RESEARCH ARTICLE

SIR2 and other genes are abundantly expressed in long-lived natural segregants for replicative aging of the budding yeast *Saccharomyces cerevisiae*

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Abstract

We investigated the mechanism underlying the natural variation in longevity within natural populations using the model budding yeast, *Saccharomyces cerevisiae*. We analyzed whole-genome gene expression in four progeny of a natural *S. cerevisiae* strain that display differential replicative aging. Genes with different expression levels in short- and long-lived strains were classified disproportionately into metabolism, transport, development, transcription or cell cycle, and organelle organization (mitochondrial, chromosomal, and cytoskeletal). With several independent validating experiments, we detected 15 genes with consistent differential expression levels between the long- and the short-lived progeny. Among those 15, *SIR2*, *HSP30*, and *TIM17* were upregulated in long-lived strains, which is consistent with the known effects of gene silencing, stress response, and mitochondrial function on aging. The link between *SIR2* and yeast natural life span variation offers some intriguing ties to the allelic association of the human homolog *SIRT1* to visceral obesity and metabolic response to lifestyle intervention.

Introduction

Aging, defined as a decline in performance and fitness over time (Finch, 1990), is a universal feature of organisms. Understanding why and how aging occurs, however, is a long-standing problem in biology. Until the 1990s, almost all researchers believed that the aging process was too complex to find treatments to slow it. Yet, in the past two decades, individual genes with allelic variants that dramatically extended the life span of laboratory organisms have been described (Kenyon *et al.*, 1993; Sinclair & Guarente, 1997; Anderson *et al.*, 2003; Echchgadda *et al.*, 2004; Burzynski, 2005; Partridge & Gems, 2006), leading to a paradigm shift in understanding the mechanisms of aging. Several mechanisms of aging have been suggested, including stress-resistance mechanisms, i.e. oxidative stress and heat stress resistance (Raha & Robinson, 2000; Thompson, 2006; Thompson *et al.*, 2006), the hormone insulin/IGF-1 pathway and calorie restriction (Hughes & Reynolds, 2005). Additionally, other molecular mechanisms, such as telomere shortening (Baxter *et al.*, 2004; Ishii *et al.*, 2006) and decreased ubiquitin-proteasome activity (Bulteau *et al.*, 2002; Ponnappan, 2002), may also contribute to aging. Despite these advances, most of the fundamental underpinnings of aging and longevity determination still remain elusive. For example, how does caloric restriction slow the aging process? How and when are the genes that govern aging regulated? Of the genes currently isolated, only a few are known as longevity- and aging-related loci, suggesting that many of the genes that affect aging have not yet been described. Systematic isolation of relevant loci in model organisms such as yeasts, nematodes and fruit flies is just beginning.

Experiments using transgenics and life span mutants have been the principal means of investigating mechanistic theories of aging (Bordone & Guarente, 2005; Kenyon, 2005; Hekimi, 2006; Longo & Kennedy, 2006; Braeckman & Vanfleteren, 2007; Kaeberlein *et al.*, 2007; Madia *et al.*, 2007; Tatar, 2009), and yet these experiments have not addressed the mechanisms that underlie naturally occurring variation in aging (Hughes & Reynolds, 2005), leading to two related questions. First, which of the mechanisms that can increase life span have been exploited by evolution to create naturally long-lived forms? Second, which genes and

pathways are responsible for the life span variation existing within natural populations? It is possible that the genes identified by mutant screens and transgenic studies that affect aging do not account for the life span variation that exists in natural populations. Because the identified genes have large phenotype effects, and because most seem to have deleterious effects on fertility, they might be subject to strong natural selection that tends to remove variation. Thus, identifying the genes or pathways that underlie natural variation in life span holds great promise for understanding the plasticity of aging.

The budding yeast *Saccharomyces cerevisiae* has been one of the most valuable model systems in the field of aging (Mortimer & Johnston, 1959; Gershon & Gershon, 2000; Sinclair, 2002; Piper, 2006). Indeed, the life span of yeast cells can be studied from two perspectives. Replicative aging is measured by counting the number of daughter cells that can arise from any given mother cell (Kennedy *et al.*, 1994; Kennedy & Guarente, 1996). Chronological aging is measured by quantifying the number of cells that remain viable in the stationary phase following nutrient deprivation (Fabrizio & Longo, 2007). Over the past 10 years, considerable progress has been made in the yeast aging field, and >20 longevity genes have been identified (Kaeberlein *et al.*, 2005). Homologs of at least two yeast genes have been shown to extend life span in *Caenorhabditis elegans* and *Drosophila melanogaster* (Lin *et al.*, 2001; Traven *et al.*, 2001; Sinclair, 2002; Hamet & Tremblay, 2003; Laun *et al.*, 2005; Sinclair & Guarente, 2006). Given the dearth of research on natural variation in aging, we focused on identifying the mechanisms of replicative aging relevant to natural populations. DNA sequence variation and consequent differential gene expression are major sources of naturally occurring variation in aging (Roy *et al.*, 2002). Measurement of gene expression via microarray technology has proved to be a powerful tool to investigate the biology of aging (Lu *et al.*, 2004; Yoon *et al.*, 2004; Han & Hickey, 2005; Laun *et al.*, 2005; Zahn *et al.*, 2006). Here, we performed a genome-wide transcriptional profiling of yeast strains with differential life spans, providing an insight into the regulation of gene expression and pathways related to the natural variation of aging.

Materials and methods

Yeast strains and phenotyping procedures

We used one tetrad of entirely homozygous diploid offspring strains from the parental vineyard isolate M5 with a met15::kanMX+/− marker introduced (Qin & Lu, 2006; Qin *et al.*, 2008). The parental heterozygous MET15/met15 marker becomes homozygous in the progeny, which renders the colonies either white (MET15+/+) or black (met15−/−) when they grow on lead-containing media. Hence, the four spores are named 2aW, 2bB, 2cW, and 2dB (W for white and B for black). Replicative life spans (RLSs) and chronological life spans (CLSs) were measured in the same protocols as described previously (Qin & Lu, 2006).

Tolerances to hydrogen peroxide were measured using a modified halo assay procedure (Machado *et al.*, 1997; Hacioglu *et al.*, 2010). Yeast strains were grown to the same OD values and then spread onto regular YPD plates. The plates were poured with the same volumes of media to ensure even diffusion of hydrogen peroxide in all samples. Drops of hydrogen peroxide solutions (5 μL) were added to the center of each plate. We used 30%, 15%, 5%, 2%, and 0.5% (wt% in water) hydrogen peroxide (Sigma-Aldrich, St. Louis, MO). The plates were air-dried and then moved to a 30 °C incubator. The next day, the diameters of halo rings were measured, and area was calculated assuming colonies under the visibly reasonable assumption were perfect circles. The largest halo ring in the 30% hydrogen peroxide treatment was viewed as 100% death of cells, and areas of other halo rings were normalized by the area of the largest ring. The viability of each treatment was calculated as 1 − [area/(the largest area)]. Linear regressions were then used to examine whether there are significant differences in diameters, areas or viabilities among strains.

DNA microarray construction

A set of clones containing 6225 verified ORFs from the *Saccharomyces* Genome Project was obtained from Research Genetics (Huntsville, AL), and amplified by PCR. The DNA was spotted on CMT-GAPS γ-aminopolysilane-coated glass slides (Corning, Corning, NY), using a microarraying robot with a 16-pin head (as in Townsend *et al.*, 2003).

mRNA samples

Yeast cells were cultured in 750 mL of YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) at 30 °C, shaking at 225 r.p.m., until the log phase ($OD_{600\,nm} = 0.8$). The cells were harvested by centrifugation and flash frozen in liquid nitrogen. Total RNA was extracted with hot acidic phenol/chloroform. The yield ranged from 5.5 to 6.0 mg, with an $A_{260\,nm}/A_{280\,nm}$ ratio of 2.0 ± 0.1. Pellets were stored frozen at −80 °C. The mRNA was purified from total RNA using the Oligotex Direct mRNA kit (Qiagen, Valencia, CA). Two micrograms of purified mRNA were used for reverse transcription, including 0.5 μg oligo(dT) primer. The resulting cDNA was labeled reciprocally with cyanine dyes and used for hybridization (as in Townsend *et al.*, 2003). All samples were competitively hybridized against deposited whole ORFs. Including dye-swaps, there were 12 hybridizations.

Data acquisition and analysis

Hybridized microarray slides were scanned using a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA) using the GENEPIX 4000 software package to locate spots in the microarray, and normalized by background-subtracted mean-by-mean normalization of well-measured spots (see Townsend *et al.*, 2003). Fluorescence intensity values were adjusted by subtracting background from foreground. A gene was considered well measured if the foreground fluorescence signals were higher than the background by 3 SDs of the distribution of intensities of the background pixels for that gene. The normalized data were analyzed using a Bayesian analysis of gene expression level (BAGEL; see Townsend, 2004). BAGEL is robust to the selective absence of data for a gene due to the low signal in a particular hybridization, reconciling the ratios observed between multiple samples to produce estimates and credible intervals for gene expression levels. Genes were considered significantly differentially expressed when $P \leq 0.05$.

Functional annotation

Functional annotation of genes with significant differential expression was performed using Gene Ontology (GO) (Ashburner *et al.*, 2000; Kanehisa *et al.*, 2004) and the Kyoto Encyclopedia of Genes and Genomes (KEGG). The significance of abundantly or meagerly expressed gene sets was calculated using Fisher's exact tests (as in Grosu *et al.*, 2002).

Real-time quantitative PCR analysis

In order to validate microarray results, five genes differentially expressed in four yeast strains differing in longevity were selected (FIT2, HSP30, MRP21, PRM7, and SIR2). Their expression level was analyzed by real-time quantitative reverse transcriptase (qRT)-PCR. An equal amount (2 μg) of mRNA, as had been used for microarray profiling, was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Primer pairs (see Supporting Information) were designed to correspond to each gene. The relative transcript abundance was measured using Applied Biosystems 7500 Fast Real-Time PCR System and SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) according to the manufacturer's recommendations. Transcript levels were calculated from triplicates within one plate using the comparative C_t method (ABI application notes, Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR). A housekeeping gene coding for δ-1-pyrroline-5-carboxylate dehydrogenase (PUT2) was used as the endogenous RNA and cDNA quantity control. The expression of PUT2 in all the strains studied was stable in the microarray experiment.

Complementary microarray analysis

To further validate our results, two complementary microarray experiments were performed. In the first experiment, primers were designed for 10 genes selected based on the results of the primary transcript profiling experiment (FIT2, FRE1, HOR2, HSP30, MRP21, PRM7, PRP11, SAP30, SIR2, and YPS3). Reverse transcription was performed as outlined above with a mixture of 10 primer sets. cDNA from two strains (short2 and long2) was competitively hybridized to the array with a dye-swap. In the second experiment, mRNA from both short-lived strains (short1 and short2) was pooled, reverse-transcribed, and labeled, and mRNA from both long-lived strains (long1 and long2) was pooled, reverse-transcribed, and labeled. Including two technical replicates originating from independent reverse transcription reactions and dye-swaps, there were four hybridizations. For both of these experiments, the experimental procedure and data analysis were otherwise the same as that described above.

Results

Life spans of yeast strains

The RLS assay revealed a considerable amount of RLS variation among offspring. A total of 50 cells in three experiments were assayed for each strain on YPD with 2% glucose. The mean average RLSs of the progeny were $2aW = 27.0 \pm 6.1$ (strain short1), $2bB = 25.6 \pm 3.1$ (short2), $2cW = 42.3 \pm 2.9$ (long1), and $2dB = 47.7 \pm 2.9$ (long2) cell divisions, respectively (Fig. 1a). This segregation pattern shows that *met15* deletion is not associated with life span segregation. The CLSs of these strains were measured by monitoring changes of CFU over time (Qin & Lu, 2006), and no significant differences were detected in these four spores (Fig. 1b).

Tolerances to hydrogen peroxide were measured using the halo ring procedure (detailed in Materials and methods). No significant difference was detected in the responses to hydrogen peroxide among the four spores (Fig. 1c). In addition, no differences in growth curves were observed when they were grown in liquid YPD with 2% glucose, which confirmed that the *met15* deletion does affect the overall health of cells in rich media.

Transcript profiling of short- and long-lived *S. cerevisiae* strains

A conservative criterion of non-overlapping 95% credible intervals in the Bayesian analysis was used to distinguish genes significantly differentially expressed between four yeast strains differing in life span. Overall, the difference in gene expression between the strains was relatively small, and

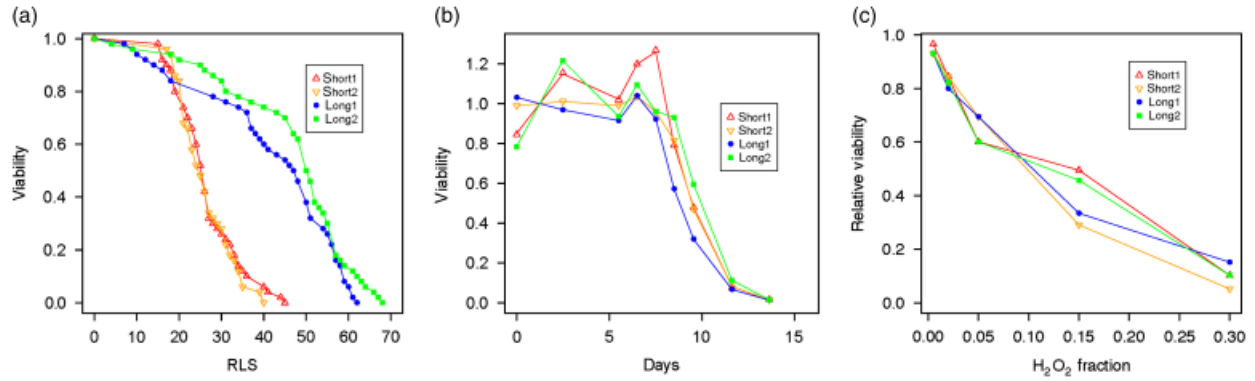

Fig. 1. The survival curves of the four segregants from a tetrad in this study. (a) RLS, measured on standard YPD plates with 2% glucose. (b) CLSss, measured in water, followed by plating assays. (c) Relative viability of the four segregants in response to exposure to H_2O_2.

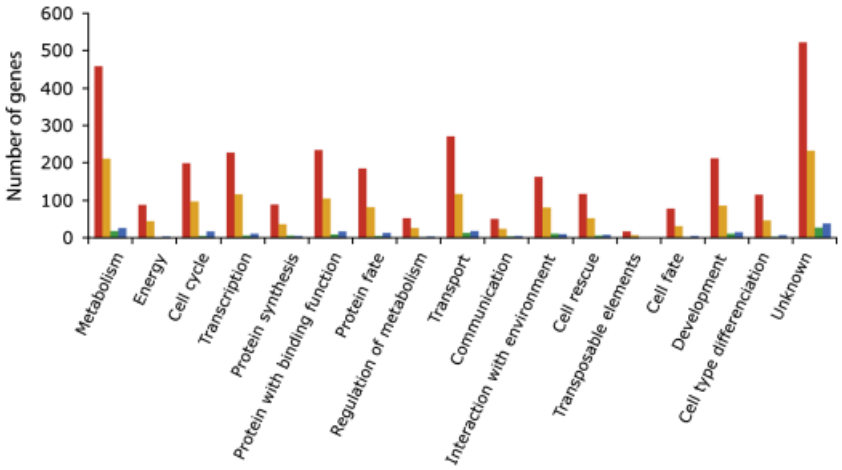

Fig. 2. Functional classification of genes differentially expressed between short- and long-lived yeast strains identified in an individual microarray profiling experiment according to three different criteria: (1) gene expression level different in at least one strain between short- and long-lived strains (orange); (2) gene expression level different in both long-/short-lived yeast strains compared with at least one short-/long-lived strain (yellow); (3) gene expression level different in both short-lived strains comparing with both long-lived strains (green) and in a microarray experiments with pooled RNA from short- or long-lived strains (blue).

many statistically significant expression changes were less than twofold. We used three different criteria to identify the genes potentially involved in longevity control: (1) gene expression level different in at least one strain between short- and long-lived strains; (2) gene expression level different in both long-/short-lived yeast strains compared with at least one short-/long-lived strain; and (3) gene expression level different in both short-lived strains compared with both long-lived strains. The numbers of genes significantly differentially expressed according to these three criteria were 1842, 842s and 82, respectively.

Functional classification of genes differentially expressed by yeast strains differing in life span

The highest number of genes with expression level different in short- and long-lived strains was that of genes classified as unknown, responsible for metabolism, transport, development, transcription, or cell cycle (Fig. 2). The functional category of cell cycle was identified as significantly different between strains naturally segregating for life span (Fisher's exact test, $P \leq 0.02$). The encoded gene products were predominantly involved in the G1–S phase transition or active in the M phase of the cell cycle (Fig. 3). Of the 82 genes significantly differentially expressed between both short- and long-lived yeast strains, 49 were abundantly expressed and 26 were meagerly expressed in both long-lived strains. The other seven significantly differentially expressed genes demonstrated inconsistent differential expression within long- and short-lived strains. Most of the genes abundantly expressed in long-lived yeast strains were involved in transport, organelle organization (mitochondrial, chromosomal, and cytoskeletal), and translation (Table 1).

Real-time qPCR validation of transcript profiling

To validate the microarray results, the expression patterns of five genes were characterized by real-time qPCR. The expression levels recorded by real-time RT-PCR were usually higher, but otherwise consistent with the microarray data (Table 2). For example, in the array, gene expression levels for heat shock protein 30 (HSP30) were determined to be 1.00, 1.07, 1.19, and 1.86 for both short and long-lived

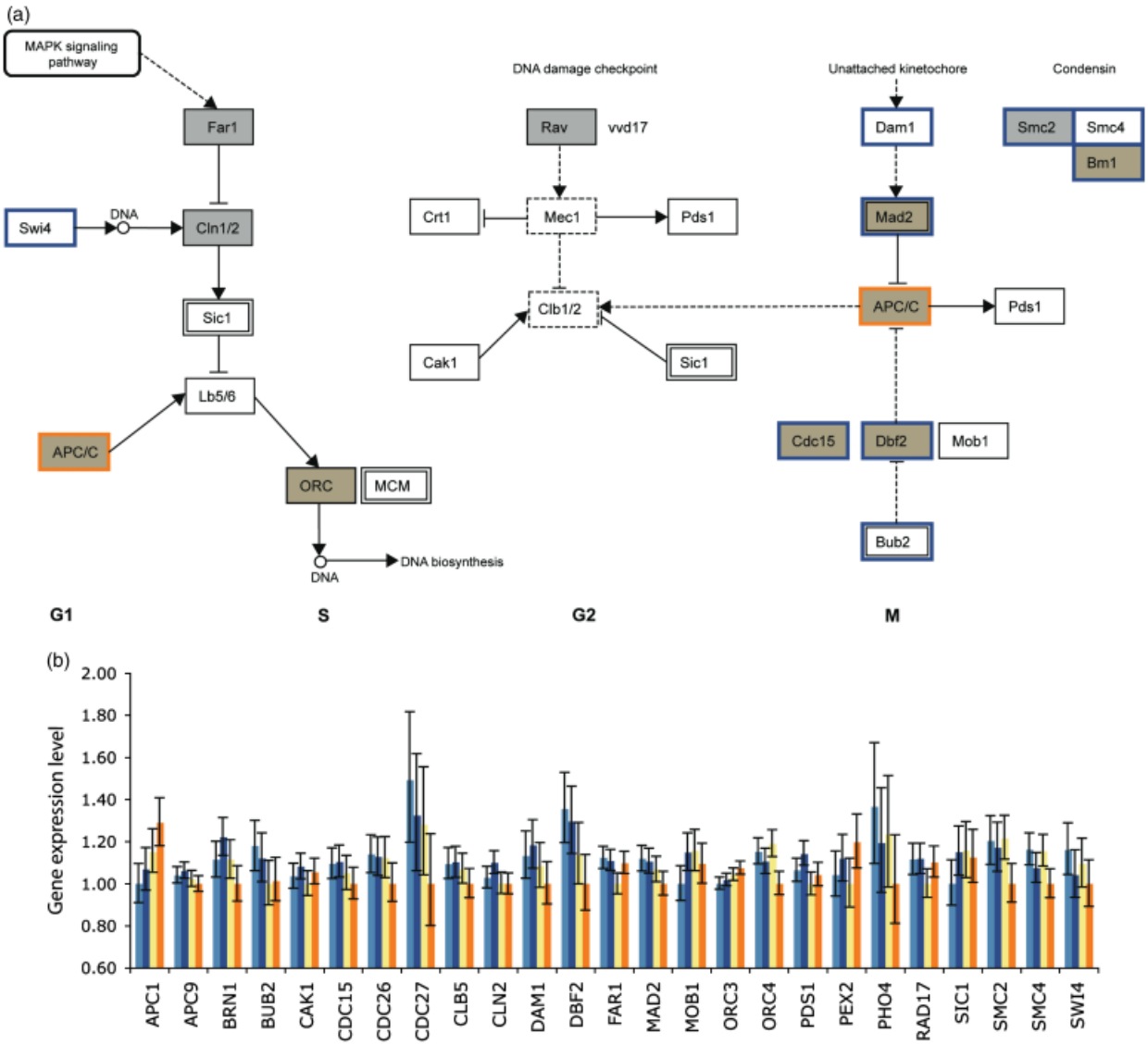

Fig. 3. The functional category of cell cycle was identified as significantly different between strains naturally segregating for life span. (a) The encoded gene products were predominantly involved in the G1–S phase transition or active in the M phase of the cell cycle (single line box, gene expression level different in at least one strain between short- and long-lived strains; gray box, gene expression level different in both long-/short-lived yeast strains compared with at least one short-/long-lived strain (gray box); double line box, gene expression level different in pooled short-lived strains compared with pooled long-lived strains. (b) Expression level of genes classified as cell cycle control related significantly differentially expressed in long-lived (blue and purple) and short-lived (yellow and orange) yeast strains. APC1, YNL172W, anaphase-promoting complex component APC1; APC9, YLR102C, anaphase-promoting complex component APC9; BRN1, YBL097W, condensin complex subunit 2; BUB2, YMR055C, cell cycle arrest protein BUB2; CAK1, YFL029C, serine/threonine-protein kinase; CDC15, YAR019C, cell division control protein; CDC26, YFR036W, anaphase-promoting complex component APC12; CDC27, YBL084C, anaphase-promoting complex component APC3; CLB5, YPR120C, B-type cyclin; CLN2, YPL256C, G1/S-specific cyclin CLN1/2; DAM1, YGR113W, DASH complex subunit DAM1; DBF2, YGR092W, cell cycle protein kinase DBF2 [EC:2.7.11.-]; FAR1, YJL157C, cyclin-dependent kinase inhibitor; MAD2, YJL030W, component of the spindle-assembly checkpoint complex; MCM2, YBL023C, minichromosome maintenance protein 2; MOB1, YIL106W, component of the mitotic exit network; ORC, YLL004W, origin recognition complex subunit 3; ORC, YPR162C, origin recognition complex subunit 4; PDS1, YDR113C, cell cycle regulator; PEX2, YJL210W, peroxisomal biogenesis factor 2; PHO4, YFR034C, transcription factor of the myc-family; RAD17, YOR368W, checkpoint protein; SIC1, YLR079W, inhibitor of Cdc28-Clb kinase; SMC2, YFR031C, component of the condensin complex; SMC4, YLR086W, structural maintenance of chromosome 4; SWI4, YER111C, DNA binding component of the SBF complex.

strains, respectively, with corresponding values of 1.00, 2.12, 2.78, and 6.06 in the qPCR experiment. Also, the expression of mitochondrial ribosomal protein (MRP21), which was similar in the four yeast strains in the microarray experiment, was confirmed by qPCR to be on a comparable level in short- and long-lived strains.

Table 1. Genes abundantly or meagerly expressed by long-lived *Saccharomyces cerevisiae* strains statistically significantly different from both short-lived strains

Gene ontology term	Abundant	Meager
Transport	*TIM12, RER1, TIM17, COY1, PAP1, FRE1, CTR3, TRS33, FRE3, FIT2, CTR1*	*GYP7, TPO2, SEE1, SFB2*
Organelle organization	*MRP21, TIM12, SIR2, RSM27, TIM17, PFD1, PET191, YKU80, MRPL23*	*LPX1*
Translation	*MRP21, RSM27, RPS30A, MRPL23*	*SUI2, RPL6B*
RNA metabolic process	*POP8, SIR2, PAP1, YKU80*	*HAC1*
Response to chemical stimulus	*CSN9, MET14, YLR346C*	*PRM7, HAC1*
Vesicle-mediated transport	*RER1, COY1, TRS33*	*GYP7, SEE1, SFB2*
Ribosome biogenesis	*POP8, MRPL23*	*RPL6B*
Carbohydrate metabolic process	*ICL1, MLS1*	*KRE2, HOR2*
Transcription	*SIR2, YKU80*	*HAC1*
Signal transduction	*CSN9, LCB3*	*HAC1, CMK2, RGS2*
Response to stress	*SIR2, YKU80*	*HSP30, HOR2, HAC1*
DNA metabolic process	*SIR2, YKU80*	
Cytoskeleton organization	*PFD1*	
Protein catabolic process	*ADD37*	
Lipid metabolic process	*LCB3*	*HAC1*
Protein folding	*PFD1*	
Cell wall organization	*PIR3*	*YLR194C*
Protein modification process	*CSN9*	*KRE2, CMK2*
Conjugation	*CSN9*	*PRM7*
Cellular homeostasis	FRE3	
Cellular amino acid and derivative metabolic process	*MET14*	*MAE1*
Membrane organization	*TIM12*	
Generation of precursor metabolites and energy		*HOR2*
Other	*ATP23, MCM16*	*PAC10*
Biological process unknown	*YAL037W, AIM1, YAR066W, YBR099C, LDB16, HVG1, YER076C, YHR214W, YIL060W, YIL169C, YAE1, YLR101C, YLR408C, AIM32, YMR144W, YOL106W, AIM42, YPL039W, YPL216W*	*YDL241W, YEL074W, YFL032W, YFL051C, GAS3, AIM38, YOR385W, YPL014W, CMR3*

Table 2. Gene expression levels in four *Saccharomyces cerevisiae* strains differing in longevity determined by microarray and qPCR

		Strain			
Gene name	Measurement	long1	long2	short1	short2
FIT2	Microarray	1.21	1.17	1.00	1.04
	qPCR	1.63	1.41	1.00	1.12
HSP30	Microarray	1.00	1.07	1.19	1.86
	qPCR	1.00	2.12	2.78	6.06
MRP21	Microarray	1.16	1.17	1.06	1.00
	qPCR	1.22	1.07	1.04	1.00
PRM7	Microarray	1.03	1.00	1.21	1.43
	qPCR	1.15	1.00	1.54	1.81
SIR2	Microarray	1.86	1.31	1.11	1.00
	qPCR	2.95	1.40	1.22	1.00

Table 3. Comparison of gene expression ratio in long2 and short2 *Saccharomyces cerevisiae* strains determined by transcript profiling of complete mRNA and profiling with gene-specific primers

Gene name	Whole transcriptome analysis	Gene-specific microarray
FIT2	1.13	2.00
FRE1	1.21	1.04
HOR2	− 1.18	− 1.37
HSP30	− 1.73	− 2.47
MRP21	1.17	− 1.09
PRM7	− 1.43	− 1.85
PRP11	1.12	1.04
SIR2	1.31	1.23
YPS3	− 1.41	− 1.72

Complementary microarray experiments

To further validate microarray profiling of yeast strains differing in longevity, 10 gene-specific primer pairs were designed and used for reverse transcription. The resulting cDNA from a short- (short2) and a long- (long2) lived strain was labeled and hybridized. The results were compared with the oligo(dT)-primed whole transcriptome analysis (Table 3). Eight genes (FIT2, FRE1, HOR2, HSP30, PRM7, PRP11, SIR2, and YPS3) had gene expression ratios that were similar in both approaches; one (MFP21) had a higher expression in the long-lived strain compared with the short-lived strain according to the oligo(dT)-based

Table 4. Comparison of microarray results with profiling individual strains and profiling combined long- or short-lived strains

Gene ID	Gene name	long1	long2	short1	short2	Long pool	Short pool
YCR021C	*HSP30*	1.00	1.07	1.19	1.86	1.00	1.38
YDL039C	*PRM7*	1.03	1.00	1.21	1.43	1.00	1.46
YDL042C	*SIR2*	1.89	1.31	1.11	1.00	1.39	1.00
YEL074W	Unknown	1.02	1.00	1.19	1.30	1.00	1.27
YJL143W	*TIM17*	1.09	1.10	1.01	1.00	1.14	1.00
YLR194C	Unknown	1.00	1.08	1.27	1.43	1.00	1.30
YLR214W	*FRE1*	1.17	1.22	1.00	1.00	1.19	1.00
YLR408C	Unknown	1.25	1.22	1.11	1.00	1.11	1.00
YLR411W	*CTR3*	1.18	1.26	1.00	1.02	1.25	1.00
YMR215W	*GAS3*	1.00	1.08	1.15	1.27	1.00	1.16
YNR018W	Unknown	1.00	1.01	1.17	1.12	1.00	1.17
YNR020C	Unknown	1.09	1.10	1.02	1.00	1.11	1.00
YOL016C	*CMK2*	1.00	1.09	1.21	1.52	1.00	1.26
YOR084W	Unknown	1.00	1.07	1.18	1.19	1.00	1.21
YPL014W	Unknown	1.00	1.06	1.18	1.55	1.00	1.33

whole-genome transcriptional profiling and a lower expression according to the gene-specific priming. Lastly, one gene (SAP30) was not well measured in the gene-specific-primed experiment and could not be compared with the whole transcriptome analysis.

Next, RNA from both short-lived yeast strains was pooled together and used for competitive hybridization with similarly pooled long-lived strains. Statistical analysis of the pooled experiment identified 127 genes significantly differentially expressed by yeast strains differing in longevity. Fifteen out of these genes were also identified by the primary whole transcriptome profiling as significantly different between both short- and long-lived strains. Although statistical significance varied between the complementary experiments, the expression levels determined by the two approaches were highly consistent (Table 4).

Discussion

Most studies of aging in yeast and other model organisms including *Drosophila* and *C. elegans* have used transgenic approaches. Here, we improved the understanding of gene expression involved in the natural variation of longevity by profiling whole-genome gene expression in young cells among four progeny from a natural yeast strain with differences in RLS. We used two microarray experimental approaches and strongly identified 15 genes with significant differential expression between long- and short-lived spores of a single tetrad in *S. cerevisiae*. The changes of expression levels in these natural variants are moderate (~20%) in comparison with those based on the comparison between gene deletions and control lab strains (up to twofold or more; see Hughes *et al.*, 2000). Because the four progeny cultures examined are primarily young cells and derive from the same diploid natural strain, their genetic backgrounds are similar and the causal differences in expression that underlie differential aging as lifespan proceeds are likely to be moderate. Nevertheless, the segregating differences allowed us to examine the segregation of effects of genes that are particularly related to the segregating difference in life span. To address concerns regarding the robustness of our measurements of these small changes, we carried out several independent validation approaches including real-time qPCR and two additional complementary microarray experiments. These results validated the main microarray experiment.

A conspicuous candidate out of the 15 genes we identified was *Sir2*, the silent information regulator 2 gene. SIR2 is a histone deacetylase, highly conserved from yeast to human, and a member of the sirtuin family. It is involved in the epigenetic regulation of gene expression, control of various metabolic processes, response to stress and, most importantly, life span (Sinclair & Guarente, 1997; Sinclair & Guarente, 2006). Previous studies suggest that *Sir2*-deletion strains have a short life span, and strains with an extra copy of *Sir2* have super-extended life spans (Kaeberlein *et al.*, 1999). An elevated ratio of NAD^+/NADH and clearance of nicotinamide can both enhance Sir2 activity (Gallo *et al.*, 2004; Reverter-Branchat *et al.*, 2007; Lu & Lin, 2009). In this report, the *Sir2* gene expression level observed was not linearly correlated with aging, but was abundantly expressed in both long-lived strains compared with the short-lived strains, suggesting cosegregation with the long-lived phenotype (Table 2). The difference in expression between the segregating phenotypes was larger than for most genes, suggesting that the *Sir2* gene plays a key role in the early mechanisms governing yeast life span.

Interestingly, the functional category of cell cycle was identified as significantly different between long- and short-lived segregants. Many of the differentially expressed genes in this category are predominantly involved in the G1–S phase transition or active in the M phase of the cell cycle. RLS is the number of cell cycles that mother cells can go through. In addition, gene silencing requires progression of cell cycle and the function of SIR2 is cell-cycle dependent (Matecic *et al.*, 2006). Hence, this finding implies that the long-lived strain may increase their life span through cell cycle-related mechanisms.

Our results on the role of *Sir2* in yeast life span are consistent with previous reports that variation of the human homolog *Sirt1* has been associated with age-related diseases. Human carriers of single-nucleotide polymorphism (SNP) rs3758391 in *Sirt1* tend to have a lower cardiovascular mortality rate (Kuningas *et al.*, 2007). Two other SNPs in *Sirt1*, rs7069102 and rs3818292, are associated with increased waist circumference and visceral fat area in obese males (Peeters *et al.*, 2008). Another SNP of *Sirt1*,

rs12413112, is associated with metabolic responses to a 9-month course of calorie restriction and increased physical activity (Weyrich *et al.*, 2008).

Several other candidate genes also offer plausible explanations for the observed variation in natural life span. TIM17 is essential for mitochondrial function and is responsible for transporting protein components of the respiratory process into the mitochondria (Neupert & Herrmann, 2007). Higher expression of TIM17 may suggest a more efficient respiratory process and hence less accumulation of reactive oxygen species in the long-lived segregants. CMK2 (calmodulin-dependent protein kinase) is predicted to be involved in stress (Ohya *et al.*, 1991), and CTR3 (copper transporter) or FRE1 (ferric/cupric reductase) regulates copper ion uptake and may in turn regulate both copper concentration and activity of superoxide dismutase (SOD1) (Georgatsou & Alexandraki, 1999; Pena *et al.*, 2000). HSP30 is a membrane heat-shock protein that can be induced by low glucose levels and ethanol stress (Piper, 1995). Higher expression levels of CMK2, CTR3, FRE1, HSP30, and TIM17 in long-lived segregants are consistent with the known effect of stress response on life span (Guarente & Kenyon, 2000; Sinclair *et al.*, 2006) and/or the mitochondrial model of cellular aging (e.g. Ugidos *et al.*). It is also plausible that post-transcriptional regulation causes the observed life span segregation. These leads could provide guidance during our search for quantitative trait loci.

In addition to gene silencing, cell cycle, mitochondrial function, and stress responses, our functional classification recognized many other pathways that are associated with the segregation of an RLS: metabolism, transport, development, transcription, chromosomal and cytoskeletal organization, and translation. Hence, a network of pathways related to aging has emerged from our comparative gene profiling study of the long- and short-lived segregants.

Our study may be compared with a recent expression profiling study using different yeast mutants (Wei *et al.*, 2009). Wei and colleagues found 63 upregulated genes and 25 downregulated genes shared in *sch9*Δ, *ras2*Δ, and *tor1*Δ mutants that have extended CLSs. None of the 15 genes that we found overlaps with the genes identified in that report. No significant correlation was found between the long/short ratio in our data and those measured by Wei *et al.* (2009). Hence, the observed natural variation here is unlikely to be related to the pathways of Sch9, Tor1, or Ras2, highlighting the importance of studying natural variation. This lack of overlap is consistent with the argument that SIR2 and TOR pathways act in parallel on life span. Notice that the change of life span in the studied tetrad is ∼50%, a change comparable to the effect on the life span of *tor1*Δ (Wei *et al.*, 2009), despite the relatively moderate changes in gene expression among the four progeny. This significant change in life span demonstrates that drastic variation of gene expression levels is not required to produce large changes in the life span of *S. cerevisiae*. Because many active pathways are different between vegetative growth and the stationary phase, it is likely many pathways are different between replicative aging and chronological aging, as argued by Laun *et al.* (2006). Interestingly, both studies show that metabolic pathways are associated with changes in life span despite the likely different underlying genetic causes of the phenotypic differences. This convergence upon metabolic phenomena is perhaps not surprising, given that calorie restriction works universally on all organisms.

Genetic variation among yeast strains isolated from vineyards and a woodland site can influence their RLS and CLS as much as twofold and fivefold, respectively (Qin & Lu, 2006; Qin *et al.*, 2008). There are also significant differences of both RLSs and CLSs between wild isolates and typical lab strains (Qin & Lu, 2006). For example, one allele of the gene SSD1 was found to confer longevity in connection with the TOR complex and in a SIR2-independent manner (Kaeberlein *et al.*, 2004b; Reinke *et al.*, 2004). In this case, the short-lived allele is recessive, and is suggested to be a null allele.

Notably, we measured gene expression in log-phase cells, which are primarily young cells, instead of examining old cells to look for causal factors of aging. Our rationale for this experimental design comes from temporal causality or Granger causality (Granger, 1969). Its application in biology can be found in many studies (Barrett *et al.*, 2010; Bressler & Seth, 2010; Fujita *et al.*, 2010; Kokal & Keysers, 2010; Sato *et al.*, 2010; Seth, 2010), including inferring causal relations from time series of gene expression data (Nagarajan & Upreti, 2010; Shojaie & Michailidis, 2010). In essence, because aging is a temporal process, we would expect causal events of aging should be found before the catastrophic decline of viability. Expression differences in surviving old cells would most likely be consequences rather than causes of aging. Further justifying this experimental design for the problem of aging, young cells have previously been assayed to discover causal expression changes for aging, and meaningful results were obtained (Lin *et al.*, 2002; Kaeberlein *et al.*, 2004b; Yiu *et al.*, 2008; Aerts *et al.*, 2009).

The present links between expression changes and life span variation are only associations regarding genes expressed in very young cells that are destined to later display differences in RLS. With this caveat in mind, it should be noted that expression profiling on replicatively aged cells, such as those enriched by a flow cytometer (Chen & Contreras, 2004), may yield different and possibly more informative results, especially with regard to the downstream determinants of aging discrepancies. We are phenotyping more segregates, and plan to identify the underlying sequence changes using a deep sequencing method to further investigate this interesting segregation of life span in a wild isolate of yeast.

Variation among yeast lab strains has contributed to some controversies surrounding the role of SIR2 in yeast. In the W303 background, SIR2 was required for life span extension by calorie restriction, whereas in the S288C background, SIR2 was found to act in parallel with the CR pathway (Kaeberlein *et al*., 2004a). These differences are often treated as experimental problems instead of inferential opportunities. Although the study of model organisms has revealed much regarding the genetic mechanisms of aging and life span, it is still unclear whether these potential mechanisms account for the natural variation in populations (Kuningas *et al*., 2008). In other words, the value of genotypic variation in yeast has not been sufficiently utilized in the field of yeast aging. It is plausible that life span is regulated through the myriad gene interaction networks that include both SIR2 and TOR. This network perspective on aging is consistent with the recent conception that gene interaction work is the key to understanding the connection between genotypic variation and phenotypic difference (Benfey & Mitchell-Olds, 2008). Hence, variation of life span in yeast offers an appealing opportunity to infer the intricacies of gene interaction networks underlying life span plasticity. The present work identifies the genes and pathways related to the natural variation of RLS in yeast. Further studies are needed to investigate the function of particular pathways or genes identified that vary in expression between individuals with differing life spans.

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Authors' contribution

Z.G. and A.B.A. contributed equally to this research. Experiments were conceived by H.Q. and J.P.T. H.Q. provided strains and performed RLS assays. Z.G. performed microarray hybridizations and qPCR experiments supervised by J.P.T. E.D.J. performed phenotyping supervised by H.Q. A.B.A. analyzed expression data supervised by J.P.T. Z.G., A.B.A., H.Q., and J.P.T. wrote the manuscript.

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