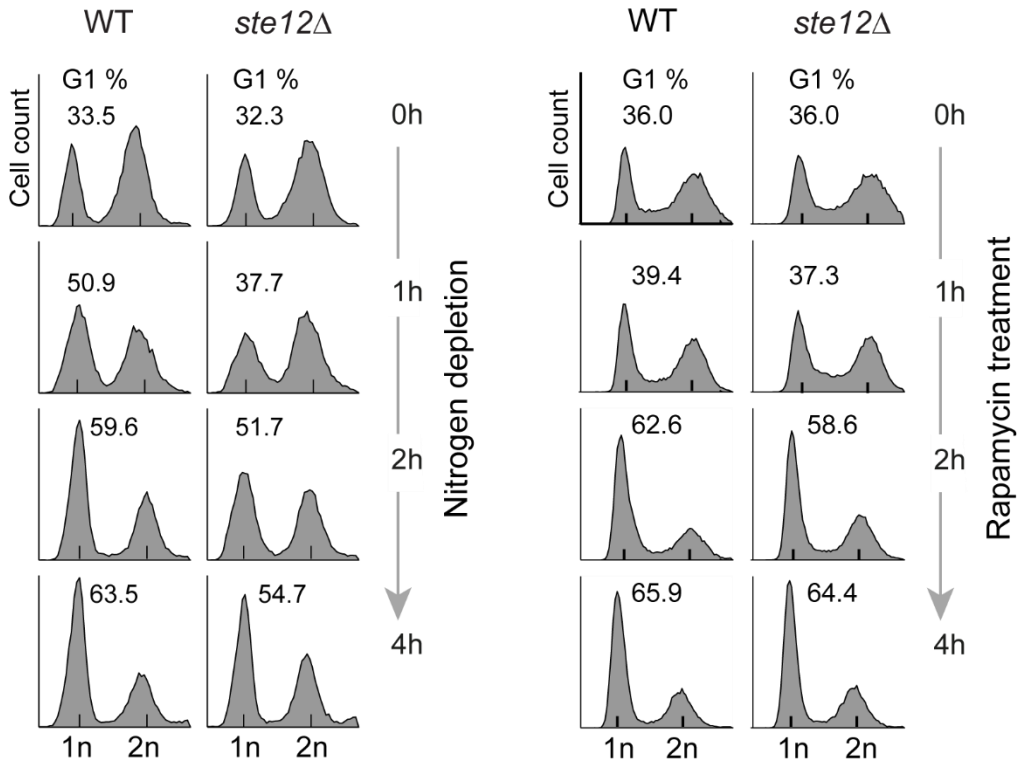


Figure 16

Cell cycle arrest throug Ste12 is independent of TOR. Cell-cycle dynamics of WT and ste12Δ populations; histograms are for cells at 0, 1, 2, and 4 hours after nitrogen starvation (left) or treatment with rapamycin (right). Cellular DNA content was measured by SYTOX-Green staining followed by flow cytometry. Numbers indicate the percentage of cells at G1 in each population.

6.9 *STE12* deletion induces transcriptional changes related to cell differentiation

To describe the downstream mechanisms of Ste12 required for lifespan extension, we sought to analyze the transcriptional changes induced by STE12 deletion under rich and dietary-restricted conditions. To do this, we extracted and enriched mRNA for RNA-seq from the WT and *ste12*Δ strains at late exponential phase in SC 2% glucose and after 4 hours of nitrogen depletion. Reads form sequencing were aligned using kallisto and then analyzed with R package sleuth. Using a 10% FDR and 2-change fold criteria only 33 genes were de-regulated in the *ste12*Δ strain under rich medium, while 26 genes changed their expression levels after nitrogen depletion (**Figure 17A-B**). Interestingly, most differential genes under both conditions were downregulated, with few genes showing up-regulation. Only 6 genes were specific under nitrogen depletion and were enriched for the Gene Ontology term Cellular component assembly involved in morphogenesis (**Appendix 3**). On the other hand 12 genes were specific for nutrient-rich condition, this set of genes were enriched for Gene Ontology terms such as Cellular response to pheromone and G-protein coupled receptor signaling pathway (**Appendix 4**).

Both gene-sets were apparently composed of genes that shared GO term enrichments (**Appendix 5**), hence, we pooled all differential genes to perform a functional kappa analysis. From this clustering analysis, only two important groups arose, namely Pheromone induced cellular fusion and Transcriptional regulating of mating (**Figure 17C**). Importantly, the larger portion of genes that were not placed in any cluster are actually transposable element sequences while a few were enriched for Response to pheromone and cell cycle arrest (**Appendix 6**). Interestingly this last GO term is represented by *FUS3* and *FAR1* in these transcriptomes. These results suggest that the transcription factor Ste12 integrates nutrient signaling and regulates downstream genes needed for cell-cycle arrest through the pheromone response pathway, which may underlie its beneficial effect on cell survivorship.

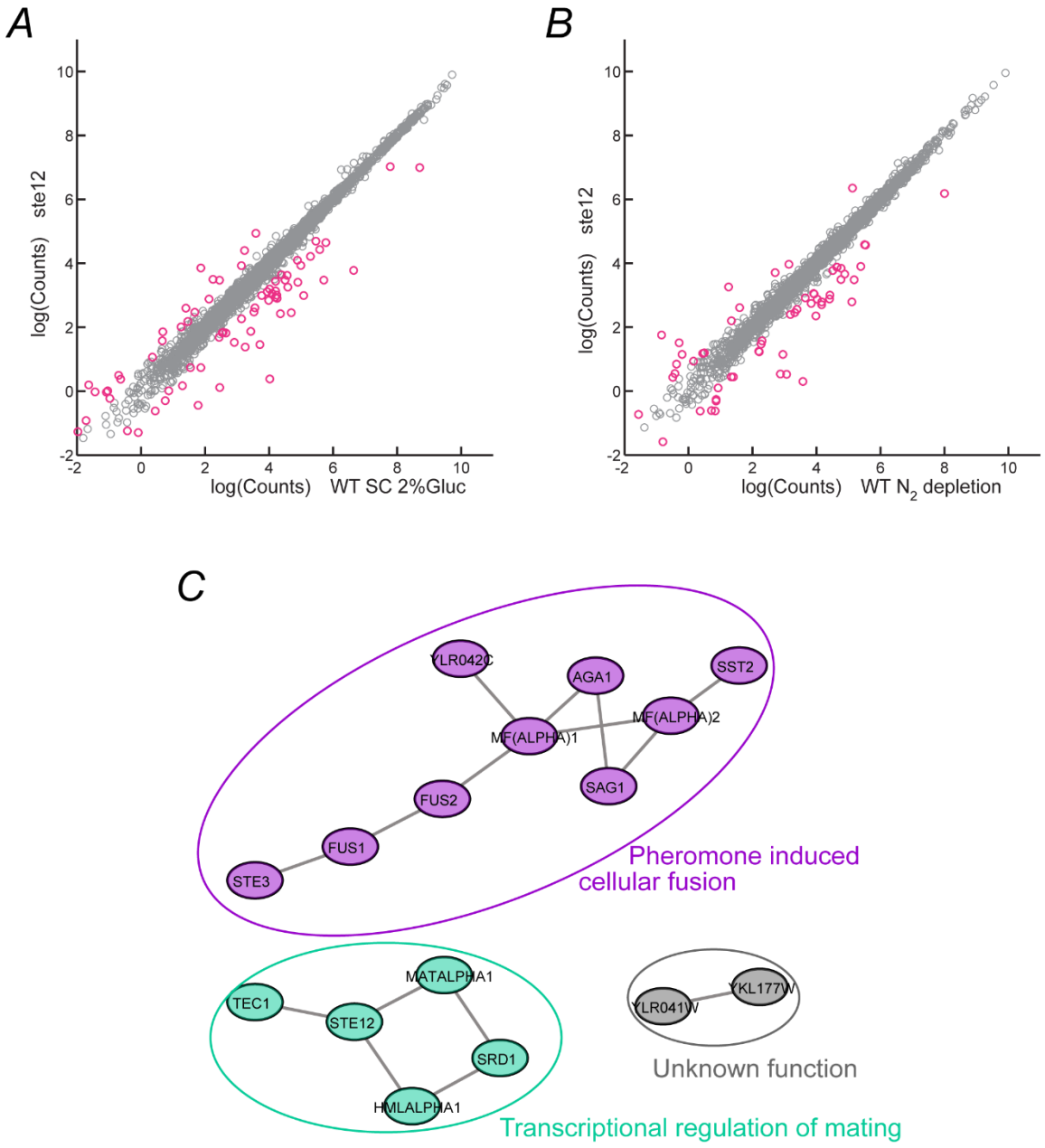


Figure 17

Transcriptomic changes associated to *STE12* **deletion**(RNA-seq data was analized for expression changes in a *ste12*Δ strain relative to the WT, under rich medium (**A**) and after 4 hours of nitrogen depletin (**B**), colored circles represent transcripts with significative expression changes. (**C**) Network representation of differentially expressed genes in the *ste12*Δ strain, edges represent functional association (kappa>0.3).

7. DISCUSSION

In this work, we have used a genome-wide approach based in a high-throughput phenotyping method to provide a quantitative and systematic description of lifespan extension by different dietary restriction. Specifically, we have screened a collection of 3,718 gene-deletion strains aged in glutamine—a preferred nitrogen source—or GABA—a limiting nitrogen condition. Our analysis revealed that nitrogen limitation modifies the lifespan effect of 473 gene deletions (DR-genes). To the best of our knowledge, this study yields the most comprehensive phenotypic compendium of genetic players involved in longevity by dietary restriction.

7.1 Functional classification of DR-genes revealed the mechanisms underlying longevity by DR.

The functional classification of DR-genes through kappa clustering analysis recapitulated several downstream cellular processes involved in longevity, providing validation of *LE* index in the form of functional relationships. Moreover, several novel cellular processes were identified, such as ribonucleoprotein complex biogenesis, cell-wall organization, nucleus localization through microtubules, and genes involved in cell cycle arrest. While it is well established that ribosomal function is downregulated in response to TOR inhibition or nutrient depletion (Hansen et al. 2007), it is not clear how the inhibition of translation might lead to lifespan extension. In this sense, our screen pointed to specific proteins involved in pre-rRNA processing (Slx9), nucleolar rRNA methyltransferase (Rrp8), nuclear export of pre-ribosomal subunits (Arx1), and translational initiation (Bud27) that might give more clues about how translation regulates lifespan. In the case of cell cycle control, we found that deletion of pheromone-responsive genes *FAR7* and *FAR8* had short-lived phenotype under dietary restriction (**Figure 6A**). In yeast, these Far proteins prevent cell cycle recovery after pheromone exposure by inhibiting *CLN1*-3 (Kemp & Sprague 2003), which may be the case during DR as well . In addition, that nucleus microtubule dynamics seems to underlie dietary restriction. In this regard, it is known that certain cellular processes needed for extended longevity, such as autophagy,

require intact function of microtubules (Köchl et al. 2006). However, the relationship between nuclear localization and lifespan extension remains unexplored. The appointment of these novel processes might shed light into the mechanisms of longevity.

Interestingly, we found that DR alleviated the deleterious effects of many mitochondrial gene deletions, accounting for most of the genes that showed enhanced lifespan extension. It is known that dietary restriction shifts metabolism towards respiration (Lin et al. 2002) but, at the same time, impaired respiration can promote longevity in yeast and nematodes through enhanced retrograde response and activation of anaplerotic pathways (Cristina et al. 2009). Noteworthy, our screens were done under low aeration; however gene-deletions of mitochondrial respiratory chain show deleterious effects, while these effects are partially buffered by DR. Although we did not follow-up on this intriguing observation, we speculate that a higher demand for respiration during dietary restriction could lead to the activation of compensatory pathways anaplerotic routes exclusively. Other feedback mechanisms may explain the alleviation of deleterious effects in other groups of genes. For instance, cell wall stress in yeast can result in the activation of unfolded protein response (Krysan 2009), a process that restores normal function by inhibiting translation and activating misfolded proteins degradation (Senft & Ronai 2015), which has been linked to longevity before. Hence, DR may activate other anti-stress responses that act in a synergistic manner, enhancing lifespan extension in several deleterious mutant strains.

7.2 Identified DR-regulators underscore the role of cell-cycle control in lifespan regulation

TFRank analysis of DR-genes as regulatory targets allowed the establishment of a set of ranked transcription factors (DR-regulators) that orchestrate the transcriptional changes in response to dietary restriction. Strikingly, most high-ranked DR-regulators were transcription factors involved in mitotic cell-cycle transitions, either by repression of Cln3 specifically in yeast daughter cells (Ace2 and Ash1) (Laabs et

al. 2003; Di Talia et al. 2009), activation of ribosomal-protein genes (Sfp1) (Marion et al. 2004), or cell differentiation in response to nutrients or pheromone (Tec1 and Ste12) (Madhani et al. 1999). Top-hit transcription factors also included, Msn2 and Msn4, two positive regulators of stress-response and lifespan extension downstream of the Tor//Ras-PKA pathways that converge on Rim15, the main protein kinase involved in cell survivorship in response to nutrients (Wei et al. 2008). Noteworthy, Rim15 is necessary for cell cycle arrest during stationary phase in yeast (Moreno-Torres et al. 2015), further providing a link between these TFs and cell cycle control. In addition, we found that several DR-regulators (Msn2, Msn4, Snf6, Tec1, and Ste12) are needed for the accurate arrest of the cell cycle in response to starvation. These results are in agreement with recent studies showing that dietary restriction extends the chronological lifespan of yeast, by regulation of the cell cycle arrest and entry into a quiescent state (Leonov et al. 2017).

Several forms of dietary restriction have been implemented in the past, raising the question whether all modes of nutrient restriction elicit the same longevity mechanisms or not. Indeed, lifespan extension by dietary-restriction depends on amino-acids balance in flies (Wu et al. 2013) and there are some exclusive nutrient sensing pathways underlying longevity in response to glucose or amino acid restriction in yeast (Mirisola et al. 2014). However, the fact that several protocols of dietary restriction result in lifespan extension in several organisms, including restriction of glucose, reduced nitrogen levels, and restriction of specific amino acids suggests that the underlying response involves overlapping mechanisms (Kennedy et al. 2007). Here, the CLS of the tested DR-regulators displayed the same direction in two different modes of dietary restriction, namely nitrogen-source or glucose-concentration limitation. This was also the case for their cell-cycle arrest defects: similar cell cycle arrest defects were observed for DR-regulators deletion strains in response to both nitrogen and carbon starvation. Thus, transcription factors herein identified as DR-regulators are likely part of the general machinery leading to extended lifespan regardless of the mode of dietary restriction. A next challenge is to describe how these regulators integrate signals from different nutrients and the extent to which their roles are overlapping or independent from one another.

Noteworthy, deletion of *ACE2* impedes mother-daughter cell separation; thus, altering the DNA content during flow cytometry, probably due to the presence of a pseudo-filamentous growth. In addition, given that Tec1 and Ste12 are master regulators of the invasive-growth pathway, and deletion of these transcription factors resulted in short CLS, it is tempting to propose that filamentous growth is a pro-longevity process. However, the yeast background used here, S288C, bears a nonsense mutation in *FLO8* that prevents it from forming pseudohyphae, making this strain incapable of invasive/filamentous growth (Liu et al. 1996). The latter suggests that the role of these TFs in lifespan regulation is exerted through an invasive growth-independent mechanism. Nevertheless, it remains to be addressed if Ste12 overexpression can override the deficiency of *FLO8* leading to pseudohyphae formation. While we did not confirm a causal link between cell cycle arrest defects and longevity in these knockout strains, their phenotypes suggest that cell cycle control or changes associated to invasive growth are involved in lifespan extension by dietary restriction.

7.3 Cell-cycle arrest in response to pheromone through Ste12 is a likely mechanism of lifespan extension

Interestingly, Ste12 appointed here as a DR-regulator was identified as a transcriptional hub during yeast stationary phase using transcriptomic evidence (Wanichthanarak et al. 2015), although no direct link to longevity was uncovered so far. We have provided compelling evidence showing that Ste12 is a positive regulator of lifespan extension in yeast. Not only is *STE12* necessary for lifespan extension under different DR protocols and experimental settings, but also *STE12* overexpression is sufficient to extend lifespan. Importantly, we confirmed the *ste12*Δ phenotypes in our screening settings under standard conditions in which CLS has been assayed elsewhere. Ste12 induces the transcription of downstream genes necessary for two cell-differentiation programs regulated by MAPK pathways, namely mating and invasive growth (Dolan et al. 1989; Roberts & Fink 1994). We also found that Fus3, a MAPK activator of the pheromone pathway, has a role in lifespan determination along with Ste12. Interestingly, a recent network-based

search for lifespan regulators provided that deletion of MAPK genes *FUS3* and *KSS1*, result in lifespan extension; moreover, they show that the mechanisms of longevity of these two strains overlap with DR response (Aluru et al. 2017). In this same vein, the deletion of a different member of the pheromone pathway, *STE11*, also displays defects in the expression of starvation-induced genes (Cao et al. 2016). All these evidences suggest that the pheromone response pathway is coopted to regulate the transcriptional response to nutrient scarcity, leading to enhanced cell survivorship during yeast stationary phase.

We also found that deletion of *STE12* results in a failure to arrest the cell cycle upon nutrient starvation. While we did not confirm a direct causal link between cell cycle arrest and extended longevity mediated by Ste12, one possibility is that upstream elements of the mating pathway are recruited to induce a cell cycle arrest in response to dietary restriction, which in turn protects against replication stress during stationary phase, leading to increased longevity (Weinberger et al. 2007). Here we found that deletion of STE12 downregulates the expression of Far1, which in the presence of pheromone arrests the cell cycle, providing a possible mechanism of longevity by DR. In addition, Far3, a member of FAR complex (Far3 and Far7-11), is a direct target of Ste12 (Lefrançois et al. 2009). The FAR complex is necessary to maintain arrest during mating (Kemp & Sprague 2003), this is also a likely mechanism for cell survivorship during stationary phase. In line with this idea, deletion of *FAR7*, *FAR8*, and *FAR11* in our primary screen resulted in diminished lifespan extension. Recently, Leonov *et al*., reported that a population of aging-resistant cells arrest in G_0 in late logarithmic phase, this arrest is lost somehow during stationary phase, suggesting there is a cell cycle maintenance program (Leonov et al. 2017). Moreover, the confirmation of *far7*Δ and *far8*Δ phenotypes and their link to the TOR pathway suggest that this complex is recruited by TOR to maintain cells arrested during stationary phase. An alternative possibility is that Ste12 mediates cell-cycle arrest and longevity through its association to Tec1. This transcription factor promotes cell cycle progression through up-regulation of Cln1 expression (Madhani et al. 1999). Hence, association to Ste12 by filamentous-

growth pathway nutrient signaling (Madhani et al. 1999) could prevent Tec1-mediated progression of the cell cycle, resulting in increased longevity.

The TOR pathway regulates cell cycle transitions upon nutrient depletion through activity of the Rim15 kinase (Pedruzzi et al. 2003), hence this pathway might be recruiting Ste12 and other DR-regulators appointed here. Moreover, Tec1 has been related before to lifespan extension in response to the Tor1-inhibiting drug rapamycin (Brückner et al. 2011), suggesting that the link between Tec1, Ste12 and lifespan extension reported here is under the control of TOR. Interestingly, the short-lived phenotype of Rim15-deficient cells seems to correlate better with other mechanisms apart from cell cycle control, such as carbohydrate storage (Cao et al. 2016). Interestingly, we found that Ste12 regulation of cell cycle is not under the control of the TOR pathway, as rapamycin treatment does not affect G1 arrest in a *ste12*Δ background, suggesting that Ste12 controls cell cycle transitions in response to nutrients in a TOR-independent manner. Given that other members of the pheromone response pathway regulate lifespan, this reinforces the idea that Ste12 is part of a distinctive pathway that promotes the emergence of a G1-arrested population in response to nutrients limitation.

Whether the longevity roles of the DR-regulators are conserved is still an open question. For instance, *STE12* has no clear homolog in animals, however, transcriptional networks can be rewired through evolution, leading to changes in the regulation exerted by specific regulators, while the downstream targets remain associated (Sorrells et al. 2015). In addition, the yeast three-kinase module regulating MAPK pheromone and invasive-growth pathways are conserved in other organisms (Widmann et al. 1999). In particular, *KSS1* and *FUS3* are key members of the MAPK pathway that regulates cell differentiation programs in yeast (Bardwell 2004), while their mammalian counterpart *MAPK1* is central to the development of several age-associated diseases in mammals (Carlson et al. 2008). Thus, the study of targets downstream the MAPK pathway in yeast might bring important insights into the regulation of aging in other eukaryotes, including humans.

8. CONCLUDING REMARKS

Dietary restriction elicits a complex response that requires the activation of several pathways and a consequent transcriptional response that involves a great amount of cellular processes. Even though the advances in the study of aging have been vast there is still the complexity of the process and the great amount of data generated call for more integrative systematic approaches.

This study provides a much-needed comprehensive picture of the mechanisms of lifespan extension by dietary restriction. The power of this approach allowed to pinpoint key links between nutrient sensing, the cell-cycle arrest machinery and longevity in yeast. In addition, we were able to identify novel genes and molecular mechanism that determine lifespan in yeast, contributing to a more complete picture of the architecture of longevity. Importantly, the strategy developed in this work provides a framework to study aging with a systems approach. Moreover, this approach can be readily applied to other genetic, environmental, or pharmacological perturbations. Surely, the information and tools generated here will continue to shed light on the mechanism of longevity.

9. PERSPECTIVES

9.1 Biological aspects of false-positive hits deserve consideration for the CLS model

The amount of data obtained in the making of this work lends itself to further research and due to the diverse nature of the information presented here, new projects could be approached with very different angles. Perhaps the first aspect that deserves further investigation is the methodologic one. The false-positive rates detected here are very low compared to other genome-wide methods, however, we did not address the nature of these spurious hits. One first possible source of false-positives is the noise observed in the dynamics of the fluorescent protein itself, this can be tested by performing dye swaps experiments, were the mutant strains would have a different fluorescent protein. To do this the original single deletion collection will have to be crossed to a parental strain with *Venus* fluorescent protein (YFP), which has been reported to be more stable and observations in the laboratory confirm better fluorescence resolution. At least part of the screen can be repeated with this new fluorophore, competed against an RFP reference, likewise, the RFP mutants should be competed against an YFP reference.

Of course, not all mutants were equally affected by fluorescence artifacts, as according to our confirmation rate most gene-deletions indeed have the effects reported here. Hence, a possibility is that the fluorescence protein stability or expression is modulated in gene-deletions for specific process such as autophagy, proteasome or others related to protein turnover. The expression of the fluorescent protein can be assessed through qPCR in a selected group of single-deletion strains. Fluorophore expression can be compared to the mean and total fluorescence from a given amount of cells in a flow cytometry run through time in stationary phase. This could be done in individual cultures to separate any effect of the competition. In addition, false-positive hits could be traced to specific strain-strain interactions. To evaluate this validated and non-validated strains could be competed against different references strains in our particular GABA and glutamine media, differences in CLS

for non-validated strains would suggest interaction between these strains and a specific reference.

9.2 The genetic wiring of lifespan extension might reveal important aspects of the genetic architecture of aging

Naturally, several questions remain to be addressed in the context of the mechanisms of lifespan extension presented here. For instance, the relationship between the cellular processes can be approached through the assessment of genetic interactions among DR-genes presented in this study. The genome-wide screen can be applied easily for the discovery of such genetic interactions. To do this we would have to select several members of the clusters presented here and performed a genetic synthetic array (SGA) (Tong & Boone 2005) to generate double knockout strains by mating. The CLS of these double-deletion strains would be assayed through our high-throughput method and compared to the effect of single-deletion mutants to establish additivity or epistasis. As our results are quantitative applying a model of epistasis (multiplicative or additive) should be very straight-forward, although perhaps several types of analysis should be tested and compared. For a smaller group of genes the reference strain for the competition can be in fact a parental single-deletion mutant, that way the comparison of effects would be easily interpretable. Moreover, this study could be focused in assessing the degree of cross-talk or modularity among pathways that regulated the response to DR. To evaluate the relation among regulatory pathways, the protein kinases and transcription factors within the DR-genes could be used to generate a set of double-deletion strains through SGA. This last approach would include a fairly small number of double mutants, lending itself for evaluation of several replicates and in turn opening the possibility of stablishing hierarchical interactions among genes based in the size of the effect (Liberali et al. 2014). Both approaches will contribute greatly to elucidate the genetic wiring of aging cells.

9.3 Ste12 mechanisms of longevity and pheromone pathway cross-talks

The results presented here regarding the DR-regulators hint at a role for cell cycle arrest in lifespan extension, however little is known about how exactly halting the cell cycle contributes to longevity. Also some studies have cast doubt on the contribution of cell cycle control to lifespan extension in general (Cao et al. 2016). To establish the importance of cell cycle arrest for lifespan extension CLS measurements should be done using several interventions known to arrest the cell cycle at different check-points. For instance, temperature-dependent mutants of cyclin 1 (*cdk1*) can be used to observe the effect on CLS of slowing down cell cycle progression at different time-points during the logarithmic, diauxic and stationary phase. Characterization of glycogen and trehalose content and oxidative stress resistance in arrested cells would allow the establishment of a link to other known features of long-lived cells (Kyryakov et al. 2012; Lass et al. 1998). Moreover, CLS and other lifespan related traits should be evaluated in mutants unable to arrest the cell cycle efficiently, for example in mutants for *CDK1* degradation targeting sites, leading to shorter G1. This strategy would allow establishing the dependence of other processes such as carbohydrate storage, autophagy, etc. on cell cycle arrest, and at the same time propose a better model of lifespan in arrested cells.

More specific questions also persist, as what is the mechanism of cooptation of the pheromone response elements in lifespan regulation? And, what is the specific mechanism by which Ste12 induces cell cycle arrest in response to DR? Importantly, the RNA-seq experiments pointed almost exclusively towards the downregulation of several elements of the pheromone pathway, including regulatory genes and cell wall-related factors, as well as factor arrest protein (Far1). The downregulation of these genes in a *ste12*Δ background should be confirmed first through qPCR. Furthermore, the CLS of single deletion of the candidate genes could be evaluated. In order to uncover the cross-talks that may exist between the pheromone and other signaling pathways to regulate CLS, a genetic interactions strategy should be applied in combination with the strategy to describe lifespan regulatory pathways described above. Regarding the implication of cell cycle arrest by the pheromone

pathway and longevity, the characterization of the CLS of double deletion of *STE12* and other members of the pheromone pathway might give a clue on the precise mechanisms. In particular, the CLS and cell cycle profiles of deletion of *FAR* genes in a *ste12*Δ background can shed light into the relation between cell cycle arrest and lifespan extension. In addition, as discussed previously the cell cycle regulation features of the FAR complex may be acting during stationary phase, suggesting that cell cycle dynamics in this phase should be measured in the *FAR* mutants to reveal important phenotypic differences with the WT. In this sense, dietary restriction should keep more cells arrested at G1 during stationary phase and this arrest should be lost in *FAR* mutants or even in *ste12*Δ. Moreover, several interventions on the cell cycle and other longevity-related interventions can be applied on single and double deletion mutants, and CLS should be characterized in these conditions to further elucidate the role of Ste12 in lifespan regulation.

Certainly, an important number of follow-up studies as the ones describe above will generate new venues for research in the near future with vital implications in the understanding of the biology of aging in particular in the discovery of new targets for lifespan regulation.

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11. APPENDIX

Appendix 1 | CLS assays, media and strains used in this study

CLS assay	Experimental setup	Media and recipes [a,b]		Strain genotypes tested
Genome screen, as (Garay et al. 2014)	Co-culture of WT and mutant strains grown in 96-deep wells with 700 µl medium/well, incubated in robotic platform at 30 °C	SC Gln	2% glucose, 0.17% YNB without amino acids and ammonium sulfate, 0.07% amino acid mix, 25 mM glutamine	*MAT*a *xxx∆::kanMX4 PDC1-mCherry-CaURA3MX4 can1∆::STE2pr-SpHIS5 lyp1∆ his3∆1 ura3∆0 leu2∆1::LEU2* WT reference *MAT*a *his3∆1::kanMX4 PDC1-Cerulean-CaURA3MX4 can1∆::STE2pr-SpHIS5 lyp1∆ ura3∆0 leu2∆1::LEU2*
		SC GABA	2% glucose, 0.17% YNB without amino acids and ammonium sulfate, 0.07% amino acid mix, 25 mM GABA	
Small-scale assay, modified from (Murakami et al. 2008)	Single-strain cultures grown and aged in 96-deep wells with 700 µl medium/well, incubated with low aeration in robotic platform at 30 °C	SC Gln	2% glucose, 0.17% YNB without amino acids and ammonium sulfate, 0.07% amino acid mix, 25 mM glutamine	Genome screen confirmation *MAT*a *xxx∆::KANMX4 PDC1-mCherry-CaURA3MX4 can1∆::STE2pr-SpHIS5 lyp1∆ his3∆1 ura3∆0 leu2∆1::LEU2* (*SpHIS5* is expressed in *MAT*a) De novo gene deletions *MAT*α *xxx∆::NATMX6 PDC1-mCherry-CaURA3MX4 can1∆::STE2pr-SpHIS5 lyp1∆ his3∆1 ura3∆0 leu2∆1::LEU2* (*SpHIS5* is not expressed in *MAT*α)
		SC GABA	2% glucose, 0.17% YNB without amino acids and ammonium sulfate, 0.07% amino acid mix, 25 mM GABA	
		SC 2% Gluc	2% glucose, 0.67% YNB without amino acids, 0.2% amino acid mix (ammonium is the nitrogen source)	
		SC 0.5% Gluc	0.5% glucose, 0.67% YNB without amino acids, 0.2% amino acid mix (ammonium is the nitrogen source)	
Standard CLS assay, as (Hu et al. 2013)	Single cultures grown in 50 ml glass tubes w/ aluminum foil cap and 10 ml of medium, incubated at 200 rpm in orbital shaker at 30°C	SC 2% Gluc	2% glucose, 0.67% YNB without amino acids, 0.2% amino acid mix (ammonium is the nitrogen source)	*MAT*α *xxx∆::NATMX6 PDC1-mCherry-CaURA3MX4 can1∆::STE2pr-SpHIS5 lyp1∆ his3∆1 ura3∆0 leu2∆1::LEU2* (*SpHIS5* is not expressed in *MAT*α)
		SC 0.5% Gluc	0.5% glucose, 0.67% YNB without amino acids, 0.2% amino acid mix (ammonium is the nitrogen source)	

a. Composition of the amino-acid supplement mix is based on the CSHL manual

Appendix 1 was taken from: Campos SE, et al., (2018) Genome-wide mechanisms of chronological longevity by dietary restriction in budding yeast. *In press*.

Appendix 2 | YNB-low fluorescence medium

PROTOCOL

1. For 1 liter of YNB-low fluorescence medium mix together:

-5 g	$(NH_4)_2SO_4$
-1 g	KH_2PO_4
- 0.5 g	$MgSO_4$
- 0.1 g	NaCl
- 0.1 g	$CaCl_2$
- 20 g	D-(+)- Glucose (Sigma G2870)
- 0.79 g	CSM (complete supplement mixture, MP)

3. Add the components to 500 ml of deionized water and dissolve
4. Add 1 militer of trace element stock*.
5. Add 1 militer of vitamin stock**.
4. Add deionized water to make 1 liter of medium.
5. Filter sterilize.

***Trace element stock**

- Mix the indicated amount of components together:

50mg	H_3BO_4
4mg	$CuSO_4$
10mg	KI
20mg	$FeCl_3$
40mg	$MnSO_4$
20mg	Na_2MoO_4

- Bring to 100 ml with deionized water.

**** Vitamin stock**

- Mix the indicated amount of components together:

0.2mg	Biotin
40mg	Calcium pantothenate
200mg	Inositol
40mg	Niacin
20mg	Para-amino benzoic acid
40mg	Pyroxidine HCl
40mg	Thiamine HCl

- Bring to 100 ml with deionized water.

Appendix 3 | GO term enrichment DR differentially expressed genes

GO_term	P-value	FDR	Gene(s) annotated to the term
cellular component assembly involved in morphogenesis	0.04936	0.64	GAS2/YLR343W:FUS2/YMR232W

Appendix 4 | GO term enrichment NR specific differentially expressed genes

GO_term	P-value	FDR	Gene(s) annotated to the term
response to pheromone involved in conjugation with cellular fusion	1.50E-05	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
cellular response to pheromone	3.54E-05	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
response to pheromone	3.93E-05	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
conjugation with cellular fusion	0.00012	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
conjugation	0.00013	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
multi-organism cellular process	0.00017	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
reproductive process	0.0003	0	FUS3/YBL016W:FUS1/YCL027W:HMLALPHA1/YCL066W:MATALPHA1/YCR040W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
reproduction	0.00038	0	FUS3/YBL016W:FUS1/YCL027W:HMLALPHA1/YCL066W:MATALPHA1/YCR040W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
cellular response to organic substance	0.00081	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C

regulation of protein export from nucleus	0.00096	0	FUS3/YBL016W:GPA1/YHR005C
signal transduction involved in conjugation with cellular fusion	0.00195	0	FUS3/YBL016W:MF(ALPHA)2/YGL089C:GPA1/YHR005C
pheromone-dependent signal transduction involved in conjugation with cellular fusion	0.00195	0	FUS3/YBL016W:MF(ALPHA)2/YGL089C:GPA1/YHR005C
response to organic substance	0.00315	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
sexual reproduction	0.00328	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
multi-organism reproductive process	0.00328	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
G-protein coupled receptor signaling pathway	0.00335	0	FUS3/YBL016W:MF(ALPHA)2/YGL089C:GPA1/YHR005C

multi-organism process	0.00473	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
regulation of mating-type specific transcription, DNA-templated	0.00888	0	HMLALPHA1/YCL066W:MATALPHA1/YCR040W
regulation of intracellular protein transport	0.01424	0	FUS3/YBL016W:GPA1/YHR005C
cellular response to chemical stimulus	0.01717	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:SAG1/YJR004C
regulation of protein transport	0.02085	0	FUS3/YBL016W:GPA1/YHR005C
regulation of nucleocytoplasmic transport	0.03308	0.01	FUS3/YBL016W:GPA1/YHR005C
regulation of establishment of protein localization	0.03308	0.01	FUS3/YBL016W:GPA1/YHR005C
protein export from nucleus	0.03777	0.01	FUS3/YBL016W:GPA1/YHR005C

Appendix 5 | GO term enrichment for all differentially expressed genes under both conditions

GO_term	P-value	FDR	Gene(s) annotated to the term
transposition, RNA-mediated	5.18E-16	0	YAR010C:FUS3/YBL016W:TEC1/YBR083W:YDR261C-D:YDR365W-B:YER138C:YGR027W-B:YJR027W:YJR029W:YLR227W-B:YML045W:YMR050C:YNL284C-A:YPR137C-A:YPR158C-D
transposition	9.13E-16	0	YAR010C:FUS3/YBL016W:TEC1/YBR083W:YDR261C-D:YDR365W-B:YER138C:YGR027W-B:YJR027W:YJR029W:YLR227W-B:YML045W:YMR050C:YNL284C-A:YPR137C-A:YPR158C-D
cellular response to pheromone	7.50E-12	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
response to pheromone	9.68E-12	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
response to pheromone involved in conjugation with cellular fusion	4.01E-11	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
conjugation with cellular fusion	1.57E-10	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
conjugation	1.73E-10	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
multi-organism cellular process	3.70E-10	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
cellular response to organic substance	1.49E-08	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
sexual reproduction	2.77E-08	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:GAS2/YLR343W:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
multi-organism reproductive process	2.77E-08	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:GAS2/YLR343W:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W

multi-organism process	7.18E-08	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:GAS2/YLR343W:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
reproductive process	7.79E-08	0	FUS3/YBL016W:FUS1/YCL027W:HMLALPHA1/YCL066W:MATALPHA1/YCR040W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:GAS2/YLR343W:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:NDJ1/YOL104C:MF(ALPHA)1/YPL187W
reproduction	1.29E-07	0	FUS3/YBL016W:FUS1/YCL027W:HMLALPHA1/YCL066W:MATALPHA1/YCR040W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:GAS2/YLR343W:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:NDJ1/YOL104C:MF(ALPHA)1/YPL187W
response to organic substance	3.84E-07	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
signal transduction involved in conjugation with cellular fusion	2.19E-06	0	FUS3/YBL016W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:FAR1/YJL157C:STE3/YKL178C:MF(ALPHA)1/YPL187W
pheromone-dependent signal transduction involved in conjugation with cellular fusion	2.19E-06	0	FUS3/YBL016W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:FAR1/YJL157C:STE3/YKL178C:MF(ALPHA)1/YPL187W
G-protein coupled receptor signaling pathway	6.77E-06	0	FUS3/YBL016W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:FAR1/YJL157C:STE3/YKL178C:MF(ALPHA)1/YPL187W
cellular response to chemical stimulus	2.05E-05	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
response to chemical	0.00042	0	FUS3/YBL016W:FUS1/YCL027W:MF(ALPHA)2/YGL089C:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:SAG1/YJR004C:STE3/YKL178C:SST2/YLR452C:FUS2/YMR232W:AGA1/YNR044W:MF(ALPHA)1/YPL187W
single-organism process	0.00456	0	YAR010C:FUS3/YBL016W:TEC1/YBR083W:FUS1/YCL027W:HMLALPHA1/YCL066W:MATALPHA1/YCR040W:YDR261C-D:YDR365W-B:YER138C:MF(ALPHA)2/YGL089C:YGR027W-

			B:GPA1/YHR005C:STE12/YHR084W:FAR1/YJL157C:YJR027W:YJR029W:STE3/YKL178C:YLR227W-B:GAS2/YLR343W:SST2/YLR452C:YML045W:YMR050C:FUS2/YMR232W:YNL284C-A:NDJ1/YOL104C:MF(ALPHA)1/YPL187W:YPR137C-A:MEP3/YPR138C:YPR158C-D
cytogamy	0.00653	0	FUS1/YCL027W:STE3/YKL178C:FUS2/YMR232W
regulation of protein export from nucleus	0.01826	0	FUS3/YBL016W:GPA1/YHR005C
regulation of termination of mating projection growth	0.0364	0	FUS1/YCL027W:FUS2/YMR232W
agglutination involved in conjugation with cellular fusion	0.0364	0	SAG1/YJR004C:AGA1/YNR044W
agglutination involved in conjugation	0.0364	0	SAG1/YJR004C:AGA1/YNR044W
adhesion between unicellular organisms	0.0364	0	SAG1/YJR004C:AGA1/YNR044W
multi organism cell adhesion	0.0364	0	SAG1/YJR004C:AGA1/YNR044W

Appendix 6 | GO term enrichment of genes not clustered by kappa analysis

GO_term	P-value	FDR	Gene(s) annotated to the term
transposition, RNA-mediated	5.27E-16	0	FUS3/YBL016W:YDR261C-D:YDR365W-B:YER138C:YGR027W-B:YJR027W:YJR029W:YLR227W-B:YML045W:YMR050C:YNL284C-A:YPR137C-A:YPR158C-D
transposition	8.60E-16	0	FUS3/YBL016W:YDR261C-D:YDR365W-B:YER138C:YGR027W-B:YJR027W:YJR029W:YLR227W-B:YML045W:YMR050C:YNL284C-A:YPR137C-A:YPR158C-D
regulation of protein export from nucleus	0.00367	0.01	FUS3/YBL016W:GPA1/YHR005C
cell cycle arrest	0.01219	0.03	FUS3/YBL016W:FAR1/YJL157C
signal transduction involved in conjugation with cellular fusion	0.01661	0.02	FUS3/YBL016W:GPA1/YHR005C:FAR1/YJL157C
pheromone-dependent signal transduction involved in conjugation with cellular fusion	0.01661	0.02	FUS3/YBL016W:GPA1/YHR005C:FAR1/YJL157C
response to pheromone involved in conjugation with cellular fusion	0.01681	0.01	FUS3/YBL016W:GPA1/YHR005C:FAR1/YJL157C:SST2/YLR452C
G-protein coupled receptor signaling pathway	0.02831	0.03	FUS3/YBL016W:GPA1/YHR005C:FAR1/YJL157C
cellular response to pheromone	0.03239	0.03	FUS3/YBL016W:GPA1/YHR005C:FAR1/YJL157C:SST2/YLR452C
response to pheromone	0.03506	0.02	FUS3/YBL016W:GPA1/YHR005C:FAR1/YJL157C:SST2/YLR452C

Genome-wide mechanisms of chronological longevity by dietary restriction in budding yeast

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SUMMARY

Dietary restriction is arguably the most promising non-pharmacological intervention to extend human life and health span. Yet, only few genetic regulators mediating the cellular response to dietary restriction are known, and the question remains which other regulatory factors are involved. Here, we measured at the genome-wide level the chronological lifespan of *Saccharomyces cerevisiae* gene-deletion strains under two nitrogen-source regimens, glutamine (non-restricted) and γ-aminobutyric acid (restricted). We identified 472 mutants with diminished or enhanced extension of lifespan. Functional analysis of such dietary-restriction genes revealed novel processes underlying longevity by the nitrogen-source quality, which also allowed us to generate a prioritized catalogue of transcription factors orchestrating the dietary-restriction response. Importantly, deletions of transcription factors Msn2, Msn4, Snf6, Tec1, and Ste12 resulted in diminished lifespan extension and defects in cell-cycle arrest upon nutrient starvation, suggesting that regulation of the cell cycle is a major mechanism of chronological longevity. We further show that *STE12* overexpression is enough to extend lifespan, linking the pheromone/invasive growth pathway with cell survivorship. Our global picture of the genetic players of longevity by dietary restriction highlights intricate regulatory cross-talks in aging cells.

INTRODUCTION

Dietary restriction—a reduction in calorie intake without malnutrition, or substitution of the preferred carbon or nitrogen source—extends lifespan in virtually all species studied in the laboratory (Mair & Dillin 2008). Dietary restriction has been associated with protection against age-associated disease in mice, including neurodegenerative disorders (Zhu et al. 1999) and cancer (Yamaza et al. 2010), promoting longer lifespan and healthier aging (Fontana & Partridge 2015). Importantly, this intervention reduces the mortality rate in non-human primates (Colman et al. 2014), and delays the onset of aging-related physiological changes in humans (Holloszy & Fontana 2007), making dietary restriction the most promising intervention targeted to extend human lifespan. Yet, we are still missing a global picture of the genetic architecture of such lifespan response, which is needed to grant a deeper understanding of the genotype-phenotype relationship of aging and longevity (Schleit et al. 2013).

The budding yeast *Saccharomyces cerevisiae* has been a pivotal model organism in the discovery of the molecular basis of aging. Two aging models are widely used in this organism: The replicative lifespan of yeast, which refers to the number of times a single yeast cell can divide, and the chronological lifespan (CLS)—a measure of the viability of a population during stationary phase through time. The latter provides a good model for inquiring the molecular changes faced by post-mitotic cells (Longo et al. 2012).

In yeast, dietary restriction results in lifespan extension at least through the modulation of the conserved TOR and Ras/cAMP/PKA pathways, which regulate cellular growth and maintenance in response to nutrient availability (Kaeberlein et al. 2005). Depletion of TOR components, Tor1 and Sch9, results in CLS extension (Powers et al. 2006). Under low nutrient conditions, the serine/threonine kinase Rim15 phosphorylates transcription factors Msn2, Msn4, or Gis1, activating a maintenance response (Fabrizio et al. 2004). However, the *msn2*Δ*msn4*Δ*gis1*Δ triple mutant still shows lifespan extension by DR (Wei et al. 2008). Moreover, transcriptomic evidence and *in silico* predictions suggest that a larger number of up- and downstream genes are involved in lifespan extension (Wuttke et al. 2012; Choi et al. 2017); most of these candidates lack direct phenotype confirmation. These observations suggest that there is an unknown number of longevity regulators that are yet to be identified.

Aging research in yeast has recently taken advantage of genome-wide approaches, enabling a comprehensive description of genes involved in lifespan regulation. For instance, a recent systematic study of replicative lifespan of most viable deletion strains revealed that translation, the SAGA complex, and the TCA cycle mediate longevity (McCormick et al. 2015). Several studies have aimed to estimate the stationary-phase survival of single-deletion mutants in parallel (Powers et al. 2006; Matecic et al. 2010; Fabrizio et al. 2010; Gresham et al. 2011; Garay et al. 2014), showing that autophagy, vacuolar protein sorting, regulation of translation, purine metabolism, chromatin remodeling, and the SWR1 complex are major determinants of longevity. However, we are still missing a direct comparison of the lifespan effects

of such gene deletions under nutrient-rich and restricted conditions, that would allow to systematically address the mechanisms of longevity by dietary restriction.

The aim of this study was to generate a global picture of the underlying genetics of lifespan extension by dietary restriction, by systematically describing gene-diet interactions in yeast. Specifically, we compared the CLS of a collection of 3,718 knockout mutants aged in media with glutamine (non-restricted) or γ-aminobutyric acid (GABA, dietary restricted) nitrogen source, for which we used a high-resolution parallel phenotyping assay (Garay et al. 2014). These screens revealed that at least 472 genes have a role in lifespan extension by GABA. Subsequent analyses uncovered the major biological processes involved and a comprehensive catalogue of transcription factors that control lifespan extension in this mode of dietary restriction. Many gene deletions of such regulators were defective in arresting the cell cycle upon nitrogen or carbon starvation, suggesting that cell-cycle control is a mechanism of chronological longevity. We focus on the transcription factor Ste12 and discuss upstream signaling pathways and downstream processes that could underlie its pro-longevity and cell cycle roles in response to nutrients.

RESULTS

Genome-wide profiling of yeast chronological lifespan under different dietary regimens

To identify gene-deletions that modify the effect of dietary restriction, we set out to establish a model of lifespan extension in response to nutrient limitation. In yeast, lifespan is extended either by limiting the concentration of glucose in the medium or by using a non-preferred source of nitrogen (Powers et al. 2006; Jiang et al. 2000). We measured the lifespan of a wild-type (WT) strain aged under different nitrogen sources by adapting an established method for quantitative analysis of yeast CLS based on outgrowth kinetics (Murakami et al. 2008) to a high-throughput robotic platform (see Experimental Procedures). The half-life of the WT strain varied substantially, from 5.6 days in the rich nitrogen source ammonium to 26.5 days in GABA (**Table 1**; **Figure S1A**). The use of GABA as the sole nitrogen source resulted in extended lifespan without major effects on the growth kinetics (**Table 1**; **Figure S1B**); therefore, we selected GABA as our dietary-restricted condition. We used glutamine in the non-restricted medium, as it resulted in one of the shortest half-lives measured and it is a preferred nitrogen source (Godard et al. 2007); ammonium sulfate was not used because of its toxicity in stationary phase (Santos et al. 2015). After repeating the experiment only under the selected conditions, we observed that our nitrogen-based dietary-restriction model resulted in a 91% extension of the CLS, from a WT half-life of 14.7 days in glutamine to 28.1 days in GABA (**Figure S1C**).

To identify the genetic determinants of lifespan extension by dietary restriction at the genome-wide level, we measured the CLS of 3,718 gene-knockout strains under

GABA (dietary-restricted) and glutamine (non-restricted) media. We used a profiling assay based on the measurement of a relative survival coefficient (*s*) of each knockout strain aged in co-culture with the WT strain (**Figure 1A**). Our model of stationary-phase survivorship assumes that yeast cells die in an exponential manner, thus the natural logarithm of the ratio of each mutant strain and the WT reference was adjusted to a linear fit; short-lived mutants had negative slopes (survival coefficient, *s*) while long-lived strains had positive slopes (see examples under the two conditions in **Figure 1B**). In addition, we obtained the *s* of 264 WT *versus* WT competitions under both nutrimental conditions, and used the mean and standard deviation of this population to calculate a Z-score for each mutant, assuming that the variance in the mutants was the same as in the WT. Finally, we used the Benjamini-Hochberg false-discovery rate (FDR) to correct for multiple hypothesis testing (**Figure 1C**). Using a FDR threshold of 5%, we scored 573 significantly short-lived and 254 long-lived single knockout strains in the non-restricted medium, while dietary restriction resulted in 510 short-lived and 228 long-lived mutants (**Supporting Data S1**). We confirmed that most mutants showed a good fit to such exponential decay model, and observed no significant differences between the distribution of errors in the fit among the different phenotypic categories (p>0.05 Wilcoxon rank-sum test; **Figure S2**).

A considerable fraction of the strains tested showed condition-specific CLS effects (**Figure 1D**). Most condition-specific effects were associated to coherent phenotypes (short or long-lived under both conditions) or neutral phenotypes under one of the conditions: only seven gene deletions were long-lived under glutamine showing the

opposite effects under GABA, while eleven gene deletions were short-lived under glutamine but long-lived under GABA.

To validate our genome-wide screens, we turned again to the small-scale outgrowth-kinetics CLS approach (Murakami et al. 2008) and measured in single-strain cultures the lifespan of a set of randomly-selected knockouts with significant lifespan effects (5% FDR). Twelve out of 16 (75%) strains that were re-tested under non-restricted glutamine medium recapitulated the CLS effects observed in the genome-wide screen (**Figure S3A**; $p<0.05$, *T*-test), while 11 out of 17 (65%) strains were consistent with the results under GABA (**Figure S3B**). Notably, the rate of validation is considerably higher compared to screening assays that have used pooled yeast deletion strains (6–31% hits validated) (Fabrizio et al. 2010; Matecic et al. 2010). To directly compare the results obtained with the conventional single-culture CLS assay and with our high-throughput screen, we modeled a survival curve from the survival coefficients of the corresponding knockout strains (**Figure S3A-B**). Together, the results of these validation experiments indicate that our profiling approach provides accurate and quantitative CLS scores under different conditions, allowing the description of gene-diet interactions.

Systematic identification of dietary-restriction genes in yeast

To gain insight into the genes that mediate the lifespan-extending effects of dietary restriction, we searched for deletion strains that showed differential relative CLS effects under the two nitrogen sources. Specifically, we sought to identify strains that showed a shorter survival coefficient under GABA compared to the non-restricted

glutamine medium (diminished lifespan extension); likewise, we scored cases where the survival coefficient was relatively larger under GABA (enhanced lifespan extension) (see examples in **Figure 2A**).

We quantitatively defined the relative lifespan extension of each knockout as $LE = \frac{s_{DR}+1}{s_{NR}+1}$ (see Experimental Procedures). Next, we compared the LE of each deletion strain to the distribution of LE in 264 independent WT replicates to obtain a *Z*-score (**Figure 2B**). After correcting for multiple tests we scored 583 mutant strains with a significant LE; for higher stringency, we filtered out strains that did not show a significant CLS effect in either condition. The final list included 472 gene-knockouts with altered dietary-restriction response (5% FDR; **Figure 2C**; **Supporting Data S2**). This comprehensive set, which we termed DR-genes, includes 219 knockouts with diminished longevity (LE<1) and 253 gene-knockouts that displayed enhanced lifespan extension (LE>1). These results indicate that many gene-environment interactions underlie longevity by nitrogen restriction in yeast.

In addition to their diminished or enhanced phenotypes based on *LE*, we classified DR-genes based on their effects under glutamine and GABA (**Supporting Data S2**). This categorization was useful to identify genes of particular cellular functions that behaved in a similar manner. For instance, DR-gene deletions with diminished longevity (LE<1) that were long-lived under both glutamine and GABA were enriched for ribosome biogenesis (5% FDR), while different functions related to autophagy and protein-targeting to vacuole were common in strains with diminished longevity and short-lived phenotypes under both conditions. On the other hand, genes of

mitochondrial translation were enriched in deletions with enhanced longevity and short-lived phenotypes under both glutamine and GABA (**Supporting Data S2**).

To estimate the sensitivity of the identified DR-genes to the mode of dietary restriction tested, specifically the source of nitrogen, we repeated our analysis using a different non-restricted medium reference. Specifically, we used data from a previously reported genome-wide screen performed in 2% glucose SC medium with ammonium sulfate as the sole nitrogen source (Garay et al. 2014), to obtain a new relative lifespan extension value for each knockout, $LE_{GABA/NH4}$. We observed a strong skew in the distribution of $LE_{GABA/NH4}$ values, for both sets DR-genes with diminished-LE ($p<10^{-28}$, one tailed Wilcoxon ranked-sum test) or enhanced-LE ($p<10^{-34}$) using the GABA/glutamine comparison (**Figure S4A**). Nonetheless, a number of LE values did show strong dependency on the nitrogen source used in the analysis.

To look into possible processes underlying media-specific effects on lifespan extension, we focused on the top 20 genes with the highest absolute difference in the LE values (**Figure S4B**). Eleven out of 20 such genes were located to the mitochondria (YeastMine, Saccharomyces Genome Database), but not significant enrichment to any GO term was found (**Figure S4C**). In this regard, the screen performed in ammonium was done in buffered medium (Garay et al. 2014), while the ones presented here are not, implying that some of the condition-specific effects could be due to different pH in the stationary phase cultures. In conclusion, these comparative analyses suggest that, while some gene deletions have diminished or

enhanced lifespan extension only under specific conditions, most DR-genes influence the lifespan response to nutrient limitation regardless of the nitrogen source used to model dietary restriction.

Functional classification of dietary-restriction genes

To systematically describe which downstream cellular functions influence lifespan extension by dietary restriction under the specific nitrogen conditions tested, we sought to classify the 472 DR-genes according to their annotated functional features. We used a kappa statistic approach (Huang, Sherman, et al. 2009) to cluster genes by shared GO terms and mutant phenotypes—as reported in the Saccharomyces Genome Database—, providing associations between the gene deletions based on their phenotypes and other genes' features reported. With this approach, genes were clustered into discrete groups even when there is no common GO association among them, allowing a non-supervised identification of the cellular processes represented. The analysis was performed separately for genes with diminished (LE<1) or enhanced (LE>1) lifespan extension (**Figure 3**; **Supporting Data S3**).

Some clusters recapitulated cellular functions previously related to lifespan regulation, such as autophagy (Meléndez et al. 2003), mitochondrial function (Aerts et al. 2009), and cytosolic translation (Hansen et al. 2007). Importantly, this classification also revealed novel processes such as maintenance of cell-cycle arrest mediated by pheromone and establishment of nucleus localization in the cell. Hence, our screen re-discovered processes previously known to influence the dietary

response and, at the same time, it allowed the identification of novel genes and processes related to longevity by dietary restriction.

Deletion of genes necessary for the maintenance of cell-cycle arrest resulted in a diminished lifespan extension. Specifically, deletion of pheromone-responsive genes *FAR7* and *FAR8* had short-lived phenotype under dietary restriction (**Figure 3A**). In yeast, these Far proteins prevent cell cycle recovery after pheromone exposure, possibly by inhibiting *CLN1*-3 (Kemp & Sprague 2003). Moreover, mutations in genes required for processing and correct localization of ribonucleoprotein complexes resulted in a strongly diminished lifespan extension. While it is well established that ribosomal function is downregulated in response to TOR inhibition or nutrient depletion (Hansen et al. 2007), our screen pointed to specific proteins involved in pre-rRNA processing (Slx9), nucleolar rRNA methyltransferase (Rrp8), nuclear export of pre-ribosomal subunits (Arx1), and translational initiation (Bud27). Likewise, deletion of *DYN1-3*, *JNM1*, *NUM1*, and *PAC1*, all involved in nuclear movement along microtubules, resulted in diminished lifespan extension. In this regard, it is known that certain cellular processes needed for extended longevity, such as autophagy, require intact function of microtubules (Köchl et al. 2006). However, the relationship between nuclear localization and lifespan extension remains unexplored.

We also found clusters with enhanced lifespan extension, such as mitochondrial function (**Figure 3B**). Dietary restriction shifts metabolism towards respiration (Lin et al. 2002) but, at the same time, impaired respiration can promote longevity in yeast and nematodes through enhanced retrograde response and activation of anaplerotic

pathways (Cristina et al. 2009). Although we did not follow-up on this intriguing observation, we speculate that a higher demand for respiration during dietary restriction could lead to the activation of compensatory pathways. Other feedback mechanisms may account for the alleviation of deleterious effects observed in other deletion strains: For example, the short-lived phenotypes of deletion of cell-wall genes *CWP1, DSE2,* and *TIR3* were largely alleviated under dietary restriction. Taken together, these findings suggest that lifespan extension in response to dietary restriction is a complex phenotype resulting from the interplay of many downstream cellular processes.

Importantly, we carried out an experiment to rule out the possibility that particular groups of DR-genes were scored due to function-specific alterations in the fluorescence signal that was used to measure the CLS of knockout strains (e.g. accumulation of fluorescent proteins in autophagy-deficient mutants). To this end, we chose several autophagy and ribosomal-protein genes, along with a set of randomly selected DR-genes (20 knockouts altogether). At different time points in stationary phase, we measured single-cell fluorescence signal of the selected strains using flow cytometry (see Experimental Procedures). The average signal increased slightly in the WT, and most mutant strains tested showed a very similar trend (**Figure S5A-B**). A number of strains did show differences in initial signal or signal change compared to the WT, including *vps51*Δ and *atg13*Δ (two out of five autophagy and vacuolar protein sorting mutants tested). However, we observed no correlation between signal changes and the measured survival coefficients

(*p*=0.092, Pearson correlation; **Figure S5B**), suggesting that specific fluorescence-signal dynamics did not introduce a systematic bias in our survival results.

A defined set of transcription factors control lifespan extension by dietary restriction

Complex phenotypic responses are frequently coordinated by transcriptional regulation of functionally-related genes. To investigate the transcriptional regulation of longevity by dietary restriction, we analyzed our set of DR-genes using an algorithm to search for the transcriptional regulators of these genes (**Table 2**). Specifically, we used TFRank (Gonçalves et al. 2011), a graph-based approach that takes advantage of all known regulatory paths in *S. cerevisiae.* A weight is assigned to each TF according to the presence or absence of target genes and their regulators in the regulatory network of *S. cervevisiae*. The weight of the transcription factor increases according to the number of direct and indirect targets to rank the transcription factors; this weight is re-evaluated through a diffusion coefficient, which takes into account the number of layers of the transcription factors hierarchy to obtain a list of prioritized regulatory players. Based on this analysis, we established a possible role in lifespan extension to the top-ranked transcription factors; which included regulators that were not in our gene-knockout screen due to gene essentiality or sterility. Transcription factors within the top 5% ranked DR-regulators included Msn2 and Msn4; these transcription factors are well known regulators of lifespan (Fabrizio et al. 2004). Intriguingly, most top-ranked transcription factors had not been previously associated to longevity in yeast. Noteworthy, the top hits (Ace2, Ash1, Tec1, Spf1, and Ste12) regulate different aspects of cell-cycle progression.

To assess the predictive power of the TFRank approach, we compared the ranks of the transcription factors that showed gene-deletion lifespan phenotypes either in glutamine or GABA media (CLS transcription factors) with that of transcription factors showing no effect on lifespan. DR-regulators with a deletion phenotype were typically ranked higher by the TFRank algorithm than regulators with no CLS effect ($p<10^{-5}$, Wilcoxon ranked-sum test) (**Figure 4A**). The average ranks for transcription factors were 63.5 and 112.9 for regulators with and without a CLS gene-deletion phenotype, respectively. This indicates that the downstream DR-genes and cellular pathways identified in our genome-wide screen are phenotypically linked to a coherent set of transcription factors.

The set of DR-genes was defined from our genome screens under a particular dietary-restriction regime based on nitrogen limitation. We thus decided to evaluate the role of those DR-regulators in lifespan extension by calorie restriction, a classic form of dietary restriction in yeast and other organisms (Mair & Dillin 2008). Specifically, we generated *de novo* deletion mutants for eight top-ranked transcription factors and measured their CLS in 2% and 0.5% glucose using the small-scale outgrowth-kinetics approach. Strikingly, all transcription-factor gene deletions resulted in altered CLS under 0.5% glucose: six strains were short lived, while the remainder strains (*ace2*Δ and *bas1*Δ) were long-lived under the glucose-restricted condition (**Figure 4B**). As expected, the same mutants showed altered CLS in SC medium with glutamine or GABA as nitrogen source (**Figure S6**). Most of the observed lifespan effects under glucose- or nitrogen-restricted conditions were moderate, which is in agreement with the fact that transcription factors in yeast

typically act upon overlapping targets, providing functional compensation to one another (Zheng et al. 2010). Together, these results show that top TFRank hits are general regulators of lifespan extension by dietary restriction in yeast.

Many of the transcription factors involved in lifespan regulation mediate cell-cycle arrest in response to nitrogen or carbon starvation

Our functional classification of DR-genes and TFRank analysis underscored the contribution of cell-cycle control as a mechanism of lifespan regulation. To further explore the association between longevity by dietary restriction and the cell cycle, we directly measured the cell-cycle status of DR-regulator mutants after nutrient depletion. Specifically, we measured the DNA content of growing cells starved for nitrogen or carbon by flow cytometry. Interestingly, most mutants (Tec1, Ste12, Snf6, Msn2, and Msn4) failed to arrest efficiently in G1 after nitrogen or carbon depletion (**Figure 5A-B**). The long-lived *bas1*Δ cells arrested faster compared to the WT, but only under carbon starvation (**Figure 5B**). The *ace2*Δ strain analysis showed events of large DNA content; this mutation affects mother-daughter cell separation, which explains the large fraction of events with >2n DNA. These findings suggest that many of the transcription factors that act as DR-regulators are involved in cell-cycle control or possibly polarized growth in response to nutrient limitation.

Ste12 is a positive regulator of longevity by dietary restriction and cell-cycle arrest in response to nutrients

Among the top hits of our DR-regulators analysis was Ste12, a transcription factor acting on genes involved in mating or pseudohyphal growth (Dolan et al. 1989;

Roberts & Fink 1994). *STE12* is an important regulatory hub during stationary phase (Wanichthanarak et al. 2015), however its role in stationary-phase survival in response to nutrients has not been confirmed, as opposed to other hits such as Msn2 and Msn4 (Fabrizio et al. 2004). To further explore the functional link of Ste12 with lifespan extension, we confirmed its role in cell survival under standard conditions of full aeration (Hu et al. 2013). While the lifespan of the *ste12*Δ strain was not affected under non-restricted 2% glucose, mutant cells showed diminished longevity in the 0.5% glucose dietary-restriction medium (**Figure 6A**). In addition, given that our method for measuring CLS in yeast relies in the ability of stationary-phase cells to re-enter the cell cycle, and that Ste12 is involved in cell-cycle arrest, we ruled out technical artifacts by using an alternative cell-viability assay. Direct staining of dead and alive cells in stationary phase showed that the alive *ste12*Δ population died faster than the WT under limited glucose and, to a lesser extent, under non-restricted conditions (**Figure S7A-B**). Together, these results confirm that the lifespan effects of the *STE12* deletion are maintained regardless of the experimental conditions and methodology used to infer population survivorship in stationary phase.

High expression of a bona fide positive regulator of lifespan is expected to cause increased cell survivorship. Hence, we generated a copper-inducible Ste12 overexpression strain with a GFP fusion to track Ste12 protein levels; the WT and *pCUP1-STE12* strains were aged under varying concentrations of copper sulfate in 2% glucose SC medium. We found that the CLS of the *STE12*-overexpression strain increased readily as a function of copper concentration under non-restricted conditions (**Figure 6B**). Increased copper concentrations had no effect on the CLS

nor growth of the WT strain (**Figure S8A-B**). Importantly, GFP signal in the nucleus increased as a function of copper concentration (**Figure S9**), confirming a link between lifespan and increased levels of Ste12. Together, these results unambiguously confirm that Ste12 is a novel positive transcriptional regulator of lifespan in yeast.

The TOR pathway regulates cell cycle transitions upon nutrient depletion through Rim15 activity (Pedruzzi et al. 2003). It was thus tempting to ask whether Ste12 mediates cell-cycle arrest in concert with the TOR pathway. To this end, we used flow cytometry to monitor the cell-cycle dynamics following treatment with the TOR-inhibiting drug rapamycin, which is enough to trigger cell cycle arrest even in nutrient-rich conditions. While we confirmed that the *ste12*Δ mutant was impaired in cell-cycle arrest upon nitrogen depletion, rapamycin treatment produced similar cell cycle profiles in *ste12*Δ and WT strains (**Figure 6C**). This result indicates that TOR-mediated cell-cycle arrest does not require the activity of Ste12, suggesting that this transcription factor integrates nutrient signals leading to cell cycle arrest independently of TOR signaling.

DISCUSSION

"The intrinsic nature of the ageing process is essentially one of systems degradation" (Kirkwood 2008). With a growing number of genetic aging factors in hand, the next great challenge is to understand how the mechanisms of aging and longevity are integrated to one another and to the environment. In this study, we have adapted

the chronological-aging paradigm in yeast to provide a quantitative and systematic description of how different dietary conditions impacts lifespan. Specifically, we have screened a collection of 3,718 gene-deletion strains aged in glutamine—a preferred nitrogen source—or GABA—a nitrogen-poor condition. Our analysis revealed that nitrogen limitation modifies the lifespan effect of 472 gene deletions (DR-genes). To the best of our knowledge, this study yields the most comprehensive phenotypic compendium of genetic players involved in longevity by dietary restriction.

The classification of DR-genes obtained with our kappa cluster analysis provided validation of *LE* index in the form of functional relationships. We note that we did not directly validate our list of DR genes by measuring survivorship with other CLS methodologies and calculating *LE* thereby. Functional clusters involved in longevity by nitrogen restriction included autophagy, cytosolic translation, peroxisome biogenesis, respiration, and mitochondrial function, along with novel factors such as processing of ribonucleoprotein complexes, cell-wall organization, microtubule-based nuclear movement, and—noteworthy—genes involved in cell cycle arrest. Furthermore, our analysis of DR-genes as regulatory targets allowed the establishment of a set of ranked transcription factors (DR-regulators) orchestrating the cellular response to nitrogen restriction. Strikingly, most high-ranked DR-regulators were transcription factors involved in mitotic cell-cycle transitions, either by repression of Cln3 specifically in yeast daughter cells (Ace2 and Ash1) (Laabs et al. 2003; Di Talia et al. 2009), activation of ribosomal-protein genes (Sfp1) (Marion et al. 2004), or cell differentiation in response to nutrients or pheromone (Tec1 and Ste12) (Madhani et al. 1999). Top-hit transcription factors also included, Msn2 and

Msn4, two positive regulators of stress-response and lifespan extension downstream of the Tor//Ras-PKA pathways that converge on Rim15, the main protein kinase involved in cell survivorship in response to nutrients (Wei et al. 2008). We revealed that several DR-regulators (Msn2, Msn4, Snf6, Tec1, and Ste12) are needed for accurate arrest of the cell cycle in response to starvation. This is in agreement with recent studies showing that dietary restriction extends the chronological lifespan of yeast, in part by regulation of the cell cycle and entry into a quiescent state (Leonov et al. 2017).

Noteworthy, deletion of *ACE2* impedes mother-daughter cell separation; thus, altering the DNA content during flow cytometry, probably due to the presence of a pseudo-filamentous growth. In addition, given that Tec1 and Ste12 are master regulators of the invasive-growth pathway, it is tempting to link filamentous growth and longevity. However, the yeast background used here, S288C, bears a nonsense mutation in *FLO8* that prevents it from forming pseudohyphae, making this strain incapable of invasive/filamentous growth (Liu et al. 1996). Nevertheless, it remains to be addressed if Ste12 overexpression can override the deficiency of *FLO8* leading to pseudohyphae formation. While we did not confirm a causal link between cell cycle arrest defects and longevity in these knockout strains, their phenotypes suggest that cell cycle control or pseudohyphae formation are involved in lifespan extension by dietary restriction.

Importantly, the lifespan phenotypes of DR-regulators were reproducible in two different modes of dietary restriction, namely nitrogen-source or glucose-concentration limitation. This was also the case for their cell-cycle arrest defects:

similar gene-deletion phenotypes were observed in the cell cycle dynamics triggered by both nitrogen and carbon starvation. This is relevant in the context that dietary-restriction responses depend on nutrient composition (Wu et al. 2013) and that different sensing pathways underlie longevity by glucose or amino acid restriction (Mirisola et al. 2014). But the fact that several protocols of dietary restriction result in lifespan extension, including restriction of glucose, reduced nitrogen levels, and restriction of specific amino acids suggests that the underlying response involves overlapping mechanisms (Kennedy et al. 2007). Transcription factors herein identified as DR-regulators are likely part of the general machinery leading to extended lifespan regardless of the mode of dietary restriction. A next challenge is to describe how these regulators integrate signals from different nutrients and the extent to which their roles are overlapping or independent from one another.

We have provided compelling evidence that *STE12* is a positive regulator of longevity by dietary restriction. Ste12 acts downstream of two cell-differentiation programs regulated by MAPK pathways, namely mating and invasive growth (Dolan et al. 1989; Roberts & Fink 1994). Not only the *ste12*Δ strain showed diminished longevity under different experimental settings, but also *STE12* overexpression was sufficient to extend lifespan under a non-restricted diet. Importantly, we confirmed the *ste12*Δ phenotypes in our screening settings under standard conditions in which chronological aging has been assayed elsewhere. We also found that deletion of *STE12* results in a failure to arrest the cell cycle upon nutrient starvation.

While we did not confirm a direct causal link between cell cycle arrest and extended longevity mediated by Ste12, one possibility is that regulatory elements of the mating

pathway are recruited to arrest the cell cycle during dietary restriction, which in turn protects against replication stress during stationary phase, leading to increased longevity (Weinberger et al. 2007). In the presence of pheromone, the cell cycle is arrested by the action of Ste12, Far1, and the FAR complex (Far3 and Far7-11) (Kemp & Sprague 2003); Far1 and Far3 are direct targets of Ste12 (Lefrançois et al. 2009). Concomitantly, deletion of *FAR7*, *FAR8*, and *FAR11* in our primary screen resulted in diminished lifespan extension. An alternative possibility is that Ste12 mediates cell-cycle arrest and longevity through its association to Tec1. This transcription factor promotes cell cycle progression through inhibition of Cln1 (Madhani et al. 1999). Hence, association to Ste12 by filamentous-growth pathway nutrient signaling (Madhani et al. 1999) could prevent Tec1-mediated progression of the cell cycle, resulting in increased longevity.

The TOR pathway regulates cell cycle transitions upon nutrient depletion through activity of the Rim15 kinase (Pedruzzi et al. 2003). However, regulation of the cell cycle shows poor correlation with the short-lived phenotype of Rim15-defficient cells; other TOR/Rim15-dependent mechanisms such as carbohydrate storage seem to play a more important role (Cao et al. 2016). In agreement, we found that Ste12 regulation of cell cycle is not under the control of the TOR pathway, as rapamycin treatment does not affected G1 arrest in a *ste12*Δ background, suggesting that Ste12 controls cell cycle transitions in response to nutrients in a TOR-independent manner. Hence, it is possible that Ste12 regulates lifespan through other mechanisms. Noteworthy, *STE11*, an upstream component of the pheromone pathway, is also implicated in cell cycle and lifespan regulation (Cao et al. 2016). This supports the

idea that that Ste12 controls cell cycle transitions in response to nutrients and that the emergence of a G1-arrested population is necessary for lifespan extension.

Whether the longevity roles of the DR-regulators are conserved is still an open question. For instance, *STE12* has no clear homolog in animals, however, transcriptional networks can be rewired through evolution, leading to changes in the regulation exerted by specific regulators, while the downstream targets remain associated (Sorrells et al. 2015). In addition, the yeast three-kinase module regulating MAPK pheromone and invasive-growth pathways are conserved in other organisms (Widmann et al. 1999). In particular, *KSS1* and *FUS3* are key members of the MAPK pathway that regulates cell differentiation programs in yeast (Bardwell 2004), while their mammalian counterpart *MAPK1* is central to the development of several age-associated diseases in mammals (Carlson et al. 2008). Thus, the study of targets downstream the MAPK pathway in yeast might bring important insights into the regulation of aging in other eukaryotes, including humans.

Our study provides a much-needed comprehensive picture of the mechanisms of lifespan extension by dietary restriction, underscoring key links between nutrient sensing, the cell-cycle arrest machinery, and longevity in yeast. Our approach can be readily applied to other genetic, environmental, or pharmacological perturbations, providing a systematic framework to describe aging networks in a simple tractable system. Other cross-talks among downstream cellular processes and their transcriptional regulators may remain to be uncovered, which will shed further light to the genetic wiring of aging cells.

EXPERIMENTAL PROCEDURES

Strains and media. Fluorescent single-gene deletion strains are prototrophic haploids (*MAT*a *xxx*Δ*::kanMX4 PDC1-mcherry-CaURA3MX4 can1*Δ*:STE2pr-SpHIS5 lyp1*Δ *his3*Δ*1 ura3*Δ*0 LEU2*) derived from crossing the *MAT*α YEG01-RFP SGA-starter with 4,340 viable deletion strains from the *MAT*a BY4741 collection from the Saccharomyces Genome Deletion Project (Garay et al. 2014). All *de novo* single-gene deletions were generated in the YEG01-RFP parental strain (*MAT*α *PDC1-mcherry-CaURA3MX4 can1*Δ*:STE2pr-SpHIS5 lyp1*Δ *his3*Δ*1 ura3*Δ*0 LEU2*) by direct gene replacement with the *natMX4* module conferring resistance to clonNAT. The Ste12 overexpression strain was generated by inserting the *CUP1* promoter and GFP-fusion construct from plasmid pYM-N4 in the 5' region of *STE12* ORF (Janke et al. 2004).

Non-restricted (NR) aging medium contained 0.17% yeast nitrogen base (YNB) without amino acids and ammonium sulfate, 2% glucose, 0.07% amino acid supplement mix (**Supporting Data S4**), and 25 mM glutamine as nitrogen source (see **Table S1** for media summary). Dietary-restricted (DR) aging medium was prepared substituting glutamine with 25 mM of GABA. All other nitrogen sources tested (ammonium sulfate, methionine, asparagine, phenylalanine, leucine, isoleucine, and valine) were also supplemented at 25 mM, as this results in an equivalent culture yield as the commonly used 0.5% ammonium sulfate. The choice of a non-preferred nitrogen source for DR instead of limited glucose concentrations

overcomes the dramatic metabolic changes due to glucose repression in yeast (Kresnowati et al. 2006), while facilitating the parallel characterization of stationary-phase cultures in low volumes, given that cell yields are similar under non-restricted and dietary-restriction conditions. SC medium used for glucose restriction was 0.17% yeast nitrogen base (YNB) without amino acids, 0.2% amino acid supplement mix, and 0.5% or 2% glucose. Outgrowth cultures for all CLS experiments were performed in low-fluorescence medium (Garay et al. 2014). No media used here contain any form of buffering.

Nitrogen-starvation medium for cell-cycle progression experiments was 2% glucose and 0.17% yeast nitrogen base without amino acids and ammonium sulfate. Glucose starvation medium contained no glucose and 0.67% yeast nitrogen base without amino acids and 0.2% amino acid supplement mix. All media recipes and preparation protocols are provided in detail in **Supporting Data S4**).

Automated competition-based CLS screens and data analysis. Fresh cultures of 4,340 tagged gene-deletion strains were replicated in 96-well plates (Corning 3585) with 150 μl of NR or DR aging medium. Deletion strains with slow growth and/or low fluorescence signal were discarded, only 4,050 strains passed this filter. In addition, several strains were lost during the subsequent steps of the automated experimental setup, resulting in the recovery of only 3,718 deletion strains that were tested under both glutamine and GABA. Saturated cultures were mixed with a CFP-labeled WT reference strain in a 2RFP:1CFP ratio for all mutants and WT controls. The latter was done to increase the dynamic range of mutant measurements, as

many of them displayed lower fluorescence than the WT. Importantly, we did not use a dye-swap strategy limiting our capacity to establish links between mutant cells yield and fluorescence intensity. Mixed cultures were then replicated by pinning into 96-deepwell plates (Nunc 260251), containing 700 μl of NR or DR aging medium, and grown at 30°C and 70% relative humidity, without shaking, in an automated system (Tecan Freedom EVO200) integrated to a multi-label plate reader (Tecan M1000). Four days after inoculation into deep-well plates, cultures were fully re-suspended and 5 μl outgrowth cultures were inoculated every other day into 150 μl of fresh low-fluorescence medium (**Supporting Data S4**) with the aid of an automated robotic arm. Absorbance at 600nm (OD_{600}) and fluorescence (*RFP* and *CFP*) measurements were taken every 150 min throughout 14 hrs with a Tecan multi-plate reader. An apparent survival coefficient, s, and its standard error, σ_s, were obtained from the slope of the linear regression (Robustfit, Matlab) of the log ratio of *RFP* to *CFP* signal at a fixed interpolation time point in the outgrowth culture (10 hrs), throughout 21 days in stationary phase. We observed no significant differences between the distribution of standard errors among the different phenotypic categories (**Figure S2**). All raw data collected in both genome-wide screens are available at www.langebio.cinvestav.mx/deluna/Campos2018/.

We have shown that the choice of reference strain does not impact CLS effects of the mutants (Garay et al. 2014). Other experimental schemes for culture mixing could be tested, such as mixing reference and mutant strains after separate aging to avoid any kind of strain-strain interaction; hence care must be taken when interpreting individual cases. While CLS effects of mutants aged under low aeration

show no drastic changes compared to highly-aerated cultures (Garay et al. 2014), we note that WT CLS decreases substantially in aerated conditions, thus mutant-specific effects might exist, specially, those involved in aerobic or anaerobic metabolism.

Scoring CLS phenotypes and lifespan extension coefficients. Short- and long-lived knockouts under NR or DR were determined by assigning a *Z*-score ($Z = \frac{s_{mut} - \mu_{wt}}{\sigma_{wt}}$) to each mutant's *s* coefficient; the distribution's mean (μ) and standard deviation (σ) of the population were taken from the measurement of 264 WT_{RFP}/WT_{CFP} independent co-cultures under either condition, assuming equal distribution of errors in the mutants and the WT. Two-tailed *p*-values were obtained from each *Z*-score to compute a false-discovery rate (FDR); we assigned significant phenotypes using a 5% FDR. While the mutants were not replicated under the same condition, the correlation of technical replicates has been measured previously (*r*=0.88, Pearson) (Garay et al. 2014).

The effect of dietary restriction on the gene-deletion strains relative to the WT was evaluated by calculating their relative lifespan extension defined as $LE = \frac{s_{DR}+1}{s_{NR}+1}$, where s_{NR} and s_{DR} are the *s* coefficients of a given deletion strain obtained from the screen under NR and DR, respectively. A *Z*-score was assigned to the LE of each mutant compared to the distribution of LE values of 264 independent WT reference experiments; significant LE<1 and LE>1 values were assigned at a 5% FDR.

Small-scale CLS assay measured by outgrowth kinetics. Selected strains were grown individually in the indicated non-restricted or dietary restriction media for 48 hours at 30°C 200 rpm in aerated tubes, then transferred to 96-well plates. In this step, each strain tested was inoculated several times in parallel from a single culture stock, the same setup was used throughout all our single-culture assays performed in deepwell plates. These plates were replicated onto 96 deep-well plates containing 700 µl of NR or DR medium and left for the entire experiment at 30°C and 70% relative humidity without shaking. From here on, all experimental steps were performed in an automated robotic station (Tecan Freedom EVO200). After 4 days, 10 µl aliquots were taken with an automated 96-channel pipetting arm to inoculate 96-well plates containing 150 µl of low fluorescence medium. OD_{600} was obtained in a plate reader (Tecan M1000) every 1.5 hours until saturation was reached; this first outgrowth-kinetics curve was regarded as the first time point (T_0, age = 0 days). Sampling was repeated every 2-3 days for 24-28 days. From these outgrowth kinetics, we extracted the doubling time and the time shift to reach mid-exponential phase (OD_{600}=0.3) that occurred between the first day of measurements (T_0) and each day in stationary phase (T_n). Relative cell viability was calculated from these data, as reported by Murakami *et al* (Murakami et al. 2008).

Viability data points relative to T_0 were used to plot a survival curve, which was fitted (all data points) to an exponential decay model ($N(T) = N_0 e^{-rT}$) where N_0 is the percentage of viability at T_0, T is time in days, and r is the rate of death. For validation of CLS effects, mutants were taken from the RFP-tagged deletion collection (or generated *de novo*, when indicated) and viability was assayed to calculate death

rates in at least 7 experimental replicates which were compared to replicates of a WT strain; significant CLS effects were considered using a p<0.05 cutoff (T-test).

CLS assay in standard aeration conditions. Standard culture conditions were used, as described (Hu et al. 2013). In brief, pre-inoculums from three different colonies of each strain were set in 5 mL SC medium for 24 hours, these were diluted 1:100 v/v in 10 mL SC with 2% glucose or 0.5% glucose aging medium in 50 mL tubes for 48 hours with shaking (200 rpm) at 30 °C. Viability at each measuring point was obtained by monitoring the change in outgrowth-kinetics parameters with time in stationary phase using three technical replicates for each colony tested, as described above.

Visualization of functional clusters. Gene Ontology (GO) associations and phenotype terms were downloaded from the *Saccharomyces* Genome Database (SGD, last updated December 2016) to build two m by n matrixes, where m is the number of DR genes (219 and 253 for LE<1 and LE<1, respectively) and n is the number of GO and phenotypic terms (1,748). Each term was used to evaluate the overall agreement between gene-pairs to calculate Cohen's *kappa* ($kappa = \frac{\mathrm{Pra}(a)-\mathrm{Pr}(e)}{1-\mathrm{Pr}(e)}$). Where *Pra*(*a*) is the number of associated-terms and not associated-terms that each gene-pair shares, divided by the total number of terms downloaded from SGD in the matrix (denominator is the same for any gene pair), and *Pr*(*e*) is the hypothetical probability for each member of the gene-pair to be associated by chance.

A matrix representing the agreement between each gene-pair was built with the *kappa* values. Gene-pairs that showed *kappa*>0.35 were regarded as likely similar, according to previous reported thresholds for large datasets (Huang, Lempicki, et al. 2009). Gene-pairs formed in the first step were used as cluster seeds to form larger groups of genes; *ie*. groups of genes that shared at least 50% of their members merged in subsequent iterative steps, thus creating larger groups in each iteration until only groups with dissimilar members remained. Clusters with at least four elements were manually named by inspection in the SGD and GO enrichment. All procedures related to Kappa calculation and analysis were performed with Matlab; input genes gene-association data, and Matlab scripts are available at www.langebio.cinvestav.mx/deluna/Campos2018/. Network representation was created using Cytoscape; edges between nodes represent kappa agreement above the established threshold (*kappa*>0.35).

TFRank analysis. TFRank was used at http://www.yeastract.com/ formrankbytf.php using the *TF Rank* algorithm option (Gonçalves et al. 2011), with a heat diffusion coefficient of 0.25. All DR-genes were introduced as Target ORF/Genes, with all transcription factors selected (*n*=197). The output file includes all transcription factors reported, ranked and weighted for regulation of the input DR-genes list (**Supporting Data S5**).

Flow-cytometry analysis of stationary-phase fluorescence signal dynamics. Twenty deletion strains and four WT replicates were aged in deep-well plates containing with SC medium with GABA nitrogen source. All cultures were inoculated

at different times to achieve ages from three to nineteen days in the same plate. Cell cytometry (LSRFortessa™, Becton Dickinson) was performed after inoculating 5 μl of the original cultures into 150 μl of YNB-low fluorescence medium; this outgrowth was incubated for 11 hours at 30 °C. With the aid of a high-throughput sampler, 20,000 events were collected for each culture in the 96 well-plate (20 mutants and four WT replicates, eight time points each); mCherry was excited with a 561-nm laser, fluorescence was collected through a 586/15 band-pass filter.

Alive/dead staining assay. We used the same scheme of 96 deep-well plates (one plate per replicate) to age cells in SC with 0.5% or 2% glucose. Each day, a single well of each strain was collected. Cells were centrifuged, washed, and dyed with LIVE/DEAD® FungaLigth™ Yeast Viability Kit, following manufacturer's instructions. Propidium iodide (IP) and Syto®9 fluorescence were measured by cell cytometry (LSRFortessa™, Becton Dickinson) at early stationary phase (4 days after inoculation) and at different time-points until 21 days in stationary phase. IP was excited with a 591-nm laser, fluorescence was collected through a 586/15 band-pass filter; Syto9 was excited with a 488-nm laser, and fluorescence was collected through 525/50 band-pass and 505LP emission filters. Cell-viability percentage was obtained by staining dead and alive cells with SYTO9 and subtracting the number of dead-cell events only as stained by propidium iodide.

Cell-cycle assays. WT and mutant strains were grown in flasks containing 50 ml of NR aging medium at 30 °C and shaken at 200 rpm until mid-logarithmic phase ($OD_{600} \cong 0.5$). Cells were centrifuged, washed twice with sterilized water, and

transferred to nitrogen or glucose starvation medium. For rapamycin-induced arrest, 10 nM rapamycin was directly added to mid-log phase cell cultures. Samples were taken at the moment of either transfer to starvation medium or rapamycin addition (time 0) and 1, 2 and 4 hours after that time point. Fixation and dying with SYTOX™ Green were performed as described elsewhere. Cells were analyzed by flow cytometry (LSRFortessa™, Becton Dickinson); SYTOX Green was excited with a 488-nm laser, and fluorescence was collected through a 525/50 band-pass filter.

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Conflict of interest

The authors declare that they have no competing interests.

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Table 1. Chronological lifespan of the WT strain aged under different nitrogen sources

Nitrogen source	Death rate, *r* [a]	Half life, days [a]	Doubling time, hours [b]
Ammonium	-0.137 ± 0.010	5.6 ± 0.4	2.70 ± 0.11
Methionine	-0.061 ± 0.005	11.4 ± 0.9	3.59 ± 0.09
Glutamine	-0.057 ± 0.004	13.1 ± 1.0	2.64 ± 0.09
Asparagine	-0.049 ± 0.004	15.3 ± 1.0	2.69 ± 0.09
Phenylalanine	-0.046 ± 0.006	15.9 ± 1.8	2.91 ± 0.12
Leucine	-0.041 ± 0.003	18.0 ± 1.3	4.31 ± 0.02
Isoleucine	-0.041 ± 0.004	18.3 ± 2.2	3.05 ± 0.12
Valine	-0.039 ± 0.004	18.9 ± 2.1	2.76 ± 0.07
GABA	-0.029 ± 0.005	26.5 ± 4.4	2.82 ± 0.07

a. Death rate and half-life are obtained from fitting survival curves to an exponential decay model; values are the average ± S.E.M. from at least 5 experiments.

b. Obtained from the rate during the exponential phase of growth.

Table 2. A catalogue of transcription factors regulating dietary-restriction genes in yeast (DR-regulators, top 5% rank)

Rank	TF	Weight[a]	% Regulated[b]	Description
1	Ace2	3.15	75.5	Involved in G1/S transition of the mitotic cell cycle; activates cytokinetic cell separation
2	Ash1	2.94	50.4	Negatively regulates G1/S transition of mitotic cell cycle; activates pseudohyphal growth
3	Tec1	2.81	63.0	Transcription factor targeting pseudohyphal-growth genes and Ty1 expression
4	Sfp1	2.55	66.3	Regulates ribosomal-protein genes, response to nutrients and stress, and G2/M transitions of the mitotic cell cycle
5	Ste12	2.45	58.2	Activates genes involved in mating or pseudohyphal-growth pathways
6	Bas1	2.36	44.9	Involved in regulating the expression of genes of purine and histidine biosynthesis
7	Snf6	2.19	37.4	Subunit of the SWI/SNF chromatin remodeling complex
8	Msn2	2.10	54.3	Regulation of transcription in response to a wide variety of stresses
9	Yrm1	1.79	44.5	Transcription factor involved in multidrug resistance
10	Gcn4	1.76	46.7	Activator of amino acid biosynthetic genes; responds to amino acid starvation
11	Ixr1	1.73	26.4	Transcriptional repressor that regulates hypoxic genes during normoxia
12	Abf1	1.723	45.3	DNA binding protein with possible chromatin-reorganizing activity
13	Msn4	1.65	44.0	Regulation of transcription in response to a wide variety of stresses
14	Rap1	1.53	41.4	Essential DNA-binding transcription regulator; role in chromatin silencing and telomere length

a. TFRank weight (Gonçalves et al. 2011).

b. Percentage of dietary-restriction genes regulated by the transcription factor.

SUPPORTING INFORMATION LISTING

Table S1. Chronological lifespan assays, aging media, and strains used in this study.

Fig. S1. The CLS of yeast is affected by the quality of the nitrogen source in the aging medium.

Fig. S2. Validation of CLS hits from the genome-wide screen.

Fig. S3. Relative lifespan extension analysis under an alternative nitrogen-rich condition.

Fig. S4. Changes in fluorescent-protein signal as a function of time in stationary phase.

Fig. S5. CLS of gene deletions of transcription factors that regulate dietary-restriction genes under different nitrogen sources.

Fig. S6. Alive/dead cell staining confirms a role of Ste12 in lifespan extension by dietary restriction.

Fig. S7. WT cells are not affected in their survival nor growth by copper treatment.

Fig. S8. Confocal microscopy of STE12 overexpression cells.

Data S1. Genome-wide CLS screens under two dietary regimens, complete data set (XLSX).

Data S2. Data set of 472 DR-genes identified in this study (XLSX).

Data S3. Functional clusters of DR-genes with diminished or enhanced lifespan extension (XLSX).

Data S4. Media recipes and detailed preparation procedures (XLSX).

Data S5. TFRank output files (XLSX).

FIGURE LEGENDS

Figure 1. Genome-wide profiling of chronological-lifespan under two dietary regimens. (**A**) Schematic representation of the screening strategy; an RFP-labeled deletion strain ($x\Delta$) is aged in co-culture with a CFP-labeled WT strain; a survival coefficient (s) relative to WT is obtained for each mutant under each aging condition. (**B**) Examples of the survival coefficients obtained; selected long-lived and short-lived mutants under glutamine (non restricted, upper panel) and GABA (dietary restriction, lower panel) are shown. For comparison purposes, initial offsets were corrected to start at zero. (**C**) Cumulative distributions of s values for the 3,718 single deletion strains under glutamine (grey circles) and GABA media (green circles), 264 WT replicas were included (black and green crosses). Right, Z-scores were obtained (only glutamine is shown) by comparing the mutants' survival coefficient (s) to the s distribution of WT-reference experiments; multiple-testing correction was used to define hits at a 5% FDR. (**D**) Venn diagrams show the overlapping short- and long-lived mutant strains scored under glutamine (NR, dark grey) and GABA (DR, green).

Figure 2. A comprehensive compendium of dietary-restriction genes. (**A**) Selected knockout strains with differences in their selection coefficients obtained under glutamine (non restricted) and GABA (dietary restriction), resulting in diminished (red panels) or enhanced (blue panels) lifespan extension. (**B**) A relative lifespan extension, LE, was calculated for each mutant strain (circles) and compared to that of the WT replicates (crosses) to score statistical significance. (**C**) Scatter plot of the survival coefficients of 3,718 deletion strains under non-restricted medium (horizontal axis) and dietary restriction (vertical axis); data points above or below the diagonal are colored according to the lifespan extension (LE) significance (q). Data points shown with special symbols are the selected examples in panel B of significantly diminished (red) or enhanced (blue) lifespan extension.

Figure 3. Functional clustering of dietary-restriction genes. Network representation of functional clusters of genes with (**A**) diminished (LE<1) and (**B**)

enhanced (LE>1) lifespan extension. Edges denote agreement between pair of genes ($kappa$>0.35), which suggests functional association between the genes; clusters were formed between at least four associated genes. Node color code indicates decreasing or increasing ranks of mean LE for each cluster.

Figure 4. Altered chronological lifespan in strains deleted for dietary-restriction regulators. (A) Plots indicate the rank of transcription factors (TFRank-analysis of DR-genes, *n*=197) with a gene-deletion effect (CLS TFs, *n*=40) and without an effect (non-CLS, *n*=79 out of 119); only 119 transcription factors measured under both dietary conditions are considered. Solid black lines indicate the average rank of each group. **(B)** Survival curves of WT and gene-deletion strains aged in SC medium with 2% glucose (non-restricted, grey lines) or 0.5% glucose (dietary restriction, blue lines). Deletion strains (discontinuous lines) are for genes coding for transcription factors Ace2, Ash1, Tec1, Ste12, Snf6, Msn2, Msn4, and Bas1. Error bars are the S.E.M. (*n*=5).

Figure 5. Several dietary-restriction regulators mediate cell-cycle arrest in response to nutrient limitation. Histograms show the DNA content of populations of WT and transcription-factor mutant cells at 0, 1, 2, and 4 hours after nitrogen (**A**) or carbon (**B**) depletion. Cellular DNA content was measured by SYTOX-Green staining followed by flow cytometry. Numbers indicate the percentage of cells at G1 in each population.

Figure 6. Ste12 is a positive regulator of longevity by dietary restriction. **(A)** Survival curves of WT and *ste12*Δ strains aged in fully-aerated tubes with 10 mL SC containing 0.5% or 2% glucose. Lines shown in the same color are colonies from three independent transformation events; WT are *his3*Δ neutral insertions. (**B**) Survival curves of the WT, *ste12*Δ, and *pCUP1::GFP-STE12* overexpression strains aged in SC 2% glucose in non-induced conditions (orange) or induced with 2μM (+), 5μM (++), or 15 μM (+++) copper sulfate. Error bars are the S.E.M. (*n*=7). (**C**) Cell-cycle dynamics of WT and *ste12*Δ populations; histograms are for cells at 0, 1, 2,

and 4 hours after nitrogen starvation (left) or treatment with rapamycin (right). Cellular DNA content was measured by SYTOX-Green staining followed by flow cytometry. Numbers indicate the percentage of cells at G1 in each population.