The complex processes and interactions that regulate aging and determine lifespan are not fully defined for any organism. Here, taking advantage of recent technological advances in studying aging in budding yeast, we discovered a previously unappreciated relationship between the number of copies of the ribosomal RNA gene present in its chromosomal array and replicative lifespan (RLS). Specifically, the chromosomal ribosomal DNA (rDNA) copy number (rDNA CN) positively correlated with RLS and this interaction explained over 70% of variability in RLS among a series of wild-type strains. In strains with low rDNA CN, Sir2 expression was attenuated and extrachromosomal rDNA circle (ERC) accumulation was increased, leading to shorter lifespan. Suppressing ERC formation by deletion of Fob1 eliminated the relationship between rDNA CN and RLS. These data suggest that previously identified rDNA CN regulatory mechanisms limit lifespan. Importantly, the RLSs of reported lifespan-enhancing mutations were significantly impacted by rDNA CN, suggesting that changes in rDNA CN might explain the magnitude of some of those reported effects. We propose that because rDNA CN is modulated by environmental, genetic, and stochastic factors, considering rDNA CN is a prerequisite for accurate interpretation of lifespan data.

Significance

Genes encoding ribosomal RNA (rDNA) are organized into a repetitive array in eukaryotic genomes. The copy number of these genes often varies and is responsive to genetics and environment. Here, we show that variation in copy number at the rDNA locus is capable of altering replicative lifespan in yeast. These results indicate that considering rDNA copy number, and conditions that could potentially change this dynamic chromosome locus, is critical for evaluating lifespan. We propose that this rDNA locus represents the kind of repeated element in eukaryotic genomes that escapes easy detection, yet has phenotypic consequences, in this case lifespan.
genetic or environmental factors contributing to the variability. In addition, a genome-wide screen measuring the RLS of deleted nonessential genes found a significant discrepancy between strains with opposing mating types carrying the same gene deletion (23). This discrepancy was mostly attributed to statistical error due to a low number of cells analyzed, but was not fully explored. Given this large degree of variability, which likely impacts the interpretation of any lifespan measurement, we wanted to address the underlying cause. Here we show that the chromosomal rDNA copy number is an important determinant of replicative lifespan in yeast and can explain a large part of the reported variability in lifespan.

**Results**

**rDNA Array Length Strongly Correlates with Replicative Lifespan of Wild-Type Strains.** A recently developed microfluidic system, which uses automated hardware and a machine-learning algorithm for analysis (1), was employed to obtain large quantities of RLS data for an extended series of wild-type yeast strains. Although these strains were derived from the same diploid parent strain, dramatic variability in RLS was observed (Fig. 1A, spontaneous variation). This phenomenon has been previously reported, but the underlying cause has largely remained unknown (22).

To address whether genetic differences might explain the observed variability in lifespan, whole-genome sequencing (WGS) was performed and the lifespan data were compared with the occurrence of single-nucleotide polymorphisms (SNPs) and other types of polymorphisms (Fig. 1 B and C). Among all SNPs and copy number variations (CNVs), the clearest signal correlating with lifespan came from the rDNA locus, which showed a gradual increase in CN along with enhanced median RLS. To test the validity of rDNA CN estimates obtained by WGS, the size of the rDNA arrays was confirmed by electrophoresis of intact arrays (contour-clamped homogeneous electric-field electrophoresis [CHEF]) (SI Appendix, Fig. S1).

To further examine the impact of rDNA CN on lifespan, a large series of wild-type strains with variable rDNA CNs were constructed by two different means (Fig. 1 D and E; experimental variation). First, a diploid wild-type strain was sporulated to spontaneously create variability. Second, to obtain strains with more extreme CNs, a construct replacing random numbers of rDNA repeats was introduced (Materials and Methods) (13). When assessing lifespans for this entire panel of strains and comparing it to rDNA CN, there again was a strong correlation between the two in strains with CNs <150 repeats, while CNs above 150 did not increase RLS any further. Cox proportional hazard estimates indicate that each loss of 10 rDNA repeats (below 150) results in a 20 ± 0.2% increase in hazard (95% confidence interval), explaining over 70% of variation observed in median lifespan. In summary, a large part of the variability in lifespan among these wild-type strains is explained by differences in rDNA CN.

Environment and genetics can impact many phenotypes, including RLS (24). This led us to ask whether the correlation between lifespan and rDNA array length was also observed under different growth conditions and genetically different strains. Lifespan experiments and CN estimation in three different media types were performed: minimal medium, synthetic complete medium (contains amino acids, etc.), and rich medium (see Materials and Methods). Under these conditions, there was again a strong correlation between CN and lifespan (SI Appendix, Fig. S2). In addition, it is worth noting that while the correlation remains strong in all three media, growth in YNB generally produced longer RLS for a given rDNA CN and YPD a shorter RLS.

Together with the observation that the impact of rDNA CN on RLS was similar in a another strain (25) (SI Appendix, Fig. S3), we conclude that rDNA CN impacts RLS under multiple disparate scenarios. Finally, we confirmed that rDNA CN impacts RLS when lifespan was measured with the traditional microdissection assay (SI Appendix, Fig. S4) (26).

**ERC and Sir2 Levels Underlie the Positive Correlation between Lifespan and rDNA Array Size.** During log-phase growth of yeast cells, there is a dynamic equilibrium between ERCs and the number of chromosomal rDNA copies (13). ERC levels anticorrelate with rDNA CN, but no further connection to aging and lifespan has been established. To test whether ERC levels were affected by rDNA CN in aging cells, a modified version of the miniature-chemostat aging device (MAD) (Materials and Methods) (27) was used to obtain large numbers of aged cells (~6 × 10^8 cells per sample). Seven wild-type strains with variable rDNA CNs were aged for 24 h, the total DNA was extracted to run on an agarose gel, and a Southern blot was performed to detect both forms of rDNA (the chromosomal array and ERCs; Fig. 2 A and B). As a control, seven fob1Δ strains of comparable rDNA CNs were included, a mutant in which ERC levels are drastically reduced, because ERC accumulation during aging is attenuated (11). In samples from aged wild-type cells, ERCs appeared in more than four isoforms (labeled 1 to 4 in Fig. 2A), a similar pattern to that previously described (13, 28). These isoforms were strongly reduced in the aged fob1Δ controls (Fig. 2A), as well as in young cells (SI Appendix, Fig. S5). When ERC levels were quantified by integrating the signal from all major species, a strong anticorrelation between ERC levels and rDNA CN in wild-type strains was observed (Fig. 2B). Consistent with lifespan (Fig. 1E), ERC levels did not decrease any further in strains with rDNA CN >150. In contrast to wild type, ERC levels remained low in fob1Δ strains, irrespective of rDNA array length or age. These data suggest that ERC accumulation with age is dramatically higher in wild-type cells with short rDNA arrays, which may explain why these cells have shortened lifespans.

To examine this phenomenon via another cellular phenotype, we took advantage of the large collection of time-lapse movies obtained with our microfluidic system. Previous studies have reported that yeast cells follow distinct aging trajectories, with one fraction of the population dying from rDNA/ERC-related causes and the other dying via rDNA-independent paths (mitochondrial defects, etc.) (29, 30). These two subpopulations are readily distinguishable by cell morphology, with the rDNA-related trajectory showing an elongated daughter cell morphology prior to death and the rDNA-independent trajectory correlating with round daughter cell shape. Using this phenomenon, cells were categorized by their mode of death (MoD) by distinguishing between an “rDNA-dependent MoD” and an “rDNA-independent MoD” with a machine-learning algorithm (SI Appendix, Fig. S6; see Materials and Methods for details). When calculating the hazard risk (chance of a cell to die within a division) for each of these strains, we noticed that the hazard risk increased drastically at earlier divisions in cells with lower rDNA CN (Fig. 2C). Importantly, this increased risk of dying was predominantly observed in cells of the rDNA-related MoD category, indicating that it was due to rDNA- and ERC-dependent causes of death. In contrast, other cellular phenotypes associated with lifespan, such as growth rate and cell size (31, 32), did not show a meaningful correlation with RLS in the strains examined (SI Appendix, Figs. S7 and S8).

If ERCs play a critical role for shortening lifespan and increasing hazard risk in cells with short rDNA arrays, reducing the number of ERCs should restore long lifespan even in strains
rDNA copy number explains a majority of the variability in RLS estimates. (A) Kaplan–Meier estimates for 13 wild-type strains derived from the same parent show significant variability ("spontaneous variation"); >400 observations per curve, median of ∼1,000 observations per curve, 95% confidence interval represented by shaded region. (B) Copy number variations in the rDNA locus correlate with RLS. Polymorphisms identified by whole-genome sequencing data are grouped into SNPs and CNVs of 2-kb genome bins. Forty-one polymorphisms occurring in at least two strains are shown. Strains are ordered by increasing median RLS from left to right. Color map for SNPs is gray (absent, REF) and black (present, VAR). Color map for CNVs is from low (dark blue) to high (yellow). (C) rDNA CN correlates with median RLS. Color map indicates rDNA CN and is maintained throughout the rest of the plots. Each point represents a unique lifespan experiment (microfluidic channel) with >400 cells. Ninety-five percent confidence intervals around median RLS estimate are represented as vertical bars. (D) Kaplan–Meier estimates for an extended panel of 54 wild-type strains with variable rDNA CN ("experimental variation"); >400 observations per curve, median ∼1,150 observations per curve. (E) Correlation of median RLS and rDNA CN for the same strains as in D (n = >319 per microfluidic channel, median 593 cells).

with low rDNA CN. To evaluate this idea, lifespans of a panel of fob1Δ strains were measured, where ERC abundance was low regardless of rDNA CN (Fig. 2A and B). In contrast to wild-type cells, fob1Δ cells were long lived irrespective of rDNA CN in strains with as few as 35 rDNA repeats and no correlation with rDNA CN was observed (Fig. 2D). In addition, fob1Δ strains did not show the characteristic inverse correlation of hazard rate with rDNA CN among cells with rDNA-related MoD that is apparent in wild-type strains (Fig. 2C). Together, these data strongly suggest that the enhanced accumulation of ERCs in strains with low rDNA CN is the underlying cause for the inverse correlation between rDNA CN and lifespan.

Iida and Kobayashi (21) proposed that the upstream activating factor (UAF) complex of the rRNA gene can also act as a transcriptional repressor at the SIR2 locus. They suggest that there is a competition for limiting UAF between the rDNA and SIR2 loci. Occupancy at the SIR2 locus increases with low rDNA CN and as a result, there is reduced expression of Sir2. This model could explain why ERC levels are higher and lifespan is shorter in cells with reduced chromosomal rDNA CN. To test whether
Chromosomal rDNA CN modulates lifespan via ERC levels. (A) Southern blots probed for rDNA and NPR2 (loading control). Samples were obtained from wild-type and fob1Δ strains with variable rDNA CN aged for 24 h. Arrows 1 to 4 highlight bands of ERCs, and arrow A highlights the chromosomal rDNA array. (Scale bar, 1 cm.) (B) Quantification of ERCs on blot from A shows anticorrelation between rDNA CN and ERC levels. ERC accumulation is suppressed by deletion of FOB1. Sum intensity of all four ERC bands is plotted. (C) Hazard estimates for 51 wild types and 20 fob1Δ strains derived from microfluidic experiments show increased hazard risk for cells in rDNA-dependent MoD with low rDNA CN. This effect is suppressed by fob1Δ. Color maps, indicating rDNA CN, are the same as in B (>400 cells per hazard estimate; shaded regions represent 95% confidence interval). (D) fob1Δ eliminates the correlation between rDNA CN and RLS (>400 cells per median lifespan estimate; vertical bars represent 95% confidence interval).

Since yeast cells are known to maintain rRNA expression constant despite differences in rDNA CN (33), we wanted to examine whether this was true in the strains used here. RNA-Seq on total RNA (without enriching for mRNA) was performed to measure the levels of rRNA relative to the rest of the transcriptome (SI Appendix, Fig. S9). There was no significant difference between the strains with variable rDNA CN, which is in agreement with previous results (33). Taken together, our results suggest that the predominant underlying effect of higher rDNA CN on lifespan is an increase in SIR2 expression and reduced accumulation of ERCs.

rDNA Array Size Alters the Lifespan of Known Aging Modulators. There is abundant evidence that rDNA array size can vary dramatically from strain to strain within and between strain collections (12, 19, 34). For instance, strains from the commonly used yeast knockout (YKO) collection (35) had a wide distribution of rDNA CNs, carrying anywhere between 80 and 180 rDNA repeats (12). Given this wide range of rDNA CN and its effect on lifespan, we were curious about how much of an impact rDNA
CN had on the RLS of known lifespan modulators. To this end, panels of the following deletion mutants were created, each with varying rDNA CN: \textit{gpa2}Δ, \textit{idh1}Δ, \textit{rpl13A}Δ, \textit{ubp8}Δ, \textit{hda2}Δ, \textit{ubr2}Δ, and \textit{tor1}Δ. These mutants span a range of processes implicated in aging and have previously been found to be long lived (23, 36–41).

When measuring the effect of each mutation on survival in the context of different rDNA CNs, three conclusions emerged (Fig. 4 A–H). First, the effect of rDNA array length on lifespan could be dramatic, even in the presence of a reported lifespan-enhancing mutation. Second, some of the mutants were indeed long lived relative to wild type at a normal rDNA CN (∼150). However, in other mutants no significant difference in RLS was detected at any copy number. Importantly, all of the lifespan-changing effects of the mutants were reliably observable and interpretable only when taking rDNA array length into account. Third, in some cases the relationship between rDNA CN and lifespan was modulated by the mutation. For example, the \textit{fob1}Δ mutation attenuated the effect of rDNA CN on lifespan across the entire range of rDNA CNs, in agreement with ERC accumulation causing this effect. In contrast, \textit{hda2}Δ exacerbated the relationship such that the effect of rDNA CN on lifespan was even greater than in wild type (WT). The \textit{ubp8}Δ mutant also appeared to modulate this relationship, but to a lesser extent and possibly only over a limited range of rDNA CN. Together, these data strongly suggest that the rDNA CN is a parameter essential for the accurate interpretation of replicative lifespan data, in wild type as well as in various mutant backgrounds.

**Discussion**

In this study, we discovered that the number of repeats at the rDNA locus is a major determinant of RLS in \textit{S. cerevisiae}. Strikingly, over 70% of variation in RLS between otherwise identical wild-type strains was explained by the number of rDNA copies on the chromosome (rDNA CN). Specifically, the median RLS increased by twofold, from ∼15 to ∼30 cell divisions, when the rDNA CN doubled from 75 to 150 copies (Fig. 1). Our results provide another level of understanding about RLS determination in budding yeast.

Furthermore, we offer a mechanistic explanation for this rDNA CN effect on RLS, which is consistent with all our data and builds upon important findings from others (Fig. 5). The CN of rDNA repeats within the chromosomal locus is in dynamic equilibrium with ERCs (13), a causal factor for reduced lifespan in \textit{S. cerevisiae} (7). Strains with low rDNA CN and shorter lifespan have lower levels of Sir2 expression (15, 21) and as a result accumulate ERCs at a faster rate (Figs. 1 and 2). In support of this model, we recapitulate previous observations describing the effect of rDNA CN on the UAF binding site at the \textit{SIR2} locus, \textit{SIR2} transcription, and ERC formation (13, 21) and show the effect of ERC formation on mode of death and lifespan. Importantly, the effects of rDNA CN both on RLS and on ERC formation are completely ablated in a \textit{FOB1} deletion background, where ERC formation is severely attenuated.

We observed an interaction between rDNA and RLS by generating a high-diversity rDNA copy number panel spanning from...
Fig. 4. rDNA CN affects the lifespan of known modulators of aging. (A–G) Correlation of median RLS and rDNA CN for wild-type and mutant strains as indicated (>300 cells per lifespan estimate; median = 593). Vertical gray line indicates rDNA CN of 150. (H) Cox Proportional Hazard model testing the effect of mutations on the hazard at 150 rDNA CN (left) and on the correlation between rDNA CN and RLS (right). (H, Left) Negative coefficients indicate a reduction in hazard due to the mutation at an rDNA CN of 150. Vertical bars represent 95% confidence interval, and open circles indicate that the estimated range overlaps with 0. (H, Right) More positive interaction terms indicate a weaker correlation between rDNA CN and RLS in the indicated mutant background. Coefficients are measured per mutation or per unit change for rDNA CN.

50 to 220 copies. In doing so, we were able to test the effect over a large copy number range and sample size using our high-throughput lifespan machines (1). We generated single-cell longitudinal lifespan data for >300,000 cells from >200 unique strains in >550 lifespan experiments and used high-throughput genotyping to accurately measure rDNA CN. These methods allowed for the effect of rDNA CN on yeast RLS to be discerned, which previous studies might have been underpowered to detect (12, 15, 18).

We propose that differences in rDNA CN could explain a significant amount of variation in lifespan reported in the literature (22), which has implications for interpreting previously reported lifespan effects in various mutant strains. We tested lifespans for a small set of such mutants, which all had been reported to extend RLS (23, 36–41). With the exception of fob1Δ, the chromosomal rDNA CN had a strong influence on lifespans of these mutants, particularly in the 75 to 150 copy number range (Fig. 4). And while some of the mutations indeed had an additive effect on
Fig. 5. rDNA CN affects lifespan through known regulation of established aging factors. Model shows how differences in rDNA CN affect lifespan. In cells with low rDNA CN, Sir2 expression is low due to repression by the UAF complex, which in turn causes ERC accumulation and shortens lifespan.

RLS at a defined rDNA CN (e.g., hda2Δ, ubp8Δ, and ubr2Δ), surprisingly, others did not (e.g., tor1Δ, idh1Δ, and gpa2Δ). While we cannot exclude that these deletions might have stronger effects in environments that were not tested in our microfluidic devices, we postulate that a longer rDNA array was the underlying reason for previously reported lifespan-extending effects.

There are several ways by which mutant strains could have had a different rDNA CN in an RLS study compared to their corresponding wild-type control strain. First, genome manipulations, such as transformations typically used to introduce a mutant allele, can alter the chromosomal rDNA CN (12). Second, rDNA CN may have been affected by the mutation itself, since 10% of gene deletions in S. cerevisiae result in a change in the number of repeats within the chromosomal rDNA array (19, 34, 42). Third, growth or metabolic conditions can modulate the size of the chromosomal rDNA locus. For instance, the rDNA array reaches different sizes depending on the environmental conditions (20), a phenomenon that was also observed in Drosophila (16). Thus, by comparing individual wild-type and mutant strains the contribution of a gene deletion to lifespan could easily be confused with a copy number effect and the accurate interpretation of lifespan data must account for rDNA CN. Given the current accessibility of next-generation sequencing, we envision that rDNA CN will be a necessary parameter to quantify and consider when conducting yeast RLS experiments.

As noted in the Introduction, the chromosomal rDNA array is a type of "contingency locus" that responds to cellular dynamics and environment to allow the cell to adapt to changes in its metabolic state (14). This has at least two implications: First, growth conditions and genetics can have a large impact on the RLS of a strain through modulation of rDNA CN. Second, this provides an example of antagonistic pleiotropy, as hypothesized by Williams (43). Mansisidor et al. (13) proposed that the presence of ERCs associated with short rDNA arrays provides metabolic flexibility in young cells. However, having an abundance of ERCs in young cells becomes a burden later in life.

Similar mechanisms to those reported here may be at play in metazoan systems. Prior work has demonstrated that there is extensive rDNA CN variation at both the population and single-cell level and that pathways regulating this process are conserved between yeast and mammals (44–46). It has also been demonstrated that nutritional stresses and aging can affect the level of copy number and DNA methylation of fly, mouse, and human rDNA (47–49). Importantly, variation in rDNA CN within these organisms has phenotypic consequences, including effects on mitochondrial copy number and transcriptional signatures (50). Given these findings, we suggest that rDNA loci, specifically their copy number variations, warrant further investigation into their regulation of aging in mammalian systems.

Materials and Methods

Yeast Strains and Media Conditions. All strains are in the S288C strain background (25). Strains derived directly from BY4743 were obtained by sporulation. To introduce variability in rDNA length, BY4743 was transformed with a linearized construct that contained a HYG cassette flanked by homology to the NTS1 and NTS2 regions of the rDNA repeat. This construct was analogous to the one published by Mansisidor et al. (13). To construct all other strains, a MATa and MATα clone, which was obtained by backcrossing the wild type of the prototrophic deletion collection (can1Δ::STE2pr::HIS3 his3Δ1 hsp1Δ) (51) with BY4741, was used. Genes of interest were knocked out in the resulting diploid and then sporulated. This was sufficient to introduce some variability in rDNA array length in this strain. To obtain more extreme CN variations, the HYG construct mentioned above was used.
All experiments were performed in synthetic complete (SC) medium, unless indicated otherwise. SC medium contained 1× SC mix and 1× yeast nitrogen base (YNB) with ammonium sulfate (Sunrise Science), 2% glucose, and 2% methyl-alpha-D-mannopyranoside (MMP) (Sigma Aldrich), pH 4.5. Yeast media (YNB) were identical to SC except without the 1× SC mix. rich media, yeast extract peptone dextrose (YPD), contained 1% wt/vol yeast extract, 2% wt/vol Bacto peptone, and 2% glucose. For aging experiments in the modified MAD system, SC medium with two modifications was used: biotin-free YNB (Sunrise Science) and additional 40 mM 7,8-diaminocephalagonic acid (DAPA) (27).

Cell Size and Growth Rate Measurements. Growth rate measurements were obtained from cells grown to saturation in SC overnight and then diluted to optical density 600 nm (OD) 0.01 OD was measured at 2, 3, 4, 5, 6, 7, 8, 10, 12 and 14 h after dilution. Cell size was measured in triplicate on cells grown in SC medium for 24 h at low OD (<0.05), using a Multisizer 4e Counter Couter (Beckman Coulter).

Replicative Lifespan Measurements. Replicative lifespan measurements were made as described in Thayer et al. (1). Briefly, cells were grown to saturation in the respective medium type in 24-well plates and then grown for 24 h below an OD 0.05. Cells were then loaded into microfluidics devices specifically designed to retain aging mother cells in polydimethylsiloxane cell “catchers.” Brightfield images were collected every 15 min for 96 h. Time-lapse images were analyzed by an automated computer vision pipeline, predicting number of divisions as well as death vs. censoring for each cell in the catchers. Median lifespan was estimated using the Kaplan–Meier estimate of the survival function. Model performance was validated by manual annotation of a subset of the images used in this study (SI Appendix, Fig. S10). A microdissection assay was performed as previously described (26).

Aging of Bulk Cell Populations in a Modified MAD System. To obtain large amounts of aged cells, ~10^6 cells for each strain were grown in biotin-free SC medium that contained the biotin precursor DAPA. Cultures were grown at an OD <0.05 for 24 h. Using this medium instead of regular SC prevents competition between free biotin in the medium and biotinylated cells (27). Cells were harvested and washed three times with 1× PBS and then incubated with 0.5 mg Sulfo-NHS-LC-LC-Biotin (Sigma Aldrich) per 2.5 × 10^9 cells for 30 min. Biotinylated cells were then washed three times with medium and recovered at 30°C in medium for 6 h at OD <0.05. Then, 22.5 mg of Dynabeads MyOne Streptavidin C1 magnetic beads (65001, Thermo Fisher) per 2.5 × 10^9 cells were added directly to the flasks and incubated for 10 min with occasional swirling. Next, cells were collected on a 0.22-μm filter (431098, Corning) and beaded mother cells were purified by sequential washing with a DynaMag-2 magnet (Invitrogen). Finally, cells were loaded into a modified MAD (27) prefill with medium and allowed to bind to the magnets for 5 min before the peristaltic media pumps were turned on. This system has the same basic setup as the original MAD with a glass vessel, a metallic bubble cage, circular magnets on the outside of the vessel, and three media ports (for cell loading, media influx, and media outflow). However, the dimensions of the vessel in this version of the MAD (130 mL media content) allow loading of 10^9 cells per device and thicker peristaltic marpure tubing (978.0102.00+, Watson Marlow) enables increased media flow. After 24 h, ~6 × 10^9 cells per sample were harvested.

ERC Blots. Bulk genomic DNA was extracted using a protocol similar to that in Mansisidor et al. (13). In brief, ~6 × 10^8 cells were spun down, washed in nuclelease-free H2O, and resuspended in 800 μL digestion buffer (1 mL Sorbitol, 42 mM K2HPO4, 8 mM KH2PO4, 5 mM ethylenediaminetetraacetic acid (EDTA) containing 40 μL R-Zymolyase (zymo Research). After incubation at 37°C for 1 h, 1,140 μL Proteinase K (Qiagen) and 44 μL 10% sodium dodecyl sulfate (SDS) were added. Lysates were incubated at 65°C for 1.5 h with occasional stirring. Next, SDS was precipitated out by addition of 1/10 vol of 3 M potassium acetate (pH 5.5) and centrifuged at 21,000 × g for 10 min. Totals of 1/10 vol of 3 M sodium acetate (pH 5.5) and 2 vol of 100% ethanol were added and DNA was precipitated for 1 h at −20°C. DNA was spun down at 21,000 × g for 10 min and washed twice with 75% ethanol. Pellets were resuspended in nuclelease-free H2O and genomic DNA was quantified with the Qubit dsDNA Broad Range kit (Invitrogen).

Equal amounts of DNA (1.5 μg total) were digested with BamHI overnight and then run on a 0.7% agarose gel at 35 V for 20 h. To control for loading, equal amounts of sample were loaded on a separate 0.7% agarose gel, which ran for 4 h. For Southern blotting, gels were washed sequentially (0.25 M HCl; 0.5 N NaOH and 1 M NaCl; 0.5 M Tris (pH 7) and 3 M NaCl) for 2 × 15 min each and then blotted overnight onto a nylon membrane (NJOHY000101; GE Water & Process Technologies). Then, DNA was cross-linked in a UV Stratalinker 2400 (Stratagene) and then hybridized overnight at 42°C with radioactive P32 labeled probe against RDN25 and NPR2, respectively. RDN25 probe was made by PCR with oligos 5′-TATTACGCTGCGGCAAGAATCCAGCA-3′ and 5′-TACCAGGAAGGGAATCTGATGT3′ on pDL5 as template (28). NPR2 probes were made with oligos 5′-CTACCATGTTTCTGACTCIT3′ and 5′-CTAGATGTTGGCGGATGATGA-3′ on the NPR2 vector from the centromere-based molecular barcoded yeast plasmid collection (52). Blots were used to expose storage phosphor screens, which were then imaged on a Typhoon FLA 9500 (GE Healthcare).

Whole-Genome Sequencing and Copy Number Estimation. Cells were grown to saturation in YPD overnight in 24-well plates and washed one time with H2O and then genomic DNA was extracted using the YeastStar Genomic DNA kit (D2202, Zymo Research). The input sample was normalized to 60 ng and fed into the Illumina DNA prep kit (20018705; Illumina) for transposition-based library preparation per vendor's instructions using six cycles of PCR. The resulting libraries were quality controlled and quantified on a Fragment Analyzer (Agilent) using the 50-kb extended-ladder Genomic DNA kit (DNF-467-0500; Agilent). Libraries were pooled at equal concentrations and sequenced on the Illumina NovaSeq with 150 bp pair-end reads. Reads were aligned to the S. cerevisiae genome (sacCer3, Release 64) using bowtie2 version 2.3.5.1 with default parameters. Sequence variations were called using the bcftools software package. Copy number was estimated by calculating the average read depth at three locations on chromosome 12 (Chr12): two single-copy loci (Chr12:760,000..790,000 and Chr12:160,000..190,000) and one in the rDNA locus (Chr12:451,575..468,931). Copy number was estimated to be the following: median(rDNA locus depth)/mean[median[Chr12-L1 depth], median[Chr12-L2 depth]]. Samples with large GC sequencing bias were excluded.

CHEF Gels. Approximately 10^8 cells per sample were resuspended in 50 mM EDTA and then mixed with 1% low-melt agarose (SeaPlaque GTG agarose; Lonza) in 50 mM EDTA. The agarose/cell mix was transferred to CHEF Disposable Plug Molds (1703713; Bio-Rad) and cooled for 15 min at 4°C. Plugs were incubated for 4 h at 37°C in 1 mL of spheroplasting solution (1 M Sorbitol, 10 mM Tris-HCl (pH 8.0), 14 mM beta-mercaptoethanol, 20 mM EDTA, 0.5 mg/mL Zymolyase) and then washed for 15 min in LDS solution (1% lithium dodecyl sulfate, 10 mM Tris-HCl (pH 8.0), 100 mM EDTA). Plugs were then incubated overnight in fresh LDS solution at 37°C, washed three times for 20 min with NDS solution (2 mM Tris base [pH 9.5], 1% Sarkosyl, 100 mM EDTA) and then washed five times with Tris (10 mM EDTA) (1 mM) (pH 8.0). For the digest, plasmids were washed three times for 20 min with 1 mL 1× NEB Buffer 4 + 1 × BSA (New England Biolabs) and then covered with 50 μL digestion solution (1.3 μL BamHI-HF, 1× NEB Buffer 4, 1× BSA) and incubated for 5 h at 37°C.

For gel casting, 0.8% low electroendosmosis agarose (SeaKem LE Agarose; Lonza) was melted in 0.5× Tris-borate-EDTA buffer (TBE; 1×: 45 mM Tris, 45 mM borate, 1 mM EDTA) and kept at 50°C. Plugs, including one with Hansenula wingei standard ladder (170-3667; Bio-Rad), were transferred onto the gel comb and excess liquid was dried off with a Kimwipe. The comb was then carefully placed into the casting tray and melted agarose was poured around it. Once the gel was solidified, it was transferred into a CHEF gel module (CHEF-DRII; Bio-Rad) containing 0.5× TBE buffer. The gel was run for 32 h with settings optimized for a size range of 0.44 to 2.3 Mb. Southern blot was performed as above, except the probes were made by PCR with digoxigenin-labeled dUTP using RDN25 oligos as above (11277065910; Roche). Blots were probed with Anti-Digoxigenin-AP Fab fragments (11093274910; Roche) and visualized with CDP-Star (11685627001; Roche).

RNA Extraction. Samples were processed similarly to that described in Hendrickson et al. (27). Frozen cell pellets (~10^7) were resuspended in 200 μL of
Lysis buffer (10 mM Tris [pH 8.0], 0.5% SDS, 10 mM EDTA). Following the addition of 200 μL acid phenol (pH 4.3), samples were vortexed for 30 s. Samples were incubated at 65 °C for 30 min in a thermomixer with intermittent shaking (2,000 rpm for 1 min every 15 min). A total of 400 μL of ethanol was then added and the RNA was purified using the Direct-zol RNA Miniprep Plus kit (R2071; Zymo Research) according to the manufacturer’s protocol, including the optional DNase digestion step. RNA integrity was confirmed using an Agilent Bioanalyzer.

RNA-Seq Library Preparation. RNA-Seq libraries were processed similarly to that described in Hendrickson et al. (27). No-selection libraries were generated using the NEBNext Ultra II Directional RNA Library Prep Kit (PN7760L; Illumina) without the polyA selection step. Given the concentration of RNA is much higher for total unselected RNA, 10 ng of total RNA input and the fragmentation time was increased to 20 min (vs. 15 min standard) to ensure efficient fragmentation of rRNA and proceeded with the vendor’s protocol. Traditional RNA-Seq libraries were generated using the NEBNext Ultra II Directional RNA Library Prep Kit using 250 ng of input and vendor’s protocol with polyA selection. All libraries were sequenced on an Illumina NOVASEQ with 150 × 150 paired-end reads.

RNA-Seq Data Processing and Analysis. RNA-Seq data were quantified using Salmon-0.8.1 with two separate annotation indexes, as described in ref. 27.

- open-reading frames (ORFs) as defined by Saccharomyces Genome Database (SGD), and
- “complex transcriptome” index created by aggregating ORFs, their longest annotated untranslated regions (UTRs), and all noncanonical and noncoding transcripts pulled from SGD.

Salmon was run using the “quant” command with the following settings: --libType ISR --useVheader[] --numBootstraps 30 --incompatiblePrior 0.

Salmon quantification was prepared and processed for differential expression analysis using Tximport (53). Differential gene expression analysis was performed with DESEQ2 using a Wald test and replicative lifespan as a numeric covarion. Eq. 1 defines the complete hazard function, where the baseline hazard is scaled by the sum of a number of partial hazards, described in Eqs. 2-5. Eq. 2 describes the effects of rDNA CN, modeled as two factors—the number of repeats above/below a normal copy number of 150 (dNDA1 and rDNA2, respectively). The resulting piecewise linear fit allowed the model to capture the nonlinearity observed above 150 repeats. Eq. 3 describes the contribution of any experiment-to-experiment batch effects. Eq. 4 describes the contribution of any particular mutation. Eq. 5 describes the interaction terms between rDNA CN and rDNA2, respectively.

Data Availability. Code and data used to generate the figures are available on GitHub at https://github.com/calicoro/DNAcN (56). Description of the strains used in this study and data used to generate figures (e.g., cell and growth measurements, gel quantification, and statistical analyses) are available in Dataset S1. Raw and processed images have been archived and can be made available upon request. Whole-genome sequencing is deposited at the NCBI Sequencing Read Archive (PRJNA796586) (57). RNA-Seq and ATAC-Seq data are deposited at the Gene Expression Omnibus (GSE193600) (58).

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