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## Genome-wide identification of conserved longevity genes in yeast and worms

Erica D. Smith<sup>a</sup>, Brian K. Kennedy<sup>a,\*</sup>, Matt Kaeberlein<sup>b,\*</sup>

<sup>a</sup>Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

<sup>b</sup>Department of Pathology, University of Washington, Seattle, WA 98195, USA

### Abstract

Technological advancements in invertebrate model organisms have recently made it possible to survey many or all of the genes in the genome for phenotypes of interest. In both *C. elegans* and *S. cerevisiae*, genome-wide searches for hypomorphic mutations that extend life span have been performed. The results from these screens are starting to provide a more complete view of the range of life span determinants in eukaryotes. In addition, it is becoming possible to test the premise that conserved aging genes and pathways regulate aging in disparate eukaryotic species. Here we compare and contrast the results from genome-wide aging screens and assess the likelihood that there are “public” aging mechanisms.

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### 1. Introduction

Many different model organisms have been used to study the biology of aging, including the budding yeast, *S. cerevisiae*, the nematode, *C. elegans*, the fly, *D. melanogaster*, and the mouse, *M. musculus*. Major advances in our understanding of the molecular basis of aging have been made, with dozens of interventions demonstrated to modulate life span in one or more of these organisms. Genetic analysis of various mutants has led to the identification of several important genes involved in aging, such as those in the insulin/IGF-1 signaling pathway (Kenyon et al., 1993; Duhon et al., 1996), genes important for stress resistance (Lithgow and Walker, 2002; Longo and Fabrizio, 2002) and genomic maintenance (Lombard et al., 2005), and the sirtuin family of protein deacetylases (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004). These and many other studies have demonstrated clearly that there is a genetic component to the aging process, reviewed in Kenyon (2005).

Environmental cues, as well as genetic factors, can also play a major role in longevity determination. The best characterized

environmental determinant of longevity is calorie restriction (CR). CR has been shown to extend life span in nearly every organism in which it has been tested (Walford et al., 1987, Masoro, 2005). Although several models have been put forth to explain how CR slows aging, no commonly accepted mechanism for CR has been described in any organism, and there is much interest in determining whether the mechanism underlying CR is conserved in different organisms.

Several years ago George Martin described the idea that the aging process is likely to be a combination of “public” and “private” features. Public features of aging are evolutionarily conserved among disparate organisms; whereas, private features of aging are specific to a given organism or closely related group of organisms. One example of a private feature of aging is the role of extrachromosomal rDNA circles (ERCs) in mother cell senescence of *S. cerevisiae* (Sinclair and Guarente, 1997); although ERCs are one cause of replicative aging in yeast, there is no evidence that ERCs accumulate or cause aging in any organism other than yeast. Public features of aging include the ability of CR to increase longevity in many different organisms and the observation that decreased signaling through the insulin/IGF-1 pathways can increase life span in worms, flies, and mammals. It remains to be determined, however, to what degree the aging process has been evolutionarily conserved and whether public determinants of longevity are the exception or the norm.

\* Corresponding authors. Tel.: +1 206 685 0111; fax: +1 206 685 1792.

E-mail addresses: [bkenn@u.washington.edu](mailto:bkenn@u.washington.edu) (B.K. Kennedy), [kaeber@u.washington.edu](mailto:kaeber@u.washington.edu) (M. Kaeberlein).

As part of a consortium of researchers located at the University of Washington and funded by the Ellison Medical Foundation, we are taking a multi-organism approach to identify conserved longevity genes. The underlying rationale for this approach is that if a particular gene family regulates longevity in yeast, worms, and mice, then there is a good chance this gene family will play a similar role in humans. Here we summarize the results from genome-wide longevity screens in yeast and worms, to date, and we discuss how these data are facilitating the search for conserved longevity genes.

## 2. Genomic approaches to the identification of aging genes

The rapid increase in whole genome sequencing has dramatically changed the way scientists do research in genetics, molecular biology and biochemistry, and biogerontology is no exception. The complete sequence of the yeast genome was published in 1996 (Goffeau et al., 1996), followed by the worm genome in 1998 (Consortium, 1998), the fly in 2000 (Adams et al., 2000), and the mouse in 2002 (Waterston et al., 2002). In the years since these genomes were published, a plethora of genomic tools have been developed, including databases such as Saccharomyces Genome Database (SGD; Hong EL et al., 2006), Wormbase (2006), Flybase (Grumbling and Strelets, 2006), Biobase Biological Databases (2006), and even a database devoted to cataloging aging genes and interventions (Kaeberlein et al., 2002). The impact of these databases, combined with technologies, such as microarrays and other genomic tools, on progress in the biology of aging is unquestionable (reviewed in Kaeberlein, 2004).

In yeast, several genomic deletion collections are now commercially available (Winzeler et al., 1999). In these collections, yeast strains are genetically identical, except for a single gene deletion in each strain. Of note, there are genetic anomalies, including aneuploidy (Hughes et al., 2000) and suppressor mutations, in a small portion of the strains in the collection. Deletion collections are available in both haploid mating types,  $a$  and  $\alpha$ , as well as homozygous and heterozygous diploids. In total, more than 20,000 unique single-ORF deletion strains have been created (Winzeler et al., 1999). Thus, researchers have the ability to screen for a phenotype of interest across the entire yeast genome in a gene-by-gene manner.

In *C. elegans*, a deletion collection similar to those available in yeast has not been developed; however, there are two independently derived RNAi libraries that are commercially available. The first is a genomic library generated by the Ahringer lab and contains over 16,000 unique bacterial strains expressing double-stranded RNA corresponding to individual genes (Kamath et al., 2003). The second library, generated by the Vidal lab, utilizes a similar expression vector and the same bacterial strain, but contains inserts derived from the ORFeome cloning project and does not include introns (Reboul et al., 2003; Rual et al., 2004). Both RNAi libraries are arrayed so that high-throughput screening is possible, where worms are fed individual clones to specifically down-regulate expression of individual genes and a phenotype of interest can be assayed.

### 2.1. Genome-wide longevity screens in yeast

We have taken a genomic approach to identify conserved determinants of longevity. Two different models of aging are commonly studied in yeast: replicative and chronological (Kaeberlein, 2006b). Replicative life span (RLS) is defined as the number of daughter cells that an individual mother cell can produce, and has been suggested as a model for the aging of mitotically active cells or stem cells. Chronological life span (CLS) is defined as the length of time that a yeast cell can survive in a non-dividing state (typically stationary phase), and has been suggested as a model for aging of post-mitotic cells. Together, both aging models make yeast a powerful system in which to study the genetics of aging, because RLS and CLS can be measured independently, allowing direct comparisons to be made between the genes regulating life span in dividing versus non-dividing cells.

#### 2.1.1. Yeast replicative life span

The first longevity screen of the yeast deletion collection to be described was our characterization of the replicative aging properties of 564 single-gene deletion mutants from the *MAT  $\alpha$*  ORF deletion collection (Kaeberlein et al., 2005). Due to the labor-intensive nature of the RLS assay, which involves microdissection of daughter cells away from the mother cells, an iterative method was developed for classifying mutants as likely to be long-lived or not, based on RLS measurements of five-cell sets (Kaeberlein and Kennedy, 2005). From this analysis, 13 single-gene deletion mutants were found to have a significantly increased RLS relative to control cells (discussed below), while approximately 20% of the single-gene deletion mutants analyzed were significantly short-lived. The genome-wide RLS screen is currently ongoing, with RLS data having been obtained thus far for more than 2500 single-gene deletion mutants.

Among the 13 verified long-lived mutants identified from the yeast RLS deletion set screen, both known and novel aging genes were identified. For example, deletion of the replication fork block gene, *FOBI*, was previously shown to increase RLS through regulation of ERC formation (Defossez et al., 1999), and *fib1 $\Delta$*  was identified from our RLS screen as long-lived (Kaeberlein et al., 2005). Deletion of the gene coding for the nutrient responsive kinase Tori also increased RLS. Although *tor1 $\Delta$*  cells had not been previously found to have long RLS in yeast, decreased TOR activity had been shown to increase life span in both worms and flies (Kapahi et al., 2004; Vellai et al., 2003).

In addition to *tor1 $\Delta$* , several strains lacking other components of the TOR signaling pathway were also found to be replicatively long-lived in our screen. These included two gene deletion strains lacking protein components of the large ribosomal subunit, *rpl31a $\Delta$*  and *rpl6b $\Delta$* . TOR promotes ribosome biogenesis by regulating transcription of ribosomal proteins in yeast, and decreased TOR activity is known to result in down-regulation of many ribosomal proteins (Martin et al., 2004). In follow-up studies, we have observed long RLS in additional ribosomal protein mutants, leading us to speculate

that regulation of ribosome function and translation could be one mechanism by which TOR activity determines longevity in dividing cells (Kristan Steffen, Vivian MacKay, B.K.K. and M.K., unpublished).

### 2.1.2. Yeast chronological life span

In a parallel screen, a semi-quantitative measure of CLS was determined for approximately 4800 strains contained in the homozygous diploid deletion collection (Powers et al., 2006). In order to screen such a large number of strains, a high-throughput CLS assay was developed where cells were aged in 96-well microtiter plates, rather than culture tubes or flasks. Viability over time was determined by measuring optical density after a fixed period of outgrowth. At each time point, this involved first diluting a small volume (~1  $\mu$ L) from the aging culture into a larger volume (~200  $\mu$ L) of rich media. The inoculated culture was then allowed to undergo growth for about 16 h, followed by measurement of optical density at 600 nm using a plate reader. The optical density in each well correlated with the number of viable cells contained in the initial inoculum. This process was repeated at multiple time points, and the relative survival for each strain was calculated as the ratio of the optical density in each well relative to the mean optical density across the entire deletion set at each timepoint. Individual wells in the 96-well plates that showed an increase in this ratio over time corresponded to deletion strains that were living longer, relative to the average of the entire collection of strains.

Interestingly, several gene deletions resulting in decreased TOR activity were also identified from the CLS deletion set screen (Powers et al., 2006). In addition to *tor1 $\Delta$*  cells, cells lacking the nitrogen responsive transcription factor *GLN3*, the ammonium permeases *MEP2* or *MEP3*, the amino acid permease *AGP1*, or an enzyme involved in lysine biosynthesis, *LYS12*, had increased CLS. Removal of asparagine or glutamine, preferred nitrogen sources in the media, as well as pharmacological inhibition of TOR, using either rapamycin or methionine sulfoximine, also increased CLS. Together, these results demonstrate that decreased nutrients, specifically nitrogen sources in the media, or decreased signaling through the nutrient-responsive kinase TOR can increase chronological life span in yeast.

Unlike the case for RLS, however, we have found no evidence that altered ribosome function is important for TOR-mediated CLS extension in yeast (Powers et al., 2006). Instead, we are focusing on two additional TOR-regulated processes, Msn2/4-mediated stress response and autophagy, as potentially important for increased CLS in response to nutrient depletion. The Msn2/4-mediated stress pathway has been previously proposed as an important regulator of CLS (Fabrizio et al., 2001), perhaps through transcriptional up-regulation of antioxidant genes, such as superoxide dismutase and catalase (Fabrizio et al., 2003, 2004). No evidence supporting a role for autophagy in yeast longevity has been reported; however, autophagy has been shown to be important for life span extension from reduced insulin/IGF1-like pathway signaling in *C. elegans* (Melendez et al., 2003).

### 2.1.3. Putting longevity genes into context

One common approach to place longevity-determining genes into pathways is to use perform epistasis studies in which two or more interventions are combined and the resulting effect on longevity is observed. We have subjected several of the yeast gene deletions identified in the above screens to this type of analysis, particularly as it relates to the mechanism of life span extension from CR in the yeast replicative aging model. For example, CR increases life span additively with deletion of *FOB1* or overexpression of *SIR2*, as well as in *sir2 $\Delta$  fob1 $\Delta$*  double mutant cells, suggesting that CR acts in a distinct replicative aging pathway from Sir2, Fob1, and ERCs (Kaeberlein et al., 2004; Longo and Kennedy, 2006). In contrast to this, CR does not further increase the RLS of *tor1 $\Delta$*  or *sch9 $\Delta$*  cells, but deletion of *FOB1* results in an additive RLS increase in combination with deletion of *TOR1* or deletion of *SCH9* (Kaeberlein et al., 2005). Thus, multiple epistasis experiments support the hypothesis that CR acts through a genetic pathway involving the nutrient-responsive TOR and Sch9 kinases.

## 2.2. Genome-wide longevity screens in *C. elegans*

The discovery of RNA interference (Fire et al., 1998; Guo and Kemphues, 1995) and RNAi through feeding (Timmons and Fire, 1998) in *C. elegans* has rapidly transformed the use of reverse genetics in the study of aging in this model. Two independent, genome-wide screens have been carried out using the RNAi library generated by the Ahringer lab (Kamath et al., 2003) with longevity as the target phenotype (Hamilton et al., 2005; Hansen et al., 2005). Initial results from screening of chromosomes I and II led both groups to conclude that genes involved in mitochondrial function are an important class of longevity genes in *C. elegans* (Dillin et al., 2002; Lee et al., 2003). In subsequent screening of the entire RNAi library, these groups both found genes involved in metabolism, signal transduction, and gene expression to regulate aging in *C. elegans* (Hamilton et al., 2005; Hansen et al., 2005). In both studies, epistasis analysis was used to place genes into novel or known aging pathways. The Kenyon lab examined epistasis with *daf-2* and *eat-2* pathways (Hansen et al., 2005), while Lee and co-workers looked at the requirement for *daf-16* or *sir-2.1* (Hamilton et al., 2005). Nearly all of the 23 novel longevity genes identified by Hansen et al. (2005) mapped to either the *daf-2* or *eat-2* pathway. Hamilton et al. (2005) examined a subset of the 89 longevity genes identified in their screen and found many genetic interactions with *daf-16* or *sir-2.1*, but 20% of those analyzed did not map to either of these pathways. These results indicate that although many genes may fall into known aging pathways, there are still novel pathways to be discovered in the regulation of longevity.

Interestingly, despite some common trends in important classes of gene function shared between the two RNAi longevity screens, the actual genes identified were quite disparate. Together, these studies identified RNAi clones that increase life span corresponding to approximately 130 genes (Dillin et al., 2002; Lee et al., 2003; Hamilton et al., 2005;

Hansen et al., 2005). Of these, only three were shared between the two screens: *cco-1* (F26E4.9), *drr-1* (F45H10.4), and *nuo-4* (K04G7.4). Differences in experimental design and variability in RNAi efficiency likely account for much of the lack of replication (Lee, 2005); however, the degree of dissimilarity is surprising. This may suggest that neither screen effectively identified a high percentage of target genes and that additional longevity-determining genes remain to be found. In addition, independent validation of the identified genes will be important, in order to weed out the inevitable false positives from the data sets. In the future, additional genomic tools will likely aid longevity studies using RNAi to target gene expression in worms. For instance, inducible hairpin RNAi constructs may reduce the variability observed from one RNAi clone to the next and make it possible to identify genes involved in tissue-specific regulation of aging, such as neurons (a tissue refractory to traditional RNAi methods) (Johnson et al., 2005).

### 3. Public pathways regulating longevity in yeast and worms

In addition to characterizing the genes that regulate aging in yeast and worms, genomic longevity studies in these organisms provide an opportunity to identify public features of aging in a genome-wide manner. One approach we have taken is to identify *C. elegans* orthologs corresponding to the long-lived yeast deletion mutants identified from our deletion set screens, then use RNAi to knock-down the function of the *C. elegans* ORF and measure the effect on life span (Fig. 1). In some cases, such as Tori, a longevity-determining role for the *C. elegans* ortholog has already been established (Vellai et al., 2003).

We are also taking advantage of the completed RNAi longevity screens in *C. elegans* (Hamilton et al., 2005; Hansen et al., 2005), by using a reverse approach to that described above (Fig. 1). In this case, we use a combined list of the *C. elegans* ORFs identified from both RNAi screens to identify candidate yeast orthologs. For those yeast orthologs that are non-essential, the corresponding deletion strain is removed from the deletion collection and both CLS and RLS is determined.

One difficulty with either of these approaches is how to separate orthologs from homologs. A typical definition for identifying ortholog pairs is to require a reciprocal best match using BLASTp. In this approach, yeast protein Abc1 is an ortholog of worm protein ABC-1 only if a BLASTp search of Abc1 against the worm genome identifies ABC-1 as the best match and a BLASTp search of ABC-1 against the yeast genome identifies Abc1 as a best match. This approach, however, excludes genes that have multiple orthologs in another species. For example, yeast Sch9 can arguably be called orthologous to four worm proteins: AKT-1, AKT-2, AKT-3, and SGK-1. The reciprocal best match approach also excludes small gene families that have high homology, such as the isocitrate dehydrogenases. Our approach has been to initially define orthologs using a reciprocal BLAST best match approach, but to also include closely related homologs in our analysis using a variety of parameters.

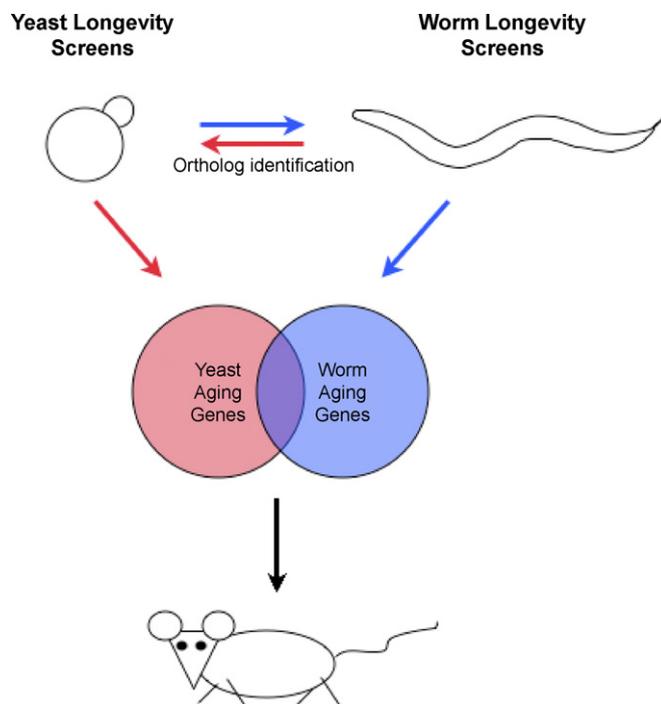


Fig. 1. A genome-wide, multi-organism approach to finding conserved mechanisms of aging. As part of a consortium of researchers at the University of Washington, we are screening the *S. cerevisiae* genome for genes that regulate longevity. The *C. elegans* orthologs of candidates identified in these yeast screens are being targeted by RNA interference in order to determine whether these genes have a conserved role in life span determination. We are also examining the life span of yeast strains that have deletions in the orthologs of genes identified in genome-wide longevity screens in worms. Genes shown to regulate aging in both yeast and worms will then be analyzed in mammals by generating gene knockouts in the mouse.

A comparative analysis of longevity among *C. elegans* and yeast ORFs is a reasonable place to begin searching in a large-scale manner for public pathways of longevity. These two organisms are highly evolutionarily divergent, relatively easy to assay for life span, and have proven track records in biology of aging research. One potential caveat to this type of yeast/worm comparison, however, is that all tissues in the adult worm (except the germline) are post-mitotic. Thus, it is possible that many genes identified in the yeast RLS screen may not play an important role in the adult worm life span, but may in fact be important regulators of aging in mitotic cells of higher eukaryotes. By using the yeast CLS assay in addition to the RLS assay, we may therefore find more conserved aging genes between yeast and worms. Overall, we are confident that this approach will be useful, as we and others have already identified a number of ortholog pairs that have a similar longevity phenotype in yeast and worms.

### 4. Conclusion

Along with others, we have adopted the idea that human aging can be better understood by first identifying public features of aging that are conserved among evolutionarily divergent organisms (Kaeberlein, 2006a). Genome-wide longevity screens in yeast and worms are providing insight into

mechanisms of aging in these simple eukaryotes. Comparative analysis of longevity-determining ortholog pairs has identified several genes worthy of further testing in mice, and future efforts will uncover additional candidates. It is our hope that this approach will provide an improved understanding which genes and pathways are likely to be relevant targets for intervention into aging and age-associated disease in humans.

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