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Functional genomics of dietary restriction and longevity in yeast

Sergio E. Campos, Alexander DeLuna*

Unidad de Genómica Avanzada (Langebio), Centro de Investigación y de Estudios Avanzados del IPN, 36824, Irapuato, Guanajuato, Mexico

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ABSTRACT

Dietary restriction-limitation of calories or other specific nutrients in the diet-is the sole non-genetic intervention known to extend the lifespan of a wide range of model organisms from yeast to mammals. Cell biology studies on the responses to dietary restriction have provided important clues about the mechanisms of longevity; however, a comprehensive genome-wide description of lifespan by dietary restriction has been mostly absent. Large-scale genetic analysis in the budding yeast *Saccharomyces cerevisiae* offers a great opportunity to uncover the conserved systems-level mechanisms that give way to longevity in response to diet. Here, we review recent advances in high-throughput phenotyping of the replicative and chronological life spans of yeast cells, which have contributed to our understanding of longevity by dietary restriction and the cellular crosstalks of nutrient-sensing regulation.

1. Introduction

Aging is the greatest risk factor for a plethora of chronic human diseases, including diabetes, cancer, and Alzheimer-disease (Lopez-Otin et al., 2013). In this regard, several genetic, pharmaceutical, and nutritional interventions that promote longevity have been researched in an effort to translate them into treatments for health and lifespan extension (Humfrey, 1998; Wanke et al., 2008; Morselli et al., 2009; Fontana and Partridge, 2015). One of such treatments is dietary restriction (DR), which has been proven to prevent a number of age-associated changes in a wide-range of organisms (Fontana and Partridge, 2015). This anti-aging intervention is usually achieved by restricting calories from the diet or by the restriction of other specific nutrients such as methionine or other amino acids (Kaeberlein et al., 2007; Wu et al., 2013). DR extends the lifespan of laboratory models such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae*, allowing the implementation of cellular and genetic techniques to dissect the conserved genetic bases of longevity by DR (Gems and Partridge, 2013). Moreover, there is evidence that DR decreases age-related morbidity in mice and rhesus monkeys, while in humans DR results in increased insulin sensitivity and lower cholesterol and blood pressure, suggesting that this intervention increases human health span, *ie*. the number of years people remain free of chronic diseases throughout their life (Holloszy and Fontana, 2007).

Aging in general has been associated to nutrient-signaling through the conserved TOR pathway, as shown by the drastic effects in lifespan displayed by mutants of genes encoding components of the TOR pathway in yeast, worms, and flies (Fabrizio et al., 2001; Kenyon et al., 1993; Tatar et al., 2001). Moreover, down-regulation of the TOR pathway through DR and genetic or chemical perturbations often leads to general lifespan extension through the activation of a cell-maintenance program in most organisms studied so far (Powers et al., 2006; Wei et al., 2008; Gems and Partridge, 2013). The latter highlights the link between nutrient sensing regulation and the anti-aging effects of DR. In this same vein, the deregulation of nutrient sensing is considered one of the hallmarks of aging and it is becoming increasingly clear that other hallmarks are tightly connected with it (Lopez-Otin et al., 2013). For instance, histone modification–specifically demethylase activity–may influence lifespan in nematodes by targeting components of the insulin/IGF-1 pathway (Jin et al., 2011). Likewise, phosphorylation of histone H3 is associated with induced transcription in response to nutritional stress in yeast; this response is regulated by protein kinase Sch9 in a Tor1-independent manner (Oh et al., 2018). In yeast, the Sir2 NAD^+-dependent histone deacetylase is needed to extend replicative lifespan by DR, implying a link between specific epigenetic modifications and DR (Lin et al., 2000). Other hallmarks of aging have been linked to nutrient sensing as well. For instance, telomere attrition in yeast is regulated by Bul2, a component of an ubiquitin ligase complex involved in trafficking of amino acid permeases; hence, amino acid availability may determine telomere length in this organism (Kwan et al., 2011). However, it is not yet clear how do epigenetic alterations and telomeres dynamics interact with other aging-processes that are regulated by nutrient sensing pathways and the degree of connectivity within these interaction networks.

* Corresponding author.
E-mail addresses: sergio.e.campos@cinvestav.mx (S.E. Campos), alexander.deluna@cinvestav.mx (A. DeLuna).

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In what follows, we review recent advances in aging genetics research in the budding yeast, in particular experimental approaches that provide systems-level views of lifespan extension by DR, contributing to a better understanding of aging and longevity. By systematically addressing the phenotypes of specific genetic interventions along a given set of conditions, functional genomics provides a comprehensive and integrated view of the processes that affect lifespan in response to the diet. For instance, the discovery that the TOR pathway regulates lifespan was uncovered by a genome-wide analysis of yeast lifespan (Kaeberlein et al., 2005; Powers et al., 2006), highlighting the relevance of systematic screens.

2. Gene-diet interactions shed light on mechanisms of dietary restriction

To identify the mechanisms of lifespan extension by DR, many studies have relied on the evaluation of the effects of genetic perturbations (e.g. gene deletions) under dietary restricted and non-restricted conditions. Genetic perturbations that mimic the effects of DR can be classified if such perturbation results in increased lifespan under non-restricted conditions, but display lesser lifespan effects as nutrients become limited. The latter can be exemplified by deletion of *TOR1* in yeast, that results in lifespan extension, while the beneficial effect of depleting Tor1 becomes less noticeable under DR (Johnson et al., 2013). On the other hand, deletion of components of genetic pathways necessary for lifespan extension in response to DR show large deleterious effects during full nutritional restriction. Such is the case of autophagy; in *C. elegans* knockdown of autophagy-essential protein VPS-34 blocks the life-extending phenotype of *eat-2* mutants, which are a model for DR as these worms are long-lived due to reduced food intake (Hansen et al., 2008). Using these phenotypic comparisons several DR-related genes have been reported in laboratory models where genetic tools are available (Mair and Dillin, 2008).

While it is true that deleting genes from well conserved TOR and other longevity-linked pathways has a great impact in the magnitude of effects of DR (Powers et al., 2006; Greer and Brunet, 2009), it is becoming increasingly clear that lifespan extension is achieved by regulation of a myriad of interacting cellular processes. Hence, the complexity of this phenotype requires high-throughput and sensible approaches to address the problem from the genetics perspective.

3. The budding yeast as a model for aging genomics

The budding yeast *S. cerevisiae* is an amenable model organism where a great number of genetic tools are available. Notably, the ease with which genetic transformation can be performed has enabled the generation of large-scale strain collections. These include the yeast knockout collection that features gene deletions for nearly every gene in the yeast genome, the GFP-tagged ORF collection for global subcellular localization, and the Tet-promoter collection that allows the regulation of essential genes, among others (Giaever et al., 2002; Huh et al., 2003; Mnaimneh et al., 2004). Using these tools, a number of forward genetic screens have allowed the understanding of gene functions, gene-gene and gene-environment interactions with a genome-wide perspective (Giaever and Nislow, 2014). However, it is only until very recently that genome-wide methodologies have been devised for a more comprehensive and quantitative description of genes and cellular processes involved in lifespan regulation in yeast.

In yeast, two different models to study aging have been established. The first being replicative lifespan (RLS), which refers to the number of divisions that mother cells are able to undergo before damage accumulation prevents further replication (Kaeberlein, 2010). RLS is classically assayed by micro-dissecting yeast cells on a nutrient-rich agar plate under a microscope; typically, more than 20 “virgin” or undivided cells are separated for each strain to be tested, then these cells are allowed to divide. After division, daughter cells are separated with a microneedle, counted, and taken to a separate area of the agar plate (Steffen et al., 2009). It has been shown that this form of aging is the product of asymmetric cell division, whereby the mother cell manages to retain most of the cellular damage, allowing daughter cells to reset their lifespan (Kennedy et al., 1994). Notably, cells from higher eukaryotes also show a limit in their replicative capacity (Hayflick and Moorhead, 1961) and this limit has been proposed to be a tumor suppressor mechanism and a contributor to organismal aging in mammals (Shay and Wright, 2011; Rodriguez-Brenes et al., 2015). Paradoxically, replicatively-aged cells show a clear link to carcinogenesis (Campisi, 2013). The relevance of replicative cell aging in animals has turned yeast RLS into a crucial model in aging research, as it is an accessible model of aging in mitotically active cells.

The second model of aging in yeast is chronological lifespan (CLS), comprising the time yeast cells remain viable in stationary phase, when almost all nutrients are depleted from the growth medium. At this stage, most cells develop a typical set of features including G1 arrest, thickening of the cell-wall, and resistance to stress (Singer et al., 1993). It is during this stage that yeast cells will start losing viability, showing some features of aging that are conserved in other organisms, such as mitochondrial dysfunction, genomic instability, and loss of proteostasis (Longo et al., 2012a). Many of the factors that have been found to affect CLS in yeast influence the lifespan of mammalian cells as well, making CLS a good model of aging for post-mitotic cells (Longo et al., 2012b). CLS is classically evaluated by counting colony forming units, typically in a petri dish, from an outgrowth sample of an aerated liquid culture which is left incubating for the entire experiment taking usually from several days to two months (Hu et al. 2013).

The study of both aging models in yeast has uncovered many evolutionary conserved aging factors such as the lifespan-extending effect of downregulating the RAS/PKA and TOR pathways, the role of sirtuin-like proteins in aging, and the anti-aging effect of rapamycin and spermidine, which are currently being investigated in mammals (Gems and Partridge, 2013). In what follows, we review advances in the discovery of the mechanisms of DR by means of large-scale genetic studies and discuss how these approaches can further contribute to understand the complex genetic architecture of longevity.

4. Modeling longevity by dietary restriction in yeast

Lifespan extension by DR is usually achieved in yeast by reducing glucose concentration from 2% to 0.5% or lower. Under high glucose concentrations, yeast cells experience transcriptional and metabolic repression of a wide range of genes involved in respiration, gluconeogenesis, and peroxisomal functions (Carlson, 1999). For instance, alcoholic fermentation is prevalent in cells grown in high glucose concentrations, even in the presence of oxygen, mainly due to excess pyruvate produced by enhanced glycolysis, which leads to saturation of the mitochondrial pyruvate-dehydrogenase complex and inhibits respiration as a consequence (Pronk et al., 1996). In this same vein, expression of tricarboxylic-acid cycle and respiratory genes along with heat-shock and anti-oxidant genes is induced when glucose levels go from 2% to 0.5% in a batch culture, indicating that there is a shift to respiration and a stress-resistance state when yeast cultures reach similar glucose levels to those used in caloric-restricted studies (DeRisi et al., 1997). In agreement with the correlation between glucose restriction and the shift towards respiration, the *CYT1*-encoded cytochrome c1 is required for RLS extension by glucose restriction, while overexpression of the respiration transcriptional activator *HAP4* results in lifespan extension in 2% glucose medium (Lin et al., 2002). Moreover, a metabolic shift to respiration has shown to be essential for CLS extension by glucose restriction, while restriction of non-fermentable sugars does not extend CLS in yeast (Smith et al., 2007; Oliveira et al., 2008; Ocampo et al., 2012). These findings indicate that enhanced respiration is a mechanism of lifespan extension in a specific form of DR, namely glucose restriction.

Importantly, other dietary protocols result in lifespan extension in yeast. For instance, Jiang et al. reported increased RLS by reducing amino acid concentration in the aging medium (Jiang et al., 2000). Moreover, substitution of the preferred amino acids asparagine and glutamate or restriction of methionine and tryptophane extends CLS (Powers et al., 2006; Johnson and Johnson, 2014; He et al., 2014), suggesting there is a common nutrient signal involved in response to these restrictions without necessarily reducing the total amount of calories in the medium. Likewise, the use of ɣ–amino butyric acid (GABA) as a nitrogen source results in up to 500% CLS extension when compared to glutamine, asparagine, methionine, or ammonium (Campos et al., 2018). In addition, replacement of glucose-exhausted medium for water in stationary phase cultures can also increase CLS of yeast cells (Fabrizio and Longo, 2003).

Although several regimens of DR have been successful in extending lifespan in yeast, it remains to be addressed whether all modes of nutrient restriction elicit the same or overlapping longevity mechanisms. In fact, lifespan extension by DR can depend on the balance of amino-acids, carbon, and nitrogen source: concentrations of glucose as high as 5% can result in long CLS when other nutrients in the medium are present in low concentrations (Wu et al., 2013). Moreover, some downstream mechanisms can be shared by different forms of DR without necessarily being activated by the same pathways. For instance, DR can elicit an increased capacity to cope with oxidative stress (Wei et al., 2008). Such resistance is brought about by at least two different nutrient-sensing pathways depending on whether glucose or amino acids are being restricted in the medium (Mirisola et al., 2014). These data suggest that there are several lifespan-extending signaling pathways. Even when some anti-aging mechanisms are activated through different signaling pathways, the fact that many protocols of DR 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).

Importantly, DR might affect yeast RLS and CLS in different ways given the metabolic differences observed in proliferating and non-dividing cells. For instance, there seems to be an important number of genes associated to DR that are exclusive to RLS or CLS, as reported in DR gene database GenDR (http://genomics.senescence.info/diet/) (Wuttke et al., 2012). Moreover, the presence of extrachromosomal rDNA circles, a specific hallmark of RLS, is reduced by DR (Riesen and Morgan, 2009); although, there is no evidence for this mechanism to play a role in CLS regulation. In addition, stationary-phase cells are usually exposed to higher concentrations of acetic acid (Burtner et al., 2009). Nonetheless, there are observations suggesting that both yeast lifespan models should be taken into account when studying the response to DR. For example, RLS is affected in CLS-aged cells. Hence, both models of aging describe similar types of cellular damage (Murakami et al., 2012). In this regard, the TOR and PKA pathway seems to be a central part of aging regulation in response to DR in both RLS and CLS (Kaeberlein et al., 2005; Powers et al., 2006). Both pathways have even been implicated in lifespan regulation in response to nutrients in mammals, highlighting the usefulness of both yeast lifespan models for aging research (Longo et al., 2012a)

5. Large-scale analysis of replicative longevity by dietary restriction

High-throughput genetic screening of yeast cells under different DR protocols have revealed some of the core pro-longevity mechanisms that are activated by different forms of restriction (Table 1). Such studies also provide an important starting point to assess the conservation of aging mechanisms in other organisms, including humans. RLS in yeast was one of the first models of aging to be explored since it was shown that yeast cells have a finite number of divisions (Mortimer and Johnston, 1959). Although RLS was studied more intensively during the

Table 1
Dietary-restriction factors revealed by large-scale phenotypic screens in yeast.

Proteins and pathways	Lifespan extension effect[a]	Aging Model	Screening method	Reference
Agp1, Gln3, Lys12, Mep2, Mep3	Negative	CLS	OD of outgrowth from stationary-phase	Powers et al., 2006
Ade4	Negative	CLS	Microarray profiling of pooled strains	Matecic et al., 2010
Fet3, Nfu1	Positive			
Vacuolar protein sorting	Positive	CLS	Microarray profiling of pooled strains	Fabrizio et al., 2010
Peroxisomal transport, Strand invasion	Negative	CLS	Bar-code sequencing of pooled strains	Gresham et al., 2011
Autophagy, Chromatin organization, CVT pathway, Endosome to vacuole transport, Filamentous growth, Fungal-type cell wall, Ion transport, Mitochondrion degradation, Reproductive process	Positive			
Amino acid biosynthesis, Cell wall, Chromosome segregation, Electron transport, Nucleic acid binding, Peroxisome, Ribosome biogenesis, RNA transport	Positive	CLS	Bar-code sequencing of dead cells sorted by flow cytometry	Davey et al., 2012
Mitchondrial-protein homeostasis, Vacuole pH homeostasis	Positive	RLS	Microdissection	Schleit et al., 2013
Swr1 Complex	Negative	CLS	Outgrowth competition, pairwise	Garay et al., 2014
Ser1	Negative	CLS	Outgrowth kinetics	Jung et al., 2018
Rim15	Positive			
Cell differentiation, ER to Golgi transport, Fungal-type cell wall, Golgi to vacuole transport, Mitochondrial membrane	Negative	CLS	Outgrowth competition, pairwise	Campos et al., 2018
Autophagy, Cell-cycle recovery in response to pheromone, Histone modification, Mitochondrial part, Nucleus localization, Peroxisomal transport, Ribosome biogenesis	Positive			

[a] Observed gene-deletion phenotype under nutrient-rich and DR conditions was used to define the intervention effect on lifespan extension by DR. "Negative" stands for factors whose role opposes lifespan extension by DR; "positive" factors are those whose role is necessary for lifespan extension by DR.

earlier years of aging research in yeast (Kaeberlein et al., 2007), the laborious micromanipulation required to separate and count daughter cells under a microscope limits the lifespan characterization to small scales (Longo et al., 2012a). Nevertheless, the continued efforts of some research groups have been successful at generating large datasets using this classical approach. For instance, an RLS screen of 166 gene-deletion mutants under 2% and 0.5% glucose revealed that the vacuole and the mitochondria play an important role in lifespan extension by DR (Schleit et al., 2013). In particular, genes that regulate vacuolar pH and oxidative stress response are required for full RLS extension by DR. Conversely, they also show that although deletion of some genes involved in mitochondria proteostasis results in short-lived RLS phenotypes under nutrient-rich conditions, however under DR these defects in lifespan are partially alleviated in these mutant strains (Schleit et al., 2013), suggesting that DR buffers the deleterious effects of mutations that would otherwise result in severe defects.

The classic microdissection method has been used to systematically determine RLS for most of the viable gene deletion strains. Analysis of these data allowed a genome-wide view of replicative aging in yeast, revealing the role of cytoplasmic translation, the SAGA complex, mannosyltransferase activity, and the TCA cycle in longevity under standard nutrient-rich conditions (McCormick et al., 2015). In this same study, authors showed that lifespan extension by DR also requires nuclear tRNA regulation: the analysis of the long-lived mutant *los1*Δ underpinned the involvement of tRNA nuclear transport in longevity, illustrating the relevance of such a compendium of gene-deletion effects in RLS for further studies. However, given the amount of time and effort that is required to produce such phenotypic information, the comprehensive evaluation of gene-nutrient interactions using this experimental setup does not seem viable in the short term. Moreover, it was found in a different study that lifespan extension observed under DR falls within the variability of the RLS observed under standard conditions obtained from 41 different studies, questioning previous evidence for lifespan extension under DR using this methodology (Huberts et al., 2014). In this same work, it was shown that RLS variation decreases when a large number (> 100) of cells are dissected; however, no study has reported RLS of yeast under DR for such a large number of experimental replicates using microdissection. Given the similarities between yeast and human aging (Janssens and Veenhoff, 2016), which include several mechanisms regulated by DR such as mitochondrial damage, redox imbalance, and loss of proteostasis (Mair and Dillin, 2008), it is of utmost importance to establish the effects of DR with methods offering higher resolution.

New methodological approaches aimed at increasing the throughput and resolution of RLS phenotypic measurements have been developed. One such method is the mother enrichment program, which allows RLS determination as a function of population viability by targeting essential genes through the expression of Cre recombinase under the control of the daughter-cell-specific *SCW1* promoter, In this experimental system, two essential genes are disrupted in daughter cells when estradiol is present, allowing the exclusive assessment of mother-cell viability when estradiol is removed (Lindstrom and Gottschling, 2009). The mother enrichment program has been used to uncover some aspects of aged cells. For instance, a proteomic screen in old mother cells obtained using the mother enrichment program revealed that some proteins accumulate in large cytoplasmic structures during aging, while in aged mammalian tissues these protein aggregates had been observed only in the extracellular matrix, suggesting that the intra-cellular accumulation of these proteins may contribute to cellular aging (Thayer et al., 2014). In addition, the mother enrichment program has also revealed that mitochondrial fragmentation is present during early stages of aging in mother cells and that this damage is related to early increases in vacuolar pH halting autophagy activity, while DR promotes longevity by promoting vacuolar acidification (Hughes and Gottschling, 2012). While the system has limitations for large-scale genetic analyses, as synthetic constructs must be introduced in all of the mutant strains of interest, the mother enrichment approach does lend itself for parallel characterization of RLS under multiple environmental or dietary conditions. However, further studies should address the possibility that the introduction of the mother enrichment system may affect some features of aged yeast cells, generating important differences with other RLS methodologies.

Recent methods to measure RLS have taken advantage of microfluidics chips to trap mother cells by mechanical pressure below a soft micro-pad, immobilization of biotinylated mother cells against an avidin-covered chip, or trapping cells in micro-cages by hydrodynamic pressure (Lee et al., 2012; Crane et al., 2014; Xie et al., 2012). In these setups, smaller or un-labeled daughter cells are washed away with constant medium flow, while live imaging is used to count the number of cell divisions in each mother cell. One great advantage of these assays is that senescence and other molecular markers can be tracked during live imaging, which is useful when looking for correlations between aging of mutant strains and status of relevant cellular processes (Lee et al., 2012; Xie et al., 2012). The measurement of RLS through microfluidics is a powerful approach that can be used for systematic characterization of DR in a vast number of conditions, providing high-throughput single-cell data. One of such microfluidics devices, based in the separation of mother cells with a microneedle followed by trapping under PDMS micropads and counting of daughter cells through imaging, was used to evaluate the effect of DR (0.5% glucose) in over 2000 WT cells, showing that DR does not extend RLS robustly (Huberts et al., 2014). However, medium is constantly flowing in microfluidic devices, washing out any metabolites surrounding yeast mother cells, which can lead to confounding results, especially in DR studies where the decreasing amount of nutrients through time might have an impact (Mei and Brenner, 2015). In a different microfluidics device based in trapping yeast mother cells in micro-chambers where a relatively low flow of medium washes daughter cells, it was found that moderate and extreme DR (0.5% and 0.05% glucose respectively) does extend RLS (Jo et al., 2015). Given the relevance of yeast RLS for the study of senescence in mammalian cells, it is worth considering the factors that account for such discrepancies in the DR protocols that have been tested using microfluidic-based RLS methods. Also, it may be worth to consider testing different types of nutritional restriction, given that one of the first studies of RLS extension by DR showed that 0.5% glucose could promote longevity only when amino acid levels were also lowered (Jiang et al., 2000). Thus, it remains to be addressed whether other DR protocols lead to RLS extension with the methods reviewed here, such as the mother enrichment program and microfluidic approaches.

6. Genomewide analysis of chronological longevity by dietary restriction

CLS has been classically assayed by sampling aerated yeast cultures after reaching stationary phase, by plating on nutrient-rich agar medium to measure cell viability as a function of the number of colony forming units through time (Longo, 1999; Fabrizio and Longo, 2003; Hu et al. 2013). Even though this assay can estimate cell viability with accuracy, it is also very time and resource-consuming, limiting the number of yeast strains or conditions that can be assayed in parallel.

In an effort to increase the throughput of the conventional colony-forming unit assay, other methods have been developed. For instance, CLS of ˜4800 single deletion strains was determined by using the correlation of optical density readings of serial dilutions of yeast cultures and the number of viable cells inoculated in fresh media, which were monitored once a week for 7 weeks in a 96 well-plate format (Powers et al., 2006). Such an approach led to the discovery that the TOR pathway has a role in yeast CLS and that decreased TOR activity increases CLS. This line of evidence in particular has been fruitful in producing translational anti-aging interventions in mammals, such as treatment with the TOR inhibiting drug rapamycin (Laplante and Sabatini, 2012). However, CLS phenotypes, specifically long-lived

phenotypes from this screen, were not reproducible with this approach (Murakami et al., 2008). In a different study, a more rigorous analysis method based in the parallel measurement of outgrowths in microtiter plates, where the change in the amount of time needed to reach a specific OD and its relation to population-doubling time were used to more precisely determine CLS; this is achieved by monitoring the growth kinetics throughout each day of measurement. This study uncovered that Sir2 does not have a prominent role in CLS extension by DR, which accounts for one of the important differences between RLS and CLS (Murakami et al., 2008). Recently, this method has been adapted to a 384 platform and optimized with the use of growth kinetic and CLS analysis software, demonstrating widespread CLS variation in 33 yeast natural isolates (Jung et al., 2015). In addition, this same methodology has been applied to phenotype the CLS of 488 meiotic segregants from a cross of two different yeast strains, revealing allelic variation at the *RIM15* locus (Jung et al., 2018), which has been shown to be one of the main modulators of the anti-aging response upon downregulation of TORC1 after nutrient depletion (Wei et al., 2008). This result suggests that there may exist an important natural variation in the response to DR in yeast. Importantly, there is also phenotypic variation in the response to DR within different mice strains (Liao et al., 2010). In this regard, loci affecting the variation of the response to DR in yeast natural populations may represent interesting targets to explore in mammalian models. These screens have proven the power of automated aging yeast culture assays for the discovery of important aging and longevity factors.

Another powerful alternative to characterize the CLS of thousands of strains has taken advantage of the molecular-barcode tagged yeast deletion collection (Winzeler et al., 1999). These experiments rely on estimating survival of pooled deletion mutants by microarray profiling or bar-code sequencing of viable single-gene deletion strains of *S. cerevisiae* cells in stationary phase (Matecic et al., 2010; Fabrizio et al., 2010). These studies have allowed the discovery of new aging-related processes in yeast such as fatty acid transport, tRNA methylation, cytoskeleton function, and the purine biosynthesis pathway. Importantly, the study by Matecic et al. (2010) targeted DR-unresponsive strains by comparing the CLS of the entire deletion collection under 2% and 0.5% glucose. This setup appointed 41 strains that were not capable of full lifespan extension under DR. Yet, only two candidate strains were successfully confirmed, namely deletions of *NFU1* and *FET3* genes which are involved in iron homeostasis and may be relevant for mitochondrial respiration during DR (Schulz et al., 2007; Schleit et al., 2013). In the study by Fabrizio et al. (2010), deletion of mitochondrial and autophagy genes, specifically vacuolar sorting proteins, were enriched among the short-lived strains, while only five deletion strains where confirmed as long-lived, including genes involved in amino acid and sphingolipid synthesis and cell proliferation (Fabrizio et al., 2010). However, the validation of these mutant strains was done in water–cultures were washed and transferred to water after reaching stationary phase (Fabrizio et al., 2010). Given that this treatment itself leads to lifespan extension (Fabrizio and Longo, 2003), it is hard to differentiate from regular aging and the effects of DR. The slim number of validated results in these studies indicates that this CLS-determination strategy results in an important number of false-positive hits, suggesting the co-culture of thousands of strains can lead to unexpected strain-strain interactions that may obscure the estimation of the rate of survival of the targeted strains. It has also been suggested that minor changes in medium composition or strain background may result in important differences in CLS phenotypes (Smith et al., 2016).

The pooling and genotyping strategy could yield better results when combined to massive parallel sequencing. Indeed, barcode sequencing has been used to characterize the CLS of 4497 single deletion mutants under different DR regimes, namely phosphate and leucine starvation (Gresham et al., 2011), showing that the survivorship of gene deletion strains is partially dependent on the DR regime implemented. In particular, deletion of mitochondrial genes resulted in deleterious effects in survival under phosphate starvation, while deletion of peroxisomal and cell-differentiation genes had an effect only under leucine restriction. Interestingly, deletion of chromatin organization and autophagy genes had an effect in either DR regime.

Survivorship of yeast populations in stationary phase has also been assayed by taking advantage of live/dead stain procedures. In particular, CLS has been assayed using the fluorescent dye propidium iodide to stain dead cells followed by flow cytometry, showing that the effect on survivorship of specific gene deletions can be determined through this method (Ocampo and Barrientos, 2011). A different method combined barcode sequencing of only dead cells sorted by cell cytometry after nutrient starvation from an original pool of 6000 gene-deletion yeast diploid strains, recapitulating the need for autophagy and peroxisome activity as well as ribosome breakdown to extend lifespan in response to low nutrients (Davey et al., 2012). Given the sequencing and interaction biases that may arise in screens where thousands of strains are being pooled, this sorting scheme in which only a fraction of the population is sequenced could enhance the resolution of pooling methods. Strains with statistically significant phenotypes in this study were compared to those of previous pooling studies showing an overlap of only 91 strains with reduced CLS in at least two studies (Davey et al., 2012). In addition, a comparison between the CFU method and propidium-iodide staining showed that CLS is only comparable under glucose exhausted medium but not when cells are transferred to water after diauxic phase (Ocampo and Barrientos, 2011). This implies there are important caveats to be considered when comparing the effects of different DR protocols using different experimental methods.

Our laboratory has introduced a high-resolution CLS assay based in the parallel co-culture of mutant and wild-type strains labeled with fluorescence markers (Garay et al., 2014). CLS of the mutant relative to the WT is estimated by measuring fluorescence of both strains in an outgrowth taken from aging cultures in a 96-well plate format through several days. The throughput of this assay has been greatly increased with the implementation of an automated robotic system. This assay was used to determine the CLS of 3878 single deletion strains, uncovering the role of novel aging factors in yeast such as the chromatin remodeling complex Swr1 (Garay et al., 2014). Interestingly, deletion of several genes of the Swr1 complex result in longer lifespan, but fail to extend lifespan under DR, suggesting that DR downregulates Swr1 or its targets. In addition, this method allowed the description of a genetic network of autophagy during aging based in epistatic phenotypes, revealing several functional associations between components of the autophagy machinery and the phosphatidylinositol-phosphate pathway (Garay et al., 2014). A different study revealed that sphingolipid biosynthesis is tightly linked to autophagy regulation (Thevissen et al., 2010), while the competition-based method showed a link between cortical ER protein *ARV1*, implicated in sphingolipid metabolism and autophagy, recapitulating the relevance of lipid homeostasis and aging in yeast (Garay et al., 2014). Interestingly, a genetic interaction between lipid metabolism and vacuolar ATPase components has also been reported (Wilms et al., 2017). In addition, Sch9, the main effector of TORC1 in yeast, has been shown to regulate the pH within the vacuole, and that the extended lifespan of *sch9*Δ depends upon a functional vacuolar ATPase, indicating that Sch9 monitors vacuolar function during stationary phase in yeast (Wilms et al., 2017). Still, many questions remain on how autophagy is regulated during aging in yeast and in particular in response to DR, which can be explored through the characterization of the genetics interactions between autophagy and other relevant processes during aging through the use of high-throughput methods to phenotype CLS.

More recently, we have shown that our experimental platform can be used to describe gene-environment interactions in stationary phase, allowing a genome-wide description of longevity in response to DR (Campos et al., 2018). By screening 3718 single deletion yeast strains under contrasting dietary regimes, over 400 genes were found to have an impact on longevity in response to DR. These genes were classified

into functional clusters that recapitulate some well-described processes such as loss of proteostasis (autophagy defects) and mitochondrial dysfunction (Campos et al., 2018). This systematic study confirmed that DR enhances longevity essentially by regulating cellular processes akin to those known as hallmarks of aging (Lopez-Otin et al., 2013). Moreover, analysis of these DR genes implied a role of the pheromone-response regulator Ste12 in CLS extension by DR, which was further confirmed (Campos et al., 2018). In this same vein, a link between TOR and other elements of the pheromone pathway, MAPKs *FUS3* and *KSS1* was revealed by gene-network analysis (Aluru et al., 2017). It is known that TOR plays an important role in cell cycle arrest through Rim15 and Igo1/2 proteins (Moreno-Torres et al., 2015). Importantly, G0 arrest has been shown to be a hallmark of quiescent yeast cells during stationary phase, and failure to enter the G0 program results in reduced survival (Leonov et al., 2017). In addition, GTP-binding protein Ras2 has been shown to impact RLS and CLS in response to nutrients (Sun et al., 1994; Fabrizio et al., 2003). It has been suggested that Ras2 regulates stress responses through the MAPK pathway, which includes Ste12 and Tec1, in particular to regulate invasive growth pathway (Jazwinski, 1999). Ras2 has also been shown to regulate cell cycle by regulating Rim15 in response to nutrients (Pedruzzi et al., 2003). In these conditions Ras2 activates Tec1/Ste12 heterodimer to initiate invasive growth (Mösch et al., 1999). These set of evidence suggests that cell-cycle control exerted by the pheromone pathway may be coopted by a nutrient response pathway. However, the interaction among the elements of the pheromone pathway has been originally explored in non-aged cells. Thus, a deeper study of aged-cells is needed to understand how the pheromone pathway and others typically associated to proliferating cells operate in aging cells. To grant a comprehensive understanding of how DR can modify lifespan, research on the subject will benefit from moving towards the characterization of the network of genetic and environmental interactions that shape the genetic architecture of aging in response to limited nutrients.

7. Concluding remarks

Using the budding yeast, large amounts of phenotypic data have led to a comprehensive view of gene-gene and gene-environment interactions of the eukaryotic cell. However, most efforts have been directed at studying cells in a proliferative, exponential-growth state, and we are yet to describe the functional and genetic rewiring of aging cells. For instance, our understanding of the pheromone pathway stems largely from studies in non-aged cells, and recent data suggests that elements of this pathway could play different roles during aging. We have herein reviewed the genetic architecture of longevity by DR, where recent advances illustrate that nutrient limitation elicits a complex response involving different signaling pathways, transcription factors, and many cellular processes, leading to extended cell survivorship and longevity. A comprehensive picture of the mechanisms of lifespan extension by DR will require the fine mapping of DR-related genes and their functional associations to determine the interplay and hierarchy between different processes that ultimately lead to longevity. To close the gap in the knowledge of the architecture of longevity by DR we will have to rely in high-resolution and high-throughput phenotyping assays that allow a characterization of lifespan in an ever growing set of conditions. The budding yeast will most certainly keep playing an important role in providing a simple cellular model amenable to high-throughput, quantitative phenotyping assays to reveal novel aspects of the biology of aging cells.

Author contributions

S.E.C. and A.D. wrote the manuscript; both authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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