Natural genetic variation in yeast longevity

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The genetics of aging in the yeast Saccharomyces cerevisiae involves the manipulation of individual genes in laboratory strains. We have instituted a quantitative genetic analysis of the yeast replicative lifespan by sampling the natural genetic variation in a wild yeast isolate. Haploid segregants from a cross between a common laboratory strain (S288C) and a clinically derived strain (YM1445) were subjected to quantitative trait locus (QTL) analysis, using 3048 molecular markers across the genome. Five significant, replicative lifespan QTL were identified. Among them, QTL 1 on chromosome IV has the largest effect and contains SIR2, whose product differs by five amino acids in the parental strains. Reciprocal gene swap experiments showed that this gene is responsible for the majority of the effect of this QTL on lifespan. The QTL with the second-largest effect on longevity was QTL 5 on chromosome XII, and the bulk of the underlying genomic sequence contains multiple copies (100–150) of the rDNA. Substitution of the rDNA clusters of the parental strains indicated that they play a predominant role in the effect of this QTL on longevity. This effect does not appear to simply be a function of extrachromosomal ribosomal DNA circle production. The results support an interaction between SIR2 and the rDNA locus, which does not completely explain the effect of these loci on longevity. This study provides a glimpse of the complex genetic architecture of replicative lifespan in yeast and of the potential role of genetic variation hitherto unsampled in the laboratory.

[Supplemental material is available for this article.]

Aging is determined by genes, environment, and chance. The budding yeast Saccharomyces cerevisiae (baker’s yeast) has been used as a model organism in the genetics of aging and longevity since the early 1990s (Jazwinski 2004), and lifespan has been analyzed in two different ways in this organism. Replicative lifespan (RLS) refers to the number of times an individual cell divides, while chronological lifespan (CLS) denotes the time spent in stationary culture before a cell loses viability. The number of genes implicated in the RLS and in the CLS of yeast grows continuously (Steinkraus et al. 2008), and a search of the genome database reveals a substantial number of genes that compensate for this dysfunction (Liu and Butow 2006; Miceli et al. 2011). A comparison of four strains showed varying effects on RLS, spanning RLS extension through curtailment. The genetic architecture of replicative lifespan in yeast and of the potential role of genetic variation hitherto unsampled in the laboratory.

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genes. The question of what genes can affect lifespan is quite different from the question of what genes actually do normally affect lifespan. This is highlighted by the recent demonstration that 65% of the long-lived mutants in a laboratory strain showed reduced fitness relative to the wild type (Delaney et al. 2011). Similar results have been obtained in the *Caenorhabditis elegans* model system (Jenkins et al. 2004). The other major genetic models used in aging research, *Drosophila melanogaster* and *Mus musculus*, are also inbred and adapted to the laboratory, raising identical concerns, while the focus on natural genetic variation in human aging is an obvious contrast.

Natural yeast variation has been queried to identify quantitative trait loci (QTLs) and genes that play a role in heat resistance, multidrug resistance, ethanol tolerance, acetic acid production during alcoholic fermentation, gene expression, spontaneous mitochondrial genome instability, proteome variation, and telomere length (Winzeler et al. 1998; Steinmetz et al. 2002; Brem et al. 2005; Gatbonton et al. 2006; Foss et al. 2007; Hu et al. 2007; Marullo et al. 2007; Dimitrov et al. 2009). Most of these studies have utilized gene expression microarrays that can query several thousands of single-nucleotide polymorphisms across the yeast genome (Winzeler et al. 1998). Here, we have used this strategy to map QTLs associated with RLS in *S. cerevisiae*. We sampled the natural genetic variation in a clinically derived *S. cerevisiae* strain, YJM789, by analyzing haploid segregants generated from a cross with the standard laboratory strain S288c (Steinmetz et al. 2002). The genomic sequences of both YJM789 (Wei et al. 2007) and S288c (Goffeau et al. 1996) have been determined. YJM789 and S288c differ at 60,000 bp throughout their genomes.

### Results

#### Genetic variation in longevity genes

To investigate the role of natural genetic variation associated with longevity, we crossed the laboratory strain S96 (*MATa ho lys5*), a derive of S288c, and the strain YJM789 (*MATa ho::hisG lys2 cyh*), a haploid segregant of the homozygous diploid strain YJM145, which was derived from the clinical isolate YJM128 (Steinmetz et al. 2002). The S96 × YJM789 diploid was sporulated, and 54 tetrads were dissected into their four haploid spores. On germination, the resulting 216 haploid segregants were characterized. We established 39 of these haploid segregants, each from parental strain S96 (Table 1). This agrees with the positive results of the LOD score of the QTL from parental strain YJM789, as it is part of QTL 1.

| Strain  | mRLS  | QTL 1 | QTL 5 | Strain  | mRLS  | QTL 1 | QTL 5 |
|---------|-------|-------|-------|---------|-------|-------|-------|
| I-1A    | 50.0  | YJM789 | S96   | VI-1D   | 30.2  | S96   | S96   |
| II-5D   | 47.3  | YJM789 | S96   | V-4A    | 29.6  | S96   | S96   |
| V-6A    | 45.6  | YJM789 | S96   | III-5A  | 29.2  | S96   | S96   |
| I-3C    | 42.4  | YJM789 | S96   | III-9B  | 28.9  | YJM789| S96   |
| I-7D    | 40.4  | YJM789 | S96   | V-1A    | 28.4  | S96   | S96   |
| VI-4D   | 38.4  | YJM789 | S96   | II-3D   | 28.3  | S96   | S96   |
| III-2A  | 38.3  | YJM789 | S96   | III-4B  | 28.3  | S96   | YJM789|
| II-3A   | 37.0  | YJM789 | S96   | I-5B    | 28.2  | S96   | YJM789|
| II-1B   | 35.4  | YJM789 | S96   | II-2B   | 28.1  | S96   | S96   |
| VI-3C   | 34.3  | YJM789 | S96   | IV-1C   | 27.5  | S96   | YJM789|
| VI-8C   | 33.5  | YJM789 | S96   | IV-3A   | 27.0  | S96   | YJM789|
| II-9A   | 33.3  | YJM789 | S96   | IV-6A   | 25.4  | S96   | S96   |
| I-6D    | 32.9  | YJM789 | S96   | I-7B    | 25.2  | S96   | YJM789|
| VI-6B   | 32.6  | S96   | YJM789 | III-1B  | 24.6  | S96   | S96   |
| V-6B    | 31.7  | YJM789 | S96   | II-6C   | 22.3  | S96   | YJM789|
| IV-2C   | 31.3  | S96   | S96   | II-7C   | 21.6  | S96   | YJM789|
| III-8A  | 31.2  | YJM789 | S96   | II-8A   | 20.9  | S96   | S96   |
| III-7C  | 31.2  | YJM789 | S96   | III-6C  | 18.3  | S96   | S96   |
| VI-9B   | 31.1  | YJM789 | S96   | III-6C  | 30.7  | S96   | S96   |
| I-2C    | 30.8  | YJM789 | S96   | Diploid | 32.7  | both  | both  |
| III-3B  | 30.7  | S96   | S96   |         |       |       |       |

The 39 recombinant lines are haploid segregants from diploids generated by crossing strains S96 and YJM789. S96 and the S96 × YJM789 diploid are shown for comparison. All haploids are mating-type a (*MATa*) and can be micromanipulated, as can S96 and the diploid. Mean RLSs and alleles of QTL 1 and QTL 5 are indicated. The upper left gray box indicates the seven strains with the longest mRLS; the lower right gray box indicates the four strains with the shortest mRLS.

#### Table 1. Mean RLS of 41 lines/strains and the parental origin of the longevity QTL

| Strain  | mRLS  | QTL 1 | QTL 5 |
|---------|-------|-------|-------|
| I-1A    | 50.0  | YJM789 | S96   |
| II-5D   | 47.3  | YJM789 | S96   |
| V-6A    | 45.6  | YJM789 | S96   |
| I-3C    | 42.4  | YJM789 | S96   |
| I-7D    | 40.4  | YJM789 | S96   |
| VI-4D   | 38.4  | YJM789 | S96   |
| III-2A  | 38.3  | YJM789 | S96   |
| II-3A   | 37.0  | YJM789 | S96   |
| II-1B   | 35.4  | YJM789 | S96   |
| VI-3C   | 34.3  | YJM789 | S96   |
| VI-8C   | 33.5  | YJM789 | S96   |
| II-9A   | 33.3  | YJM789 | S96   |
| I-6D    | 32.9  | YJM789 | S96   |
| VI-6B   | 32.6  | S96   | YJM789 |
| V-6B    | 31.7  | YJM789 | S96   |
| IV-2C   | 31.3  | S96   | S96   |
| III-8A  | 31.2  | YJM789 | S96   |
| III-7C  | 31.2  | YJM789 | S96   |
| VI-9B   | 31.1  | YJM789 | S96   |
| I-2C    | 30.8  | YJM789 | S96   |
| III-3B  | 30.7  | S96   | S96   |

The QTL with the largest effect on RLS is located on chromosome IV (QTL 1, LOD score = 9.27) (Fig. 1A,B). It contains 12 genes within a region of 18 kbp (Fig. 2A). The 13 recombinant lines with the longest RLS contain QTL 1 from parental strain YJM789, whereas the seven strains with the shortest RLS contain QTL 1 from parental strain S96 (Table 1). This agrees with the positive signal in the lower graph of Figure 1B. The LOD score of the QTL located on chromosome XII (QTL 5) is 3.68 (Fig. 1A,C). It comprises >1 Mbp of DNA, including 23 protein-coding genes, one tRNA, some autonomously replicating sequence (ARS) consensus sequences (ACs), and the rDNA cluster (Fig. 2B).

**SIR2 is part of QTL 1**

One of the genes contained in QTL 1 is SIR2, a well-known regulator of RLS in laboratory strains of *S. cerevisiae* (Kaeverlein et al. 2005).
In S96 and YJM789, Sir2p has a length of 562 amino acids (AA) but differs in five AA: P65T, V92E, D158V, K320N, and M424V. None of the five altered AA possesses an assigned function (Garcia and Pillus 2002; for review see Sauve et al. 2006; Choy et al. 2011; Froyd and Rusche 2011). Nevertheless, SIR2 was chosen as a candidate for gene swap experiments. The SIR2 open reading frame (ORF) of strain S96 was substituted by the YJM789 allele between +75 and +1323. This part of the gene contains all five polymorphisms. We then compared the RLS of S96 and S96 possessing the SIR2 gene of YJM789 (S96 sir2Δ::SIR2[YJM789]). The mean RLS of S96 was 31.7 generations, and for S96 sir2Δ::SIR2 (YJM789) it was 46.9 generations, which is 48% longer (Fig. 3A).

We next conducted the reverse experiment. Strain YJM789 displays filamentous growth; it cannot be micromanipulated nor can its RLS be determined. Therefore, the recombinant line I-1A was used for another SIR2 ORF-swap experiment. I-1A has the longest mRLS (50.0 generations) of all analyzed recombinant lines; it has the YJM789 allele of QTL 1 (Table 1). We generated strain I-1A sir2Δ::SIR2(S96) by substituting the SIR2 (YJM789) by the S96 allele. Strains S96 and S96 sir2Δ::SIR2(YJM789) served as additional controls in the subsequent RLS determination. Strain I-1A had a mRLS of 49.5 generations, and its corresponding mutant I-1A sir2Δ::SIR2(S96) had a mRLS of 41.6, a decrease of 16% (Fig. 3B). S96 and S96 sir2Δ::SIR2(YJM789) had a mRLS of 30.7 and 43.7 generations, respectively, an increase of 42% in the latter strain in this experiment (Fig. 3B). These results were reproducible (data not shown) and in agreement with the previous experiment (Fig. 3A). From both RLS determinations, we conclude that one or more of the AA polymorphisms in SIR2 are responsible for the large differences in mRLS.

Figure 1. Composite interval mapping of RLS QTL. (A) The upper panel depicts all QTL in all 16 yeast chromosomes, each indicated by a roman numeral. (B) QTL 1, 2, and 3 on chromosome IV have LOD scores of 9.27, 5.02, and 2.60, respectively. (C) QTL 4 and 5 on chromosome XII have LOD scores of 2.70 and 3.68, respectively. In all panels, the top graphs show the LOD score (y-axis) versus genetic distance in centimorgans (x-axis). A LOD score above the genome-wide significance threshold of 2.5 is evidence for a QTL. The lower graphs show the "additive effect." Positive or negative values indicate that the parental YJM789 or S96 allele, respectively, is associated with longer RLS. The open and closed arrowheads in A indicate the positions of AMN1 and MATA on chromosomes II and III, respectively. The QTL are numbered in B and C.
The effect of the SIR2 allele on RLS was confirmed by reciprocal hemizygosity analysis (RHA) (Steinmetz et al. 2002). This was performed by generating diploid strains (S96 YJM789) in which either the S96 or the YJM789 SIR2 gene was deleted and replaced by the KanMX drug-resistance marker, which does not affect RLS. To examine the potential effect of copy number, an additional diploid that had two copies of the YJM789 SIR2, one of which replaced the S96 allele, was created. The hemizygous SIR2 allele from S96 (strain YFS90) curtailed RLS compared with the heterozygote (strain YFS97), while the hemizygous SIR2 allele from YJM789 (strain YFS70) extended it (Supplemental Fig. S3). An additional copy of the YJM789 allele of SIR2 in the diploid (strain YFS77) had no further effect on this RLS extension. Thus, SIR2 is the major RLS locus in QTL 1, and its alleles from the two parental strains YJM789 and S96 show incomplete dominance (semidominance). Indeed, the heterozygous diploid had a mRLS and maximum RLS of 30.6 and 57 generations, respectively, which was very close to the arithmetic average of those values for the hemizygous strains YFS70 and YFS90. These results suggest that the SIR2 from S96 not only cannot fill the role of the YJM789 allele in determining RLS but that it has a deleterious effect, perhaps by competition at the protein level.

We know that aging processes and the production of extrachromosomal rDNA circles (ERCs) in yeast are connected (Sinclair and Guarente 1997). High levels of ERCs are responsible for accelerated aging. To investigate whether the AA polymorphisms in Sir2p influence ERC production, we conducted Southern blot analyses with a probe directed against the rDNA in the strains S96, S96 sir2D T SIR2 (YJM789), I-1A, and I-1A sir2D T SIR2 (S96). The levels of ERCs were quantified using the ACT1 gene as reference (Fig. 4). S96 has a shorter mRLS than S96 sir2D T SIR2 (YJM789) and showed higher levels of ERCs. Similarly, I-1A sir2D :: SIR2(S96) has a shorter mRLS than I-1A and its ERC levels were higher. This experiment suggests that RLS is indirectly proportional to the levels of ERCs. It is known that overexpression of SIR2 increases RLS (Kaeberlein et al. 1999). We did not alter the endogenous SIR2 promoter region in any of the strains we generated. Nevertheless we performed qRT-PCR to determine the SIR2 expression level in
S96 and YJM789. No significant differences were found (Fig. 5). This suggests that the differences in mRLS are caused by the altered AA of Sir2p.

QTL 5 consists mainly of the rDNA locus

In addition to QTL 1, other QTLs impact RLS, the most prominent of which is QTL 5. About 97%–99% of QTL 5 consists of tandem repeats of rDNA depending on the copy number of the rDNA array (Fig. 2B). This and the effect of ERCs on RLS in laboratory strains (Sinclair and Guarente 1997) drew our attention to this locus. Of the 39 recombinant lines, the seven with the longest RLS contain QTL 5 from S96, while the four shortest-lived lines contain QTL 5 from YJM789 (Table 1). This suggests that the S96 variant of QTL 5 may prolong the RLS compared with the YJM789 variant. Therefore, we decided to create strains in which the rDNA cluster of strain YJM789 is substituted by the S96 rDNA cluster. Because the rDNA cluster segregates as a unit during meiosis (Petes et al. 1978), this was accomplished by introgressing the S96 rDNA cluster into YJM789 through seven successive backcrosses and the selection of the S96 rDNA cluster after each backcross. Finally, strains which contained statistically 99.25% of their genome from S96 were selected for both versions of the rDNA cluster. These strains were MATa to allow a comparison with all previously analyzed strains.

An RLS analysis was performed with the congenic strains YFS7 and YFS9. The former contains 99.25% DNA from YJM789 including the rDNA, whereas the latter differs by carrying the rDNA cluster from S96 (Table 2). The mRLS of YFS7 was 33.8 generations, and the mRLS of strain YFS9 (YJM789 background with RDN1 from S96) was 40.0 generations. The difference in RLS was significant (P = 0.030). The RLS of 40 cells each was determined. For strain S96, the mRLS was 29.8 generations, and for strain YFS1 (S96 background with RDN1 from YJM789) it was 24.6 generations. The difference in RLS was significant (P = 0.032). The x-axis shows the RLS in generations and the y-axis shows the percentage of surviving cells.

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The rDNA repeats of S96 and YJM789 are different

To gain insight into the effects of the rDNA cluster on mRLS and ERC production, we compared the sequences of the rDNA of strains S96 and YJM789. The rDNA sequence of strain S96 can be accessed in the Saccharomyces Genome Database (http://www.yeastgenome.org/). We sequenced the rDNA of YJM789 in our

Figure 3. Cumulative survival curves. (A) The RLSs of 49 cells of strain S96 and 60 cells of strain S96 sir2Δ::SIR2(YJM789) were determined. The mRLS of S96 was 31.7 generations, and for S96 sir2Δ::SIR2(YJM789) it was 46.9 generations. The difference in RLS was significant (P < 5 x 10^-6). (B) The RLS of 81 cells each was determined. For line I-1A, the mRLS was 49.5 generations; for strain I-1A sir2Δ::SIR2(S96), it was 41.6. The difference in RLS was significant (P = 0.0079). For S96, the mRLS was 30.7 generations, and for S96 sir2Δ::SIR2(YJM789) it was 43.7 generations. The difference in RLS was significant (P < 5 x 10^-6). I-1A is the recombinant line with the longest RLS. (C) The RLS of 45 cells each was determined. For strain YFS7 (YJM789 background with RDN1 from YJM789) the mRLS was 33.8 generations, and for strain YFS9 (YJM789 background with RDN1 from S96) it was 40.0 generations. The difference in RLS was significant (P = 0.030). (D) The RLS of 40 cells each was determined. For strain S96, the mRLS was 29.8 generations, and for strain YFS1 (S96 background with RDN1 from YJM789) it was 24.6 generations. The difference in RLS was significant (P = 0.032). The x-axis shows the RLS in generations and the y-axis shows the percentage of surviving cells.
SIR2 (Kaeberlein et al. 1999), and the SIR2 SIR2...conclusion arises from the comparison of strains S96 and YFS1 (Fig. 2009). Comparison of strains YFS7 and YFS9 suggests a decrease in decreased ARS function to result in lower ERC levels (Ganley et al. Fig. S6), which would most likely alter its function. We would expect corresponding sequence of YJM789 is a C instead of a T (Supplemental S4, S5). Two polymorphisms are located in the 107-bp ARS1200. ITS1...sequence. On the other hand, polymorphisms were found in described genes TAR1 of the rRNA-encoding genes...polymerase II transcript...patterns of the rRNA-encoding genes RDN25, RDN58, RDN18, and RDN5 are identical in both strains, as are the ORF of the polymerase II transcribed genes TAR1, YLR154W-E, YLR154W-F, and the ET52-1 sequence. On the other hand, polymorphisms were found in ITS2, ITS1, ET52-1, NTS2-1, and NTS1-2 sequences (Supplemental Figs. S4, S5). Two polymorphisms are located in the 107-bp ARS1200. Compared to the 11-bp ACS, the ninth base pair of the corresponding sequence of YJM789 is a C instead of a T (Supplemental Fig. S6), which would most likely alter its function. We would expect decreased ARS function to result in lower ERC levels (Ganley et al. 2009). Comparison of strains YFS7 and YFS9 suggests a decrease in ARS function for the ARS from strain YJM789, but the opposite conclusion arises from the comparison of strains S96 and YFS1 (Fig. 4). SIR2 has been shown to suppress ERC levels in a laboratory strain (Kaeberlein et al. 1999), and the SIR2 from YJM789 is more effective in this regard (Fig. 4). However, the parental origin of SIR2 is only one of the differences between the strains in the comparisons of YFS7 with YFS9 and S96 with YFS1 in Figure 4. Thus, it appears that genetic variation outside the SIR2 and rDNA loci also contributes to ERC levels. In any case, RLS does not appear to simply be a function of ERC levels (Figs. 3, 4).

The 9.1-kb rDNA repeat in S. cerevisiae gives rise to several primary transcripts (Supplemental Fig. S5). We determined the transcript levels of 5S rRNA and 18S rRNA, which are transcribed by polymerase III and I, respectively, because we found polymorphisms in spacers and nontranscribed sequences that might affect their transcription (Supplemental Figs. S4, S5). The polymerase II transcript TAR1 was examined for comparison. Strains harboring rDNA clusters from S96 showed an increase in the 5S rRNA transcripts but not in 18S rRNA or TAR1 transcripts (Fig. 6). This suggests that polymorphisms in the NTS2-1 region, which is the promoter region of RDN5, are responsible for its altered transcription levels. It is not immediately obvious, however, how this would affect RLS or ERC levels.

We also examined the rDNA copy number quantitatively in the parental strains S96 and YJM789, as well as in several of the other strains and lines generated in this study (Supplemental Fig. S7). Total rDNA and genomic rDNA paralleled each other, with the difference attributed to the ERC copy number, which was also determined in this analysis. Total rDNA copy number did not correlate with mRLS (Supplemental Fig. S7). Strain YFS9 had 88% as many ERCs as genomic rDNA copies. Yet, its mRLS was 81% that of the longest-lived line (I-1A) and 63% longer than that of the shortest-lived strain that was analyzed for rDNA copy number (YFS1). Thus, there was no direct relationship between ERC levels and mRLS.

**Discussion**

We have found five QTLs associated with RLS, representing genetic differences between a laboratory yeast strain and a clinically derived strain. These QTLs account for two-thirds of the variation in RLS in the cross between these strains. The two QTLs with the largest effects on longevity contain genes implicated in RLS in previous studies with laboratory strains. They are QTL 1, which has the largest impact on longevity accounting for 50% of the genetic effect, followed by QTL 5, which accounts for 20%. The naturally occurring variant of the SIR2 gene was responsible for the majority of the effect on RLS of the first of these QTL, as determined in the gene swap experiments and confirmed by RHA (Fig. 3A,B; Supplemental Fig. S3). Interestingly, it was the laboratory variant of the RDN1 (rDNA) locus that accounted for the majority of the effect of the second QTL, as demonstrated by the reciprocal introgression experiments (Fig. 3C,D). The results suggest that the wild SIR2 variant compensates for the negative effect of the wild RDN1 variant on RLS. One negative effect of rDNA is the generation of ERCs, which curtail RLS (Sinclair and Guarente 1997).
wild \textit{SIR2} variant appears to compensate for this, both for the wild and for the laboratory variants of rDNA, with the expected effects on RLS (Figs. 4 [S96, S96 \textit{sir2}\textit{A} :: \textit{SIR2}[YM789], I-1A, I-1A \textit{sir2}\textit{A} :: \textit{SIR2}(S96)]. This represents a gene-by-gene interaction in determining yeast longevity and an example of heterosis (hybrid vigor). Although this conclusion is supported by both the complex molecular biology of the interaction of \textit{SIR2} with rDNA (Ganley et al. 2009; Ha and Huh 2011) and evidence pointing to a role in RLS for \textit{SIR2} outside rDNA (Kaeberlein et al. 1999; Lasker et al. 2011; Wang et al. 2011).

\textit{SIR2} has been found abundantly expressed in long-lived compared with short-lived yeast strains (Guo et al. 2011). In addition, young cells contain greater than 10 times more Sir2p than older cells (Lindstrom et al. 2011). As a consequence, histone H4 lysine-16 of older cells remains heavily acetylated. This hyper-acetylation is thought to be one factor contributing to senescence (Dang et al. 2009). It was previously shown that the introduction of a second copy of \textit{SIR2} in yeast strain W303R resulted in \textasciitilde30\% extension of mRLS (Kaeberlein et al. 1999). Our qRT-PCR experiments show that the laboratory strain S96 and the clinically derived strain YM789 displayed equal levels of \textit{SIR2} expression (Fig. 5). On the other hand, a change in five AA in the \textit{SIR2} ORF led to a 48\% increased mRLS (Fig. 3A), a larger effect than seen due to over-expression of the gene (Kaeberlein et al. 1999). Figure 4 (S96, S96 \textit{sir2}\textit{A} :: \textit{SIR2}[YM789], I-1A, I-1A \textit{sir2}\textit{A} :: \textit{SIR2}(S96)] shows reduced ERC levels in strains carrying the \textit{SIR2} ORF from strain YM789 compared with strains with the S96 allele, irrespective of the source of the rDNA. We conclude that one or more of the five changed AA strengthens the silencing of the rDNA cluster, which in turn leads to stabilized rDNA and thus to increased mRLS. However, it is possible that the reduced ERC amplification and the increased RLS are independent effects of the altered \textit{SIR2} in these strains.

Four of the five polymorphic AA of strains S96 and YM789 (Pro65/Thr65, Val92/Glu92, Asp158/Val158, and Met424/Val424) are not conserved in other species, since their \textit{SIR2} homologs lack the corresponding protein domains (Sauve et al. 2006). We compared S96 and YM789 with four other \textit{S. cerevisiae} strains (FostersB, FostersO, JAY291, RM11-1a). Apart from YM789, no other strain contains Glu92 or Val158. One of the other strains contains either Thr65 or Val424. Strains like \textit{S. cerevisiae} RM11-1a or the species \textit{Saccharomyces bayanus}—but only a few higher eukaryotes—contain Asn320. Unlike the four other altered AA, only Lys320/Asn320 is located in a conserved domain of the Sir2 protein. The identity of the variant(s) critical for RLS and ERC production thus remains an open question.

The effects on the RLS and ERC of the \textit{SIR2} and \textit{RDN1} variants and their mutual interactions appear to be modified by the genetic background. The replacement of the \textit{SIR2} ORF in S96 by the one from YM789 increases the mRLS by 15 generations (Fig. 3A) in the presence of both strains of the rDNA cluster from S96. However, if the \textit{SIR2} ORF of recombinant line I-1A is replaced by the S96 variant, the difference in mRLS is only eight generations (Fig. 3B), strengthening the silencing of the rDNA cluster, which in turn leads to stabilized rDNA and thus to increased mRLS. However, it is possible that the reduced ERC amplification and the increased RLS are independent effects of the altered \textit{SIR2} in these strains.
for other quantitative traits in yeast (Steinmetz et al. 2002; Sinha et al. 2006).

The presence of the S96 rDNA cluster leads to increased mRLS (Fig. 3C,D). But what causes the RLS extension? We found many sequence changes between the rDNA clusters of S96 and YJM789. One candidate for the polymorphism that affects RLS is within ARS1200 where, in YJM789, a conserved T in the ACS is replaced by a C that may reduce the recognition of ARS1200. The activity of the replication origin of the rDNA affects the levels of ERC (Ganley et al. 2009), suggesting that the rDNA cluster of YJM789 would give rise to fewer ERCs than its S96 counterpart. This is only the case in the YJM789 background, but not in the S96 background (Fig. 4; Table 2). However, in both backgrounds, the possession of the S96 rDNA cluster increases mRLS significantly (Fig. 3). Therefore, we exclude the effect of the sequence changes in ARS1200 on ERC levels as the cause of the observed RLS extension. We postulated that the polymorphism in the ARS1200 sequence influences the transcription rate of the rDNA. The promoter region of the SS rRNA is located in the NTS2-I region, which also contains the ARS1200 polymorphism. Significantly increased transcription was only found for SS rRNA in strains harboring the rDNA cluster from strain S96 (Fig. 6).

There is another spacer of interest. The most prominent polymorphisms in the NTS2-I region are four deletions in the sequence of YJM789 (Supplemental Fig. S4). In the sequence of S96, the spacing between these deletions is ~190 bp, the distance between two nucleosomes. The NTS2-I region contains the 400-bp EXP region adjacent to the replication fork barrier (RFB) sequence (Supplemental Fig. S4). EXP is needed for FOB1-dependent repeat expansion of the rDNA (Kobayashi et al. 1992, 2001). The EXP region contains seven polymorphisms, but we saw no gross differences in rDNA unit copy number (Fig. 4). Furthermore, quantitative analysis showed that there was no direct relationship between rDNA copy number and mRLS (Supplemental Fig. S7). Two rDNA polymorphisms are close to the Fob1p binding site, which itself is conserved (Supplemental Fig. S4). Since DNA wraps around Fob1p in a nucleosome-like manner (Kobayashi 2003), adjacent polymorphisms may be sufficient to affect Fob1p function. Fob1p has multiple functions, among them replication fork arrest and rDNA silencing (Bairwa et al. 2010). Therefore, altered Fob1p binding can have different effects. ERC levels seem to be affected, but in a genetic background–dependent manner (see above). On the other hand, the increased transcription rate of the SS rRNA may lead to increased displacement of Fob1p at the RFB sites, which in turn may reduce the amount of Fob1p-mediated double-strand breaks (Wei et al. 2003). This is in agreement with the extended RLS in strains harboring rDNA from S96. Alternatively, a process involving condensin could be involved in the genetic effects described here (Machin et al. 2004; Johzuka and Horiiuchi 2009). FOB1 is ~200 Cm centromere-proximal to QTL 3 on chromosome IV, suggesting that these potential mechanisms must operate independently of any variation in FOB1 itself.

The various recombinant lines carrying the SIR2 ORF of YJM789 and rDNA of S96 display differences in mRLS up to 21 generations (I-1A and III-9B in Table 1). This suggests that QTL 2, 3, and 4 contain genes influencing RLS significantly, none of which have been identified before in the laboratory. It is likely that other RLS QTL segregate in the S96 × YJM789 cross but are masked by the major effect QTL and epistasis (Sinha et al. 2008), although our MIM analysis seems to marginalize the latter effect. The complex genetic architecture of QTL has been amply demonstrated (Steinmetz et al. 2002; Sinha et al. 2006). This architecture includes genetic interactions in cis and in trans, as well as complicated background effects. It also encompasses haplotype effects that involve tightly linked genes that alone are neither necessary nor sufficient to elicit the phenotype. These considerations suggest that our study only scratches the surface of the quantitative genetics of RLS in yeast.

The quantitative genetic analysis described here does not assess the potential contributions to RLS of the mating-type MAT locus or of loci responsible for filamentous growth. The parental strains S96 and YJM789 are MATα and MATα, respectively, and the MAT locus segregates in the cross between them. We focused our attention on MATα segregants exclusively to decrease the sample size needed to detect QTLs for RLS elsewhere in the genome, leaving any contributions of the mating-type locus for future studies. It is worth noting, however, that no QTL was detected in the vicinity of the MAT locus on the right arm of chromosome III (Fig. 1A). It is possible to draw some conclusions regarding an association of filamentous growth and RLS. Because S96 does not display filamentous growth, we searched for the presence in all 39 recombinant lines of the same region(s) of the S96 genome identified by contiguous stretches of one or more of the 3048 markers used for genotyping. Only one such region was found, encompassing 11 markers. It was on the right arm of chromosome II between base pairs 562,406 and 582,418 (Fig. 1A). 4208 bp downstream from AMNI and encompassing 13 genes. However, there was only one recombinant line that could potentially possess a crossover within AMNI, and there were no QTL for RLS on chromosome II. AMNI was identified in a large-scale analysis of filamentous growth (Jin et al. 2008). A dedicated search will be necessary to identify QTL that affect filamentous growth. In sum, we have not ruled out the possibility that the MAT locus or a filamentous growth QTL might affect RLS, and as discussed above, QTL1 and QTLS identified here may contain RLS genes in addition to SIR2 and RDN1, respectively.

S. cerevisiae is an opportunistic pathogen (Mulder and McCusker 2009). The natural variation in RLS that we have sampled comes from one clone of a clinical isolate of S. cerevisiae. A population genetic analysis has shown that three distinct groups characterize this species. These include vineyard and brewery strains, clinical and fruit isolates, and soil isolates (Diewzmann and Dietrich 2009). Interestingly, the parental strain S96 is a laboratory derivative of a yeast isolated from a rotten fig (Mortimer and Johnston 1986) and together with YJM789 belongs to the second group. This leaves the genetic variation in the other two groups unsampled in our analysis. This variation is likely to reveal additional genes and pathways that determine longevity.

Methods
Gene chip sample preparation
Genomic DNA was isolated using Qiagen Genomic-tip 100/G columns. All procedures for DNA isolation were followed as published in the manufacturer's instructions, except incubation times were extended from 30 to 45 min. Purified samples had an A_260/ A_280 of 1.7–1.9. Eleven micrograms of genomic DNA was digested with 0.165 units of DNase I (Invitrogen) for 3–10 min at 37°C. DNase I was then heat inactivated for 15 min at 95°C. A 0.5 μg aliquot of fragmentized DNA was loaded onto a 2% agarose gel to determine fragment lengths and sample homogeneity by electrophoresis. Sybr green II was added to samples to visualize the DNA, and a 25-bp oligonucleotide marker (Invitrogen) was electrophoresed alongside to locate and quantify 25- to 50-bp fragments. Digested DNA was labeled using 50 units of terminal transferase in
1× TdT reaction buffer (Roche), 2.5 mM CoCl2, and 1 nmol biotin-16-ddATP (Enzo) for 1.5 h at 37°C. To stop the labeling reaction, 25 mM EDTA was added.

Array hybridization, washing, and scanning
Reagents for hybridization, wash, and staining were purchased from Affymetrix (GeneChip HT Hybridization, Wash and Stain kit) unless otherwise stated. The Affymetrix gene chip expression analysis manual was followed for sample preparation. Each sample was included in a hybridization cocktail containing 30 pM control oligonucleotide B2, 1× eukaryotic hybridization controls (bio1, bioC, bioD, cre), 0.1 mg/mL herring sperm DNA (Promega), 0.5 mg/mL acetylated BSA (Invitrogen), and 1× hybridization buffer. Hybridization samples were heated for 5 min to 99°C, incubated for 5 min at 45°C, and centrifuged for 5 min at 20,000g, to remove any precipitate. Each Affymetrix Yeast 39B GeneChip (AYGC) was equilibrated to room temperature prior to hydration with 200 μL 1× M2S hybridization buffer and then incubated for 10 min at 45°C with rotation. The hybridization buffer was removed from the AYGC, and the hybridization cocktail was applied to the gene chip and incubated for 16 h at 45°C, while mixing on a rotisserie at 60 rpm. After hybridization, samples were inserted into the Affymetrix fluidics station. The stain solution, containing 1× stain buffer, 2 mg/mL acetylated BSA, and 10 μg/mL streptavidin-phycoerythrin (SAPE) was inserted into the sample holder of the fluidics station. The protocol for washing and staining (EuKGE-WS2v4) of the AYGC was followed according to the Affymetrix Gene chip expression analysis manual. Parameters using the Gene Chip Operating Software (GCOS) version 1.4 were as follows: fluid extension value of 450, target signal of 250, and normalization value of 1.

Data analysis
Raw output CEL files were converted from compressed binary CEL files to CEL text files using the Affymetrix CEL file conversion tool (available at https://www.affymetrix.com/support/file_download.affx?onloadforward=/support/developer/downloads/Tools/CelFileConversion.ZIP). CEL files were imported into Allelescan (available for download at http://med.stanford.edu/srgt/research/download/), and data quality was determined for parental strains and recombinant lines by using Allelescan’s quality check for controls, markers, hybridizations, and replicates. Samples were excluded from the data set if the comparative analysis indicated an outlier, as described in the Allelescan manual. A critical F-test value of 35 and marker score filter value of 0.50 was used to identify approximately 3048 molecular markers found between the two parents, constituting an estimated 3.9% of the total genetic variation between the two strains (Winzeler et al. 1998). The genotype of each haploid segregant was confirmed using the molecular marker data to determine the inherited regions from each parental strain (Supplemental Fig. S2).

QTL mapping
Molecular marker designations and crossover locations were determined using the Allelescan software. Molecular marker positions and segregant genotypes were then used to compile input files for Windows QTL Cartographer version 2.5 (available at http://statgen.ncsu.edu/qtlcart/WQTLCart.htm), and along with RLS data, they were analyzed using CIM to find DNA regions associated with RLS (Basten et al. 1994, 2004). The walking step for the mapping was set at 0.5 cM. The LOD value 2.5 was determined as the genome-wide significance threshold for statistical significance of the QTL identified. The effect of QTL on RLS and QTL interactions were further estimated using MIM provided by Windows QTL Cartographer (Zeng 1994).

RLS analysis
Lifespan analysis was performed as described (Kim et al. 1999). Briefly, virgin cells (new buds) from an exponentially growing overnight culture were used for each RLS determination. These cells were deposited in a row in isolated spots on an YPD (2% peptone, 1% yeast extract, 2% glucose) plate. The number of buds removed by microdissection prior to cell death is the cell’s RLS in generations. The Mann-Whitney test was used to assess statistical significance of differences between the lifespan of different strains in an experiment. All RLS analyses were repeated at least two times. Statistical analysis was done using Statmost (DataMost). Reported P-values are two-sided.

Construction of strains S96 sir2Δ::SIR2(YJM789) and 1-1A sir2Δ::SIR2(S96)
The ura3-1 of strain W303, obtained by PCR amplification using primers URA3-F (5′-ATTGAGGCTACTCGCCCAAT-3′) and URA3-R (5′-GGAGTTCAATCGCTTATCT-3′), was transformed into strain S96 and line 1-1A. Transformants were selected on synthetic complete (SC) medium (Guthrie and Fink 1991) containing 1 g/L 5-fluoroorotic acid (5-FOA). The SIR2 loci of strains S96 and YJM789, amplified using PCR primers 5′-TATTACGTCGACCT GCCAACCCTCAATGT-3′ and 5′-ATGGATTGTCGACTCGTTCCA CCTGCCCCCTC-3′, were ligated into plasmid pUC19 previously cut with Sall, resulting in plasmids pUCS3 and pUCM3, respectively. The SIR2 ORF of plasmids pUCS3 and pUCM3 were replaced (between the BsAI and BstXI sites) by a URA3 cassette (obtained by PCR amplification using plasmid pCM297, kindly provided by Mark Rinnerthaler (University of Salzburg, Austria), and primers 5′-AAGGGTGACTGACGTTGGTGGTGGTCGAC-3′ and 5′-CAT TACCATCCATTTGTGGGACCCAGATTCCCGTAA-3′), resulting in plasmids pUCS3 and pUCM3, respectively. Plasmids pUCS3 and pUCM3 were cut with Sall and transformed into strains S96 ura3-1 and 1-1A ura3-1, respectively. The selection of S96 ura3-1 sir2Δ::URA3 and 1-1A ura3-1 sir2Δ::URA3 transformants was done on SC-uracil medium. The SIR2 ORF of plasmid pUCS3 was replaced by the SIR2 ORF (YJM789) of plasmid pUCS3 from BsaI and BstXI sites, resulting in plasmid pUCS5M. The same was done in the opposite direction, resulting in plasmid pUCM5 containing the S96 SIR2 ORF. Plasmids pUCS5M and pUCM5 were cut with Sall and transformed into strains S96 ura3-1 sir2Δ::URA3 and 1-1A ura3-1 sir2Δ::URA3, respectively. Selection of S96 ura3-1 sir2Δ::SIR2(YJM789) and 1-1A ura3-1 sir2Δ::SIR2(S96) transformants was carried out on 5-FOA medium. Both strains were transformed by the URA3 locus of strain S96, amplified using PCR primers URA3-F and URA3-R, to restore the URA3 locus. Selection of S96 sir2Δ::SIR2(YJM789) and 1-1A sir2Δ::SIR2(S96) transformants was done on SC-uracil medium. The entire SIR2 loci of strains S96 sir2Δ::SIR2(YJM789) and 1-1A sir2Δ::SIR2(S96) were verified by sequencing. (Underlined primer sequences indicate sites for Sall, BstXI, and BsAI, respectively.)

Construction of strains YFS1, YFS7, and YFS9
Strain YJM789 was crossed with strain S96. The diploid was sporulated, and a haploid segregant was backcrossed to S96, followed by isolation of a haploid segregant. This backcross procedure was repeated five more times to introgress the YJM789 RDN1 cluster into the S96 background in seven steps, yielding strain YFS1. The introgression was carried out in the opposite direction to introduce...
the S96 RDN1 cluster into the YJM789 background, yielding strains YFS7 and YFS9. In each step, strains with yeast-like growth type and the desired RDN1 (rDNA cluster) allele were selected. This was done by PCR amplification and restriction analysis of RNN203, which is located 12.241 bp upstream of the rDNA cluster. Primers 5'-ATGACAAAGAGTGCCGTGAAAT-3' and 5'-TACGTGATTTATGA CATCGATGAG-3' were used. The resulting 333-bp product can be cut into 184 bp and 149 bp by EagI only when derived from YJM789. The rDNA cluster of the final strains was partially sequenced for verification.

ERC detection

Cells were grown overnight in YPD. DNA was isolated using the spheroplast method, as described (Medvedik and Sinclair 2007). Three micrograms of DNA was cut with SpeI, which does not cut in the rDNA, but releases a 4.1-kb fragment of ACT1 used for quantification. DNA was electrophoresed in a 0.7% agarose gel at 0.9 V/cm for 18 h and subsequently transferred to a nylon membrane, which was hybridized with the ACT1 and 35S rDNA probes. Preparation of the probes using the RediPrime kit (GE Healthcare) was performed previously (Borghouts et al. 2004). Blots were scanned using the Typhoon (GE Healthcare), and quantification was performed using ImageQuant version 5.1 software ( Molecular Dynamics). ERC copy number was quantified by normalizing to the specific activity of the ACT1 and 35S rDNA probes. The probes were prepared by nick translation, in this case, and they were ~500 bp. Total rDNA copy number was similarly determined. However, DNA was digested with KpnI, which cuts once within each rDNA repeat and once within ACT1 (Kim et al. 2006). The genomic rDNA copy number was the difference between total rDNA and ERC copy number.

qRT-PCR analysis

For the preparation of cDNA, the RNase-free DNase set (Qiagen) and the Taqman kit (Applied Biosystems) were used according to the manufacturer’s manual. For amplification of cDNA, primers 5'-CTTGAGAAAAAGCTACCATC-3' and 5'-ATTGTCACTATTACTCCCTGAATTAGGA-3' (18S rRNA), 5'-TACACCCACCTAGAGGCGT TGCAT-3' and 5'-CGAGGAGTGCTGGTTCTTTGT-3' (18S rRNA), and 5'-ACTCTGCTGACGGCTCT TACCA-3' (5S rRNA), and 5'-CTCCATCAGAGGCTTGGT-3' and 5'-CAGGCTTAAATGGAAGACGTG-3' (ACT1) were used in the same PCR. qRT-PCR were carried out in an Applied Biosystems 7300 real-time PCR machine. PCR conditions were chosen according to the manufacturer’s manual. The Newman-Keuls post hoc test was used to determine the significance of specified differences in transcript levels. P-values are two-sided.

Sequencing

The equivalent of one repeat of the RDN1 locus of strain YJM789 was sequenced. Parts of the rDNA of ~1 kb were PCR subcloned at least three times for sequencing. Subsequently, they were sequenced in both directions. Proofreading DyNazyme and Phusion polymerases were utilized (NEB). Sequences with stretches of the same base were sequenced at least an additional three times. All strains generated in this study have been verified by sequencing altered loci plus 1 kbp upstream and downstream.

Data access

Array genotyping data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE37590. The GenBank (http://www.ncbi.nlm.nih.gov/genbank/) accession number for the sequence of YJM789 rDNA is JQ277730.

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