Transcriptional rewiring of aging pathways and dietary restriction effects in different species of yeast

Significance and Aims

Aging is one of the most influential issues of our time. From the medical perspective, old age is the major risk factor to develop cancer, heart disease, diabetes, and neurodegeneration. But, paradoxically, our knowledge about the aging process is very limited. Little is known about the genetic control of lifespan and its evolutionary dynamics. To develop strategies to deal comprehensively with chronic conditions, it is essential to understand the process of aging from its most fundamental biological principles such as its underling molecular mechanisms and the way these mechanisms change over evolutionary time.

The considerable differences in longevity between related species suggest that aging rates evolve. Even when similar genes and pathways have been shown to modulate aging in very distantly related organisms, there are no genes known to have specifically evolved to cause cellular damage and aging. Changes in the rate of aging between species are rather thought to be side effects of selective pressures affecting other traits such as growth speed and fecundity. Overall, little is known about the molecular changes and processes driving aging evolution.

The yeast *Saccharomyces cerevisiae* is one of the model organisms that have contributed significantly to the advancement of biogerontology; research in this unicellular organism has led to the identification of precise molecular mechanisms of aging. In addition, *S. cerevisiae* has been successfully used to describe some of the genes and pathways that govern lifespan extension by dietary restriction. However, not much is known about these processes in related yeast species, which would shed light on the evolution and biological significance of survivorship at the cellular level.

The long-term goal of this project is to gain a comprehensive understanding of the aging process, with a special focus on its transcriptional control and evolution. We will build the foundations of this ambitious goal by exploiting the powerful yeast genetics toolbox available in the Johnson Lab at UCSF and combining it to the genome-wide aging phenotyping platform available in the DeLuna Lab at CINVESTAV. We will identify transcription factors (TFs) that control the chronological lifespan of *S. cerevisiae* and of the related, but pathogenic yeast *Candida albicans*—with special focus on those that mediate lifespan extension by dietary restriction. Comparison of the identified TFs will show how the regulatory mechanisms controlling aging have evolved. To gain deeper knowledge on the molecular basis of transcriptional rewiring of cell survivorship, we will carry out transcription profiling and ChIP-seq analyses of the identified TFs in both *S. cerevisiae* and *C. albicans*. In doing so, we will obtain insights not only on the evolutionary logic of the chronological lifespan of one of the most important models used in aging research, but also on the genetic control of cell survivorship in the major human fungal pathogen.

Our specific aims are:

Aim 1: To identify TFs that modulate chronological lifespan in response to dietary restriction in *S. cerevisiae* at the genome-wide level.

Aim 2: To evaluate the functional conservation of the TFs that control aging by identifying the TFs that modulate chronological lifespan in *C. albicans*.

Aim 3: To reconstruct and compare the transcriptional circuits controlled by the identified aging TFs in *C. albicans* and *S. cerevisiae* by defining the direct molecular targets of relevant players.

Background

Genetics of aging and the effects of dietary restriction on lifespan

Aging is the gradual deterioration of cells, tissues, and organisms as a function of time. At the cellular level, this damage can be prevented or reversed by mechanisms of maintenance and repair that influence lifespan [1,2]. As other processes, aging is subject to genetic regulation mediated by signaling pathways and TFs; their perturbation results in aging and longevity phenotypes. For example, mutations in the insulin receptor gene/IGF-1 increase longevity of *Drosophila* and *Caenorhabditis* and mice lacking the insulin receptor in adipose tissue also live longer [3-5]. Moreover, the activity of the histone deacetylase Sir2 and its mammalian homolog extend longevity in yeast and mice, respectively [6].

Calorie or amino acid restriction with optimal nutrition extends lifespan of yeast, worms, flies, and mice, among other model organisms [7,8]. Such dietary restriction (DR) results not only in longer lifespan, but also in a late appearance of most of the signs of aging. DR extends the longevity and prevents the development of diabetes, cancer, heart disease, and neurodegeneration in mice [9]. Likewise, DR initiated during adulthood has a positive impact on age-related survival of Rhesus macaques [10].

Lifespan extension by DR is the result of an active cellular response, rather than a passive biophysical phenomenon. Some of the genes and central processes that mediate this response have been described. Such is the case of the Tor kinase whose decreased activity leads to increased longevity in a similar manner than DR [11]. Both experimental conditions promote resistance to stress and induce maintenance and repair mechanisms, suggesting that these processes are related and act as part of the same signaling cascade.

Evolution of aging

Research focused on the evolution of aging aims to explain why almost all living organisms weaken and die with age. Early ideas by August Weismann [12] suggested that aging was programmed to remove old organisms and make space for the next generation. Given its teleological nature, this idea was largely discounted in the following years. Medawar's theory invokes the accumulation of deleterious mutations across generations because alleles that arise in late stages of reproduction are

effectively not subject to selection [13]. Building on this idea, it was posited that alleles that increment mortality in late reproductive phases could be selected for because they have beneficial effects during early stages of life (i.e., antagonistic pleiotropy) [14]. Resource allocation to reproduction and metabolism under favorable conditions, as opposed to maintenance and repair, has also been suggested as a similar, compensatory mechanism for aging [15]. Although some evidence exists in favor of these theories (e.g. [16,17]), conflicting results and debate still exist [18,19].

Yeasts as model organisms in aging research

The budding yeast *S. cerevisiae* is one of the model organisms that most have contributed to the advancement of aging research [20]. Genetic and environmental players identified in the budding yeast usually influence in similar ways the lifespan of invertebrates and mammal models. Furthermore, research in this unicellular organism has led to some of the most promising drug candidates to treat aging in humans, such as rapamycin, resveratrol, and spermidine [21]. Two aging paradigms have been proposed in *S. cerevisiae*: Its replicative lifespan, which is a model of aging of mitotic cells defined as the number of daughter cells produced by a parent cell prior to death. Meanwhile, the chronological lifespan (CLS) of the budding yeast is a model of aging postmitotic cells and is defined as the survival time of a population of quiescent cells in stationary phase. It is known that DR delays aging of both replicative and chronological lifespan of budding yeast cells through the inhibition of Tor, Sch9 and Ras/PKA pathways [22].

Functional genomics approaches of aging using simple model organisms like the budding yeast have the potential to provide a comprehensive and integrated view on the mechanisms of aging and longevity extension by DR. However, such approaches have been primarily limited by the lack of methods to characterize longevity accurately and at a large scale. Moreover, comparative genetic analyses that have the potential to shed light on the evolution of the regulation of aging-related pathways are practically inexistent. Comparative genetic analyses have been mostly limited to the description of aging genes and pathways in the budding yeast and in the fission yeast *Schizosaccharomyces pombe* [23,24].

The biololgy of *Saccharomyces cerevisiae* and *Candida albicans*

C. albicans, as *S. cerevisiae*, is a hemiascomycete fungus, but it belongs to a monophyletic subclade characterized by the translation of the CTG codon as serine instead of leucine [25]. *C. albicans* is a usual commensal of the human microbiota that is mostly maternally transmitted across generations. However, it is also an opportunistic pathogen causing superficial epithelial diseases in healthy individuals and systemic infections with fatal consequences in immunocompromised hosts. In terms of its prevalence and its mortality rate, *C. albicans* is the most important human fungal pathogen [26,27].

While no environmental reservoirs outside of animals are known for *C. albicans* [26], *S. cerevisiae* is often isolated in the wild from rotten fruits or tree bark. Insects are also known dispersal vectors and natural reservoirs of *S. cerevisiae* [28]. *S. cerevisiae* has been isolated from immunocompromised human patients [29], but this association is thought to be mainly a side consequence of the utilization of the budding yeast by humans for food and drink production.

It is estimated that *C. albicans* and *S. cerevisiae* diverged from a last common ancestor between 100 and 300 million years ago [30]. In terms of their genomic coding sequences, *S. cerevisiae* and *C. albicans* are as divergent as fish and humans [31]. Despite their evolutionary distance and the differences in the ecological niches they inhabit, their cellular properties are similar. They are both encountered in yeast (blastopore) and filamentous forms and most genetic, molecular, and cell biology techniques used in *S. cerevisiae* also work in *C. albicans*.

Little is known about cellular lifespan and survivorship in *C. albicans* and studies have been limited to its replicative lifespan [32]. These cellular traits have possible implications for pathogenicity [24]. This fact, together with the characteristics of *S. cerevisiae* and *C. albicans* mentioned above, make the comparative study of the molecular mechanisms regulating aging in these two species particularly interesting. We are working with two species whose phenotypic traits can be directly contrasted, but that are evolutionarily and ecologically very distinct.

Transcription factors and regulatory rewiring

The evolutionary potential of modifications in transcriptional circuits was recognized shortly after the discovery of gene regulation [33]. Changes in the timing and conditions under which a gene is expressed, rather than modifications in its coding region, can lead to new adaptive phenotypes over generations. This idea gained strength as genes and proteins of related species were compared. The differences in gene content and number did not seem sufficient to explain phenotypic diversity [34]. However, it was only with technological advances in sequencing and functional genomics that the direct empirical study of transcriptional circuit evolution was facilitated. To date, the importance of transcriptional modifications has been documented in the evolution of many different phenotypes, from mating patterns in yeasts to wing pigmentation in fruit flies [33,35].

Evolutionary changes in TFs as well as in *cis*-regulatory elements (CREs), the two main components of transcriptional circuits, have been observed in different scenarios [33]. There are many examples in fungi and animals where the set of regulated genes varied while the TFs, their binding specificity and partner proteins are conserved (e.g. [36,37]). In these cases, changes occur mainly in the CREs of target genes. In contrast, there are several examples where the genes that are regulated are conserved, but regulation is performed by a different TF (e.g. [38]). In addition, it has been shown that transcriptional circuits evolve very rapidly. When circuits are compared between closely related species, frequently there is variation in the regulated genes and/or in the TFs involved [33,34]. Such differences have even been detected among individuals of the same species [39].

As described, aging and longevity are determined by regulatory circuits composed of TFs that control sets of target genes and downstream processes. Whether such molecular regulation changes during evolution or is "hardwired" across different species remains largely unknown. Significant rewiring has been reported for the Tor1-Sch9-Rim15 pathway in *C. albicans*, but this is only one of the many pathways that control aging. Characterization and comparison of the transcriptional circuits that underlie aging in *C. albicans* and *S. cerevisiae* will shed light into how these molecular mechanisms change or are maintained over evolutionary time.

Research design and methods

Aim 1: Genome-wide identification of TFs that control chronological lifespan in **response to dietary restriction in the budding yeast *Saccharomyces cerevisiae***

Rationale and approach. We will focus our study on the genetic regulation of chronological lifespan (CLS) extension by DR. Since a large number of downstream processes are associated to longevity, we hypothesize that several TFs will be associated to this phenomenon. We will use gene knockout strains of all non-essential genes in the budding yeast *S. cerevisiae* and will ask which mutants show altered lifespan phenotypes that depend on the diet. Such DR-insensitive or hyper-sensitive strains will indicate which genes are needed to or redundant with extended longevity by DR. By further combining this functional genomics data with available regulatory knowledge of TFs and their targets in *S. cerevisiae*, we will unravel the main regulators of lifespan and DR, even in the cases in which their single-gene deletion has little or no phenotypic effect.

To this end, we will take advantage of a high-throughput screening approach that was recently developed at the DeLuna Lab [40]. In brief, each single-knockout *S. cerevisiae* mutant labeled with mCherry will be mixed with a CFP-labeled wild-type reference strain and grown to saturation. At different time intervals over ~20 days, an aliquot of the stationary phase population will be inoculated onto fresh medium and fluorescence signal will be used to estimate the relative number of viable cells at a particular age of the non-dividing culture. We will screen over 4,000 mutant strains grown on 2% glucose supplemented with glutamine or with GABA, which are non-restricted or dietary-restricted conditions of nitrogen, respectively.

We will analyze the data output in two different ways. On the one hand, we will focus on each experimental condition individually and will ask which gene knockouts have CLS phenotypes that are significantly different from the WT phenotype. On the other hand, we will take advantage of the quantitative nature of our approach and derive a DR index, I_{DR}, which will report on the mutant's phenotype on restricted nitrogen relative to the phenotype under non-restricted conditions. Genes associated to mutations with a higher absolute I_{DR} value will be those that are more likely related to the cellular response to DR. Finally, we will use the Yeastract repository [41] to ask which TFs are known to regulate targets from this high-rank I_{DR} list.

Anticipated results, potential problems, and alternative strategies. The cellular response to DR is known to depend on TFs downstream of Rim15, such as Msn2, Msn4, and Gis1 [22]. Our functional-genomics screen should confirm these TFs. Moreover, since previous studies have shown that longevity is extended by DR even in the absence of Rim15, it is expected that our comprehensive screen will reveal novel TFs and signal-transduction pathways that modulate this effect.

Our screen is not exempt of false-positive and false-negative hits, given its high-throughput nature and the specific conditions imposed by automation. We will focus on the top I_{DR} hits to run validation experiments using conventional lower-throughput CLS measuring methods [42], which should give a more direct estimate on the fraction of false-positive hits. Regarding false-negative results, it is

expected that the use of a curated repository with more than 200,000 regulatory associations will maximize the number of correctly identified TFs.

Aim 2: Functional conservation of TFs that control aging: identification of TFs that control chronological lifespan in *Candida albicans*

Rationale and approach. Aim 1 will provide a catalogue of TFs that control the cellular response to DR in *S. cerevisiae* by looking both at the direct phenotype of single TF knockouts and by characterizing the phenotypes of deletion of their targets. The first step in our functional comparative analysis, will be to characterize the single-knockout effects of a large collection of TFs in the fungal pathogen *C. albicans*.

The fact that *C. albicans* is a diploid organism that does not undergo a regular sexual cycle makes the generation of gene-knockout strains in this species considerably more laborious than in *S. cerevisiae*. Both alleles of each gene have to be knocked out independently in two successive rounds of gene disruption using two different auxotrophic or drug-resistance markers [43]. Despite this difficulty, the community working in *C. albicans*, in particular efforts by the Johnson research group, has generated a series of gene-knockout strain collections. One of these collections specifically focused on deleting genes encoding TFs [31]. The initial collection consisted of 143 homozygous gene knockout strains and further efforts by the Johnson laboratory have added gene knockout strains for 54 more TFs. In addition, the Johnson laboratory is currently working in performing the deletions for 30 further TFs. This collection encompasses most of the between 200 and 300 expected TFs in the *C. albicans* genome.

The CLS of *C. albicans* can be measured by using the conventional phenotyping approaches established for the budding yeast [44]. Given the difficulty to tag homozygous diploid strains with fluorescence markers, we will use an alternative method based on high-resolution outgrowth curves [45], which has been robotically up-scaled by the DeLuna group (data not shown). In brief, *C. albicans* strains will be individually grown to saturation in 96-deep well plates in a large number (8-12) of experimental replicates. Dietary restriction will be modeled by growing *C. albicans* in either nitrogen restricted (glutamine vs GABA or proline) or caloric-restricted cultures (2% vs 0.5% glucose). In doing so, we will identify which TFs from the fungal pathogen *C. albicans* mediate the cellular response to dietary restriction and the associated extended longevity. It is important to point out that significant rewiring has already been reported for the Tor1-Sch9-Rim15 pathway in *C. albicans* [46]. Therefore important differences are expected even between these two species of yeast.

Anticipated results, potential problems, and alternative strategies. The initial characterization of the ~227 mutant strains will provide a list of TFs with potential effects on lifespan control in *C. albicans*. This will inform on the functional conservation and divergence of such regulators with the budding yeast *S. cerevisiae*. In addition, the identity of such TFs and current knowledge about their targets will inform on which cellular processes are important for survivorship of this fungal pathogen. It will be interesting to ask which of these hits are also important for pathogenicity.

The characterization of lifespan effects in *C. albicans* will be done only by testing the possible roles of individual TFs and not on their possible targets, as will be done for *S. cerevisiae*. It is thus expected that this initial screen will yield several false negatives in the cases in which the TFs have marginal effects on lifespan. One way to overcome this possible problem is to screen all false hits under a few additional experimental conditions (e.g. drugs or environmental stresses) that could expose the effects of such TFs on lifespan. Another possible pitfall is that dietary restriction is likely to be triggered by different sources of carbon or nitrogen between the two species [24]. If this is the case, we will test different regimes of dietary restriction to achieve similar degrees of lifespan extension. All these alternative approaches are possible given the described automated screening process.

Aim 3: Reconstruction and comparison of the transcriptional circuits controlled by the TFs that control aging in *C. albicans* and *S. cerevisiae*

Rationale and approach: Aims 1 and 2 will reveal the set of TFs that control aging in *S. cerevisiae* and *C. albicans.* The objective of Aim 3 is to reconstruct and compare the transcriptional circuits of these TFs by identifying the target genes that they control. It is expected that only a defined number of TFs from *S. cerevisiae* and *C. albicans* will be involved in the lifespan response to dietary restriction. Therefore, the reconstruction of the transcriptional circuits involved in aging in the two species seems feasible.

To find the genes which promoters are directly bound by the different TFs, we will perform chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) [47]. To this end, the TFs will be tagged with an epitope that allows immunoprecipitation, but that does not interfere with protein function. The collection of bound regions will also allow determination and comparison of the binding motifs recognized by the ortholog TFs using tools such as MochiView and MEME [48,49]. Following these experiments and to find out whether TF binding triggers gene expression in each bound gene, we will perform transcriptional profiling by RNA-seq [50]. This will be done comparing the profiles in the gene knockout strains of the different TFs with the profiles of the wild-type strain in each species. These experiments will be carried out at the Johnson laboratory, where the methodologies are well established (see references [37,51]).

Anticipated results, potential problems, alternative strategies: The molecular approaches in Aim 3 will lead to the reconstruction of the transcriptional circuits underling lifespan extension by dietary restriction in the two species and their comparison will show how these circuits have changed over evolutionary time.

It is possible that epitope-tagging of the TFs interferes with the function of the protein. A solution to this problem is using different epitopes and varying the protein end that is tagged. If this does not render an active TF, it is also possible to raise an antibody specific for the TF. The Johnson laboratory is familiar with both of these possible solutions [51].

Collaborative elements

This project brings together the expertise of three research groups to investigate a fundamental biological question in the field of aging research: how do the molecular mechanisms that regulate lifespan and lifespan response to dietary restriction evolve? Given how broad this question is, each of the groups cannot tackle the problem by itself, making the collaborative nature of the project essential. The group of Alexander DeLuna at CINVESTAV has extensive expertise in functional genomics of aging using the budding yeast as a model organism. They will provide the methodological platform that they have developed to characterize lifespan in the budding yeast to study cellular aging in both *S. cerevisiae* and its implementation in *C. albicans*. At a conceptual level, they will also provide the needed expertise in the aging field that the project requires. Alexander Johnson at UCSF is an expert in *C. albicans* biology and in molecular evolution of transcriptional circuits. His group will provide the knowledge, techniques and resources needed to work with *C. albicans*, as well as to characterize transcriptional circuits across species. Alicia Gonzalez at UNAM is also an expert in the study of transcriptional circuits, but from a biochemical perspective. The expertise of her group will be essential to elucidate the detailed differences of the transcriptional circuits between the species at a finer scale. Overall, the expertise of the three research groups intersects perfectly to study the evolution of the molecular determinants of aging.

Conducting research, timeline, and project continuation

We have carefully considered how and where to execute the different parts of this research. The identification of the TFs that modulate response to dietary restriction in *S. cerevisiae* and *C. albicans* (Specific Aims 1 and 2) will be performed during the first six months of the project (July-Dec 2015) in the DeLuna lab at CINVESTAV Mexico. The *C. albicans* strain collections and the expertise needed to work with *C. albicans* will be provided by Alexander Johnson's group. This will be done through a two weeks visit of Eugenio Mancera, a postdoc from the Johnson group, to the DeLuna laboratory at the beginning of the project, followed by constant communication between the two laboratories. Once the TFs determining cellular lifespan have been identified in the two species, the transcriptional circuits that they control will be characterized first at the genome-wide level by the Johnson group at UCSF and then at a more detailed scale in the laboratory of Alicia Gonzalez at UNAM. This will be done during the six following months (Jan-June 2016). The ChIP-seq and RNA-seq experiments will be performed at the Johnson laboratory during a one-month visit by Sergio Campos, a graduate student from the DeLuna group. The visit will accelerate the acquisition of the genome-wide data and will train the student from the DeLuna group in these techniques. The analysis and integration of the data as well as writing-up the results for publication and future grant applications will be performed during the last six months (July-Dec 2016) in the three laboratories in parallel. To consolidate this last step, members of the three groups will meet at the DeLuna laboratory in Irapuato for a week during the last two months of the project.

We expect to continue the project beyond this initial 18-month grant period. Funding of our proposal will generate the initial results that will guide the next research steps and will make future funding

proposals competitive. The subject of our project is well within the scope of other general funding opportunities such as those from the NIH, NSF, or Conacyt Ciencia Básica, but also within the scope of more specific funding agencies such as the American Federation for Aging Research or Conacyt Atención a Problemas Nacionales. Eugenio Mancera, currently a postdoc at the Johnson Lab, is planning to establish his own independent research group in Mexico (late 2016, see his application for the UC MEXUS-CONACYT Postdoctoral Fellowship Program for further details). Given his interest in evolution and direct participation in this research project, we expect that the collaborating team between UCSF and Mexico will not only be maintained but will also grow in the near future.

Regional impact of the proposed project

Apart from the benefits that our findings will have to the general understanding of aging and its evolution, our project will have three main regional impacts. First, our project will facilitate the exchange of cutting edge research approaches and technologies between the regions. At the beginning this will impact mainly the research groups involved, but in the long-term dissemination of the knowledge and techniques to neighboring research groups will have a broader regional impact in the research communities. Second, the development of our project will contribute to the establishment and progress of the field of biogerontology in Mexico, which is currently inexistent in the country despite the medical, social, and economical importance of the phenomenon of biological aging. As described, we will address the problem from its most fundamental molecular and evolutionary principles. Third, understanding cellular lifespan in the pathogen *C. albicans* promises to have direct implications for treatment and prevention of infections caused by this important human pathogen. For example, the identified TFs or members of the characterized transcriptional circuits are potential targets for antifungal treatment: modifying the lifespan of *C. albicans* could inhibit infection. Our findings could be the basis for regional start-ups to develop novel antifungal treatments. All three research groups involved in this proposal are interested in pursuing such biotechnological possibilities.

Project's fit to the UC-MEXUS/CONACYT program goals and objectives

If funded, the realization of the proposed project will establish a collaboration of two Mexican research groups in two different academic institutions with a research group at UCSF that had not worked together in the past. As mentioned before, all three groups intend to continue the project beyond the scope of the UC-MEXUS/CONACYT Program by applying for other funding opportunities in the future. The results of the project funded by the UC-MEXUS/CONACYT Program will be the basis for such long-term endeavor. It is also important to mention that this collaboration will serve as a starting point for future collaborations in other research subjects. During the realization of this application, we have already started exchanging ideas about other research projects that would benefit from the collaboration between the groups. These include performing systematic epistasis analysis in pathogenic fungi or studying the evolution of duplicated genes and moonlighting protein functions.

Previous UC-MEXUS/CONACYT grants

The two co-principal investigators of this project have received UC-MEXUS/CONACYT Program Grant for Collaborative Projects in the past: Alexander Johnson with Dr. Irene Castaño at IPCYT Mexico (2006) and Alexander DeLuna with Dr. Van M. Savage at UCLA (2011). Both past projects were very successful in terms of the resulting publications and the establishment of long-term collaborations. The collaborations are currently still going on in both cases. The more recent project co-led by Alexander DeLuna was also in the field of aging, but had a totally different aim, namely to develop an experimental and theoretical platform to study genetic interactions that influence aging in *Saccharomyces cerevisiae*. This project has so far resulted in one high-impact publication [40] and one manuscript which is under review [52].

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