rDNA copy number variation alters response to common growth environments in yeast

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Abstract

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Ribosomal DNA (rDNA) in eukaryotes is maintained in hundreds of copies with significant rDNA copy number variation among individuals within a species. For instance, the rDNA copy number across wild isolates of the budding yeast *S. cerevisiae* ranges from 90 to 300 copies. We addressed two major questions concerning rDNA copy number variation: (1) why do cells maintain an rDNA copy number in this range and (2) are there functional consequences due to naturally occurring rDNA copy number variation? We investigated the effects of rDNA copy number variation on *S. cerevisiae* fitness using isogenic strains that have from 35 to 200 rDNA copies. In standard growth conditions, we found that fitness gradually increases with rDNA copy number until a plateau is reached, spanning from 98 to 160 rDNA copies, well within the

range observed across diverse *S. cerevisiae* strains. However, rDNA copy number-dependent fitness differed across environments: strains with higher rDNA copy numbers show greater fitness when presented with stressful conditions. Moreover, the gradual fitness change observed in standard growth conditions gave way to strong threshold effects. Our results suggest that there are selective pressures that drive rDNA array maintenance to a particular copy number and that cells maintain higher rDNA copy numbers to buffer against environmental stress. The similarity of *S. cerevisiae* rDNA copy number range to the ranges reported in *C. elegans*, *D. melanogaster*, and humans suggests that common mechanisms might maintain rDNA copy number across metazoans and warrant further study.

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Chapter 1. Introduction to this thesis

The nucleolus

All life must respond swiftly to changing environments to ensure survival; these responses are observable at both macroscopic and microscopic levels. Cells react to environmental conditions such as nutrient availability, temperature, reactive oxygen species, and hypoxia. A way cells cope with the changing environment is through directed actions by the nucleolus. The nucleolus is a highly conserved, distinct, and dynamic non-membrane bound nuclear organelle capable of integrating cellular responses to environmental stress (Quin et al., 2014). Its principal function is ribosome biogenesis, the complex multistep process of ribosomal RNA (rRNA) synthesis, modification, ribosomal protein processing and assembly to generate ribosomes. Some additional functions of the nucleolus include apoptosis, DNA damage stress response, cell cycle regulation, genome regulation, rRNA processing, and metabolism (Boisvert et al., 2007; Quin et al., 2014). Nucleoli generally form around actively transcribing regions of the genome known as ribosomal DNA (rDNA).

What is ribosomal DNA?

rDNA is a highly repetitive region of the genome, present in hundreds to thousands of copies, that encodes rRNA which are essential building blocks for ribosomes (Long and Dawid, 1980). The rDNA array can be divided into two structural units: (1) In humans, the 45S array, encoding the 28S rRNA, 5.8S rRNA, and 18S rRNA, and (2) the 5S array, encoding the 5S rRNA. The genomic regions containing rDNA vary between species. For example, the rDNA

encoding the three major rRNA genes (28S, 5.8S, and 18S) are found on five acrocentric chromosomes in humans and on six chromosomes in mice (Cerqueira and Lemos, 2019; Parks et al., 2018; Stults et al., 2008). In *Drosophila melanogaster*, the three major rRNA genes are found on chromosomes X and Y (Paredes et al., 2011). In *Caenorhabditis elegans*, the three major rRNA genes reside on chromosome I (Morton et al., 2023). Finally, in *Saccharomyces cerevisiae*, the 25S, 5.8S, and 18S are found on chromosome XII (Hall et al., 2022). The 5S array is found on different chromosomes in all the organism listed, except for *S. cerevisiae* where it is integrated with the other three rRNA genes on chromosome XII.

Given the nature of the rDNA, different transcriptional machinery must be dedicated to properly transcribe all the major rRNA species. The 45S rRNAs are transcribed by RNA Polymerase I (Pol I) while RNA Polymerase III (Pol III) transcribes 5S rRNA (Quin et al., 2014; Cerqueira and Lemos, 2019; Ferreira et al., 2020). Many eukaryotic organisms maintain a specific range rDNA copies for sufficient ribosome biogenesis. For instance, humans have 100 – 600 rDNA copies per haploid genome while mice have 74 to 419 rDNA copies per strain (Hall et al., 2021; Parks et al., 2018). *D. melanogaster* have 40 – 300 rDNA copies per haploid genome, *C. elegans* have 70 – 400 rDNA copies per haploid genome, and 500–2,500 rDNA copies in *Arabidopsis thaliana* (Mohan and Ritossa, 1970; Morton et al., 2020; Thompson et al., 2013). *S. cerevisiae* has 90 – 300 rDNA copies per haploid genome (Morton et al., 2020). Typically, only a fraction of the rDNA copies are actively transcribed while many other rDNA copies are silenced (Schneider 2012). The malleable nature of the rDNA array results in reversible dynamic changes to rDNA copy number in response to environmental conditions and may play a role in phenotypic

variation of organisms (Aldrich and Maggert, 2015; Ide et al., 2007; Jack et al., 2015; Kobayashi et al., 1998; Lu et al., 2018; Nelson et al., 2019; Salim et al., 2017).

Why should we care about ribosomal DNA (rDNA)?

All life must respond swiftly to changing environments to ensure survival; these responses are observable at both macroscopic and microscopic levels. Cells react to environmental conditions such as nutrient availability, temperature, reactive oxygen species, and hypoxia. A way cells cope with the changing environment is through directed actions by the nucleolus. The nucleolus is a highly conserved, distinct, and dynamic non-membrane bound nuclear organelle capable of integrating cellular responses to environmental stress (Quin et al., 2014). Its principal function is ribosome biogenesis, the complex multistep process of ribosomal RNA (rRNA) synthesis, modification, ribosomal protein processing and assembly to generate ribosomes. Some additional functions of the nucleolus include apoptosis, DNA damage stress response, cell cycle regulation, genome regulation, rRNA processing, and metabolism (Boisvert et al., 2007; Quin et al., 2014). Nucleoli generally form around actively transcribing regions of the genome known as ribosomal DNA (rDNA).

rDNA's role in cancer development can be further seen pharmacologically by the mode of chemotherapeutic agents' activity. For instance, cisplatin, a well-known platinum-based anticancer drug indicated for bladder, lung, ovarian, and testicular cancer treatment, was thought to mainly function by inducing DNA damage through platinum-DNA adduct. It was not until recently that researchers discovered that cisplatin inhibits ribosome biogenesis by displacing the upstream binding factor (UBF) from the rDNA (Drygin et al., 2010; Hamdane et al., 2015).

Other chemotherapeutics were later shown to interfere with various aspects of Pol I transcription, which also then inspired the development of selective Pol I inhibitors (Burger et al., 2010; Ferreira et al., 2020). There continue to be additional discoveries that cement rDNA as a region of the genome that plays a more active role in the growth, development, and physiology in organisms than previously assumed. The pursuit of uncovering mysteries surrounding rDNA is a source of intrigue and rDNA should be considered as a factor in phenotype and disease.

Impact of rDNA copy number variation on phenotype in metazoans

An important question that comes up is whether rDNA copy number variation is implicated in phenotypic variation in various organisms. Generally, rDNA copy number reductions well below the natural distribution in a given species lead to a wide range of phenotypes related to impaired ribosome biogenesis and organismal development. For instance, flies and chickens with extremely low rDNA copies show arrested development and cell death (Delany et al., 1994). C. elegans with deleted rDNA arrays can complete embryogenesis due to the maternal pool of ribosomes but arrest at the first larval stage (Cenik et al., 2019). A recent study from our lab characterizes developmental delays and morphological defects in C. elegans strains with lower rDNA copy number, highlighting tissue specific rDNA requirements and suggesting that rDNA copy number is important for fitness and development of metazoans (Morton et al., 2023). Additionally, substantially reduced rDNA copy numbers in D. melanogaster that still allow for sufficient ribosome biogenesis have been associated with the bobbed phenotype, characterized by delayed development, small bristles, and abdominal etching (Ritossa and Atwood, 1966, 1966). There is also a report showing transgenerational loss of rDNA copies in D. melanogaster with TOR hyperactivity tied to excess dietary yeast consumption (Aldrich and Maggert, 2015).

Changes in rDNA copy number have also been implicated in gene regulation because rDNA copy number reduction can affect the epigenetic state of the genome. Low rDNA copy number in *D. melanogaster* is associated with globally altered gene expression, mitochondrial function, nucleolar size, and morphology differences (Mohan and Ritossa, 1970; Paredes et al., 2011; Paredes and Maggert, 2009a, 2009b; Ritossa and Atwood, 1966). There is strong association between rDNA transcription and nucleolar size. Long-lived *C. elegans* mutants show reduced ribosome biogenesis and smaller nucleoli (Tiku et al., 2017). These findings were also observed in *D. melanogaster* conditions that extend fly lifespan, including dietary restriction, mTOR inhibition with rapamycin, and genetic deletion of the insulin-like peptides *ilp-2-3,5* (Tiku et al., 2017).

Impact of rDNA copy number in human disease

Changes in Pol I transcription and rDNA copy number have been well documented in human cancers. There is elevated Pol I transcription in tumorigenesis generally involving tumor suppressor gene inactivation, oncogene activation, and oncogenic signaling (Bywater et al., 2013; Drygin et al., 2011, 2010; Sharifi and Bierhoff, 2018). For instance, the tumor suppressor p53 indirectly inhibits Pol I activity via its interactions with two components of the selectivity factor 1 (SL-1), TATA-box-binding protein (TBP) and TBP-associated factor 1C (TAF1C), which prevents SL-1 association with UBF (Bywater et al., 2013). Similarly, the tumor suppressor Retinoblastoma (Rb) inhibits ribosome biogenesis by directly interacting with UBF, disrupting the interaction between UBF and SL-1 (Hannan et al., 2000). PTEN, another tumor suppressor, represses RNA Pol I transcriptional activity though its lipid phosphatase activity and inhibits Akt

activation, ultimately leading to the disruption and dissociation of the SL-1 complex from the rDNA gene promoter (Zhang et al., 2005). Loss of *PTEN* has been associated in cancers with mTOR hyperactivity. A study found that human cancer genomes with mTOR hyperactivity have fewer rDNA copies than matched normal genomes (Xu et al., 2017). Using a *PTEN* negative mouse model for leukemia, Xu *et al.* also found that, the hematopoietic cancer stem cells had fewer rDNA copies when compared to normal tissue (Xu et al., 2017).

Besides cancer, there are other human diseases that have been associated with changes in rDNA copy number and Pol I transcription. For instance, some patients with schizophrenia have increased rDNA copy number compared to healthy controls (Chestkov et al., 2018). Additional studies have pointed to increased rDNA transcription in the dorsal raphe nucleus of patients with residual schizophrenia (Krzyżanowska et al., 2015). rDNA expansion has been observed in *post mortem* samples of parietal cortex of patients that had dementia with Lewy bodies (Hallgren et al., 2014). Altered rRNA gene transcription and increased genomic rDNA content in cerebrocortical samples was noted in patients with Alzheimer's disease (Pietrzak et al., 2011). Patients with chromosomal abnormalities such as Down's Syndrome have more active ribosomal genes, due to the presence of additional copy of chromosome 21 (Lyapunova et al., 2017). There have been reports of genomic instability of the rDNA in congenital diseases linked to increased cancer risk, such as Bloom syndrome and ataxia-teleangiectasia (Killen et al., 2009).

Although the constellation of disorders that impact ribosome biogenesis (ribosomopathies) often involve genes outside of the rDNA locus, many features of ribosomopathies include increased cancer risk and tissue-specific abnormalities. For instance, Diamond-Blackfan anemia

(DBA), characterized by a chronic constitutional aregenerative anaemia with absent or decreased erythroid precursors in bone marrow, can include thumb, upper limb, craniofacial abnormalities in addition to increased risk of hematological malignancies (Draptchinskaia et al., 1999). DBA is associated with mutations in ribosomal protein S19 (*RPS19*) as well as *RPL5*, *RPL11* and *RPS10* (Kampen et al., 2020). While there have been associations with rDNA copy number and human disease, more studies are needed to make causal links between this genomic region and complex genetic disorders.

Impact of rDNA copy number variation on phenotype in *S. cerevisiae*

rDNA copy number variation also impacts phenotypes in the budding yeast *S. cerevisiae*, a unicellular eukaryotic organism. Yeast strains that have below 30 rDNA copies grow more slowly due to insufficient rRNA transcription for ribosome biogenesis (French et al., 2003; Ide et al., 2010; Mohan and Ritossa, 1970). *S. cerevisiae* strains with about 30 rDNA copies show sufficient ribosome biogenesis with no apparent growth defects but such strains show increased sensitivity to DNA damaging agents such as methyl methanesulfonate, ultraviolet radiation, and hydroxyurea (Ide et al., 2010; Kwan et al., 2023). Sensitivity to DNA damaging agents decreases with increasing rDNA copy number. Moreover, yeast strains with reduced rDNA arrays (~35 copies) show early rDNA replication and delayed genome replication (Foss et al., 2017; Kwan et al., 2023; Yoshida et al., 2014).

Much of what we know about rDNA comes from studies in yeast. At the genomic level, rDNA copy number impacts the epigenetic state of the *S. cerevisiae* genome. For instance, yeast strains with short rDNA arrays show increased chromatin silencing elsewhere that is mediated

through the actions of Sir2 (Michel et al., 2005). rDNA copy number changes have also been implicated in replicative life span: a type of lifespan assay that measures the number of asymmetric mitotic divisions that individual yeast cells can undergo. Yeast strains that are wild-type for *FOB1* with fewer rDNA copies in the endogenous chromosomal array show shorter lifespans, and yeast strains with more chromosomal rDNA copies show longer lifespans (Hotz et al., 2022). The difference in yeast lifespan is related to the level of extrachromosomal rDNA circles (ERCs) that increases when chromosomal rDNA decreases in a *FOB1* wild-type strain (Sinclair et al., 1997). rDNA copy number has no effect on replicative lifespan in strains if *FOB1* is deleted (Crane et al., 2019; Hotz et al., 2022). Given all the knowledge we gained from studies in yeast, there are still many questions regarding the role rDNA copy number in yeast fitness.

How is rDNA copy number variation measured?

Given the nature of the rDNA locus, attempts to accurately measure rDNA copy numbers have been difficult. Not only does the position of rDNA arrays differ between organisms, but the copy number, structure, sequence, and repeat length make determining rDNA copy number a challenge. There are a few major techniques used to measure rDNA copy number: (1) Contour-clamped homogeneous electric field (CHEF) gel electrophoresis, (2) droplet digital PCR (ddPCR), and 3) whole genome sequencing (WGS).

First, the gold-standard for determining rDNA copy number is CHEF gel electrophoresis. CHEF gel electrophoresis works by periodically changing the direction of the electrical fields. The alternation in electric field forces DNA molecules to change orientation and reptate through

the agarose gel matrix, enabling the resolution of large DNA molecules up to 10 megabases (Herschleb et al., 2007; Morton et al., 2020). Although rDNA copy number can be visualized directly and calculated in conjunction with Southern blotting techniques, there are notable disadvantages. CHEF gel electrophoresis takes 2-3 days outside of sample preparation and is unable to resolve DNA above 10 megabases, which limits the organisms that can be examined. For instance, humans have hundreds of rDNA copies with each repeat unit being ~43 kilobases (Cerqueira and Lemos, 2019; Stults et al., 2008), well outside the size that can be resolved by CHEF gels.

Second is ddPCR, which is another commonly used method used to analyze copy number of various repetitive elements in genomes, including rDNA copy number. ddPCR works by partitioning a sample, that contain all reagents necessary for two fluorescence assays, into thousands of individual droplets prior to PCR amplification. After PCR amplification, the number of droplets containing rDNA fluorescence signal specific for rDNA fragments are used to calculate rDNA copy number relative to the fluorescence signal of droplets containing the reference genomic sequence (Bell *et al.* 2018). ddPCR allows for rDNA copy number estimates; however, the absolute values of rDNA copy number do not always agree with estimates found in CHEF gel electrophoresis. In fact, data from our lab show that ddPCR underestimates rDNA copy number by 11 – 41% (Morton et al., 2020).

Lastly, WGS is commonly used to estimate copy number through reads aligned to the repetitive region relative to the rest of the genome. WGS accuracy in estimating copy number in large highly repetitive genomic regions can be low. Findings from our lab indicate that rDNA

copy number estimates from WGS vary significantly from CHEF gel electrophoresis rDNA copy number estimates (Morton et al., 2020), likely due to batch effects during preparation of sample libraries for WGS sequencing. As sequencing methods and imaging technologies improve, interest in repetitive elements will increase and provide more opportunities to explore the fundamental mechanisms that connect rDNA copy number and disease states.

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Chapter 2. rDNA copy number variation alters response to common growth environments in yeast

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Abstract

Ribosomal DNA (rDNA) in eukaryotes is maintained in hundreds of copies with significant rDNA copy number variation among individuals within a species. For instance, the rDNA copy number across wild isolates of the budding yeast *S. cerevisiae* ranges from 90 to 300 copies. We addressed two major questions concerning rDNA copy number variation: (1) why do cells maintain an rDNA copy number in this range and (2) are there functional consequences due to naturally occurring rDNA copy number variation? We investigated the effects of rDNA copy number variation on *S. cerevisiae* fitness using isogenic strains that have from 35 to 200 rDNA copies. In standard growth conditions, we found that fitness gradually increases with rDNA copy number until a plateau is reached, spanning from 98 to 160 rDNA copies, well within the range observed across diverse *S. cerevisiae* strains. However, rDNA copy number-dependent fitness differed across environments: strains with higher rDNA copy numbers show greater fitness when presented

with stressful conditions. Moreover, the gradual fitness change observed in standard growth conditions gave way to strong threshold effects. Our results suggest that there are selective pressures that drive rDNA array maintenance to a particular copy number and that cells maintain higher rDNA copy numbers to buffer against environmental stress. The similarity of *S. cerevisiae* rDNA copy number range to the ranges reported in *C. elegans, D. melanogaster,* and humans makes this an intriguing avenue for further studies.

Introduction

Ribosomal DNA (rDNA) encodes the sequences that are transcribed into the ribosomal RNAs (rRNAs) which provide the structural and catalytic properties of ribosomes. In eukaryotes, rDNA is maintained in tandem repetitive arrays of hundreds of copies per cell (Hall et al., 2022; Kobayashi et al., 1998; Nelson et al., 2019). rDNA's repetitive nature makes it prone to copy number variation within species: 90 to 300 copies in the yeast *Saccharomyces cerevisiae*, 70 to 400 copies in the nematode *Caenorhabditis elegans*, 80 to 600 copies in the fly *Drosophila melanogaster*, 500 to 2,500 copies in the plant *Arabidopsis thaliana*, and 100 to 600 copies in humans (Hall et al., 2022, 2021; Mohan and Ritossa, 1970; Morton et al., 2020; Thompson et al., 2013). Given the breadth of variation, both in copy number and the fraction of the genome it represents, rDNA may be an underappreciated source of genetic variation influencing fitness.

rDNA copy number variation is increasingly recognized as an influential factor in cell physiology. rRNA makes up approximately 80% of total cellular RNA, and a minimum number of rDNA copies is required to sustain the high levels of rRNA transcription required for ribosome biogenesis (Warner, 1999). For *S. cerevisiae*, this minimum number of rDNA copies is 35 (French et al., 2003; Ide et al., 2010; Kim et al., 2006; Kwan et al., 2023), a value which is significantly lower than observed in wild yeast isolates and laboratory strains (Hall et al., 2022; Morton et al., 2020). Strains with substantially reduced rDNA copy numbers sufficient for ribosome biogenesis (35-40 copies) show compromised genome replication and increased susceptibility to DNA damage (Ide et al., 2010; Kwan et al., 2023; Salim et al., 2017). In animals and humans, reductions of rDNA copy number below the naturally occurring range of

variation is associated with developmental abnormalities and disease phenotypes (Morton et al., 2023; Ritossa and Atwood, 1966; Valori et al., 2020; Xu et al., 2017). However, the intrinsic differences among tissues in multicellular animals complicates parsing out how exactly rDNA copy number variation affects cellular fitness.

We therefore wanted to investigate in yeast cells (1) whether naturally occurring rDNA copy number variation affects fitness, (2) if increases in rDNA copy number gradually affect fitness or if there are copy number thresholds, and (3) what additional biological processes are affected by substantial reduction in rDNA copy number. To tackle these questions, we generated a set of isogenic strains in the yeast *S. cerevisiae* containing between 35 to 200 copies of rDNA, which spans the range from the threshold required for ribosome biogenesis up to the natural variation present in the species. We examined these strains for effects of rDNA copy number variation on competitive fitness in different growth conditions, and used transcriptome analysis of a strain with 35 rDNA copies and a wild-type strain to identify potentially altered genetic pathways.

We found that rDNA copy number variation profoundly affects fitness in yeast. In standard laboratory conditions, strains below ~100 copies showed reduced fitness that scaled with the severity of copy number reduction. In contrast, strains with copy numbers ranging from 98 to 160 rDNA copies displayed similarly high fitness, establishing a fitness plateau that roughly coincided with the naturally occurring rDNA copy number range reported for yeast (Hall et al., 2022; Morton et al., 2020). Additional rDNA copies beyond this plateau were not beneficial: Fitness decreased in strains with more than 160 rDNA copies. Our transcriptome

analysis comparing the 35 rDNA copy number strain and a wild-type strain showed the expected altered expression of genes involved in mitigating DNA replication stress, in addition to expression changes in canonical stress response genes. We followed up on these findings with phenotyping and competition assays at increased temperature and with a nonfermentable carbon source. We found that the tested rDNA copy number variants showed profoundly different fitness in different growth conditions, and that the gradual fitness changes observed in standard growth conditions had given way to stark threshold effects. Taken together, our findings suggest that the wide range of rDNA copy numbers observed in wild yeast strains buffers the strains' exposure to varied environments.

Results

rDNA copy number affects fitness in standard growth conditions

To generate a set of isogenic *S. cerevisiae* strains with variable rDNA copy numbers we started with a *fob1Δ* strain with 35 copies (Ide et al., 2010; Kobayashi et al., 1998; Kwan et al., 2023). We screened for clones with different rDNA copy number after a transient reintroduction of *FOB1*. The strain with 200 rDNA copies was isolated by chance during a cross. We verified rDNA copy number for three clones from each strain with CHEF gel electrophoresis and Southern blotting (Figure 1A,B; Kwan et al., 2016; Morton et al., 2020; Tsuchiyama et al., 2013). We hypothesized that strains with reduced rDNA copy number would be less fit than strains with higher rDNA copy numbers and that fitness will increase as rDNA copy number increases.

All of the strains had similar growth rates (**Figure S1A**), but to magnify small phenotypic differences between strains (Conti et al., 2022) we performed competition assays to assess the relative fitness of strains carrying specific rDNA copy number variants. The competitor strain for each experiment was a GFP-tagged strain with 180 rDNA copies that was otherwise isogenic. To control for possible fitness effects of GFP, we also tested the 180 copy rDNA strain against its isogenic GFP 180 rDNA strain.

To determine the competitive fitness of each strain (in triplicate) we mixed the two starting cultures in an approximately 1:1 ratio and grew them to saturation (Gresham and Dunham, 2014). Every 24 hours, we took a sample to measure the ratio of GFP-positive to GFP-negative cells by flow cytometry. We then diluted the competition culture 1:1000 into fresh media and grew them again to saturation, allowing for approximately 10 generations of growth.

We repeated this process for approximately 50 generations. Upon completion of the growth experiments we estimated fitness by tracking the ratios of the GFP competitor strain to a given test strain in each competition over the indicated number of cell divisions. The comparison of the GFP competitor strain (180 rDNA copies) with the strain carrying the 180 rDNA copy number variant yielded an estimate of the expected fitness costs of GFP expression in each of three experimental replicates (0.08% per generation in **Figure 1C; Figure S1B,C**).

We noticed three trends in our fitness data: (1) reduced fitness in strains with fewer than 98 rDNA copies, (2) a fitness plateau for strains with 98 to 160 rDNA copies, and (3) reduced fitness in strains with more than160 rDNA copies. The strain with the fewest rDNA copies (35 copies) showed the greatest fitness defect and strain fitness gradually improved as the rDNA copy number increased from 35 to 55 (**Figure 1C**) and more gradually after 80 copies. Strains with 98 to 160 rDNA copies showed high fitness in standard growth conditions (**Figure 1C, Figure S1**), mirroring the distribution of rDNA copy genotypes in wild *S. cerevisiae* isolates (**Figure 1C, inset**). Lastly, adding rDNA copies above of this newly defined fitness plateau resulted in reduced fitness. A strain with 180 rDNA copies showed reduced fitness compared to the strains with 98 to 160 rDNA copies, but greater fitness than the strains with fewer than 98 rDNA copies (**Figure 1C**). In all three replicate experiments, strains with 200 rDNA copies showed reduced fitness compared to the GFP competitor strain, the 180 rDNA copy number strain, and the strains carrying 98—160 copies (**Figure 1C, Figure S1**). We conclude that rDNA copy number variants with fewer than 98 and more than 160 rDNA copies show reduced fitness in this S288c strain background under standard growth conditions. Overall, fitness appeared to change gradually without strong threshold effects.

rDNA expansion decelerates upon reaching the fitness plateau of ~100 rDNA copies

Strains with substantially reduced rDNA arrays increase their rDNA copy numbers back to wild-type levels when *FOB1* is re-introduced (Kobayashi et al., 1998). The re-introduction of *FOB1* and passaging cells to an rDNA copy number equilibrium enables a more nuanced analysis of the fitness effects of rDNA copy number than the preceding competition experiments. Instead of competing two strains with fixed rDNA copy numbers, this experiment relies on naturally occurring changes in rDNA copy number among the *FOB1* cells in the culture and the resulting selection among different rDNA copy number variants. Moreover, assessing fitness of wild-type *FOB1* cells avoids putative unrelated effects of the *fob1Δ* mutation. We hypothesized that the expansion of rDNA arrays upon *FOB1* reintroduction would slow when it reached ~100 copies in our growth conditions, which would be consistent with fitness being the primary driver for rDNA expansion. Alternatively, rDNA copy number may expand to the 150 rDNA copies previously reported for this strain background (Ide et al., 2013; Kwan et al., 2016; Morton et al., 2020) supporting a model where strain-specific rDNA copy number is determined by a counting mechanism (Iida and Kobayashi, 2019a, 2019b).

To conduct this experiment, we reintroduced *FOB1* into a short rDNA strain by crossing a 35 rDNA *fob1Δ* strain with a 170 rDNA *FOB1* strain, picked three 35 rDNA copy number *FOB1* spores, and passaged the resulting cultures (and the two parental strains) for 300 generations. Samples were collected every 30 generations for a total of 300 generations for subsequent CHEF gel electrophoresis and Southern blotting to determine rDNA copy number (**Figure 2A,B**).

After approximately 30 generations, spore #1 – #3 had an average of ~56 rDNA copies (Figure 2C). After 150 generations, spore #1 – #3 had ~100 rDNA copies, and then ~107 rDNA copies after 300 generations, suggesting that rDNA copy number expansion slows once ~100 rDNA copies is reached. As expected, the parental strains did not change rDNA copy number significantly after 300 generations. We conclude that there appears to be no significant fitness advantage in having more than ~100 rDNA copies in standard growth conditions, which is consistent with our observation of a fitness plateau of 98 to160 copies in standard growth conditions.

An rDNA strain with 35 copies shows altered gene expression specifically in late S-phase

Strains with substantial reduction of rDNA copy number (20-40 copies) show increased sensitivity to mutagens, defects in genome replication, and cell cycle misregulation (Ide et al., 2010; Kwan et al., 2023). Although these phenotypic effects themselves might suffice to cause the observed fitness defects, we wanted to employ RNA-seq analysis to discover possible other biological processes that may be altered between a short (35 copies) and a wild-type (180 copies) rDNA strain. Because of the known role of rDNA copy number in causing genome replication delays (Kwan et al., 2023), we collected cells in late S-phase in addition to asynchronous, logarithmically growing cells. We identified six genes that were differentially expressed (DEGs) between the 35 and the 180 rDNA copy number strains in asynchronous cultures (p-adj < 0.01), and 708 genes that were differentially expressed between these strains in late S-phase, with three genes overlapping between the two sets (**Figure 3**). Of these, *PRP11*, implicated in splicing, and *STR3*, implicated in peroxisome function, were expressed at lower levels in the 35 rDNA copy

number strain. The third gene, *HSP12,* was expressed at higher levels in the 35 rDNA copy number strain; this gene is induced in response to many stressful conditions, including heat stress, ethanol and salt stress, in addition to DNA replication stress. The small number of genes showing expression differences between the 35-copy and the 180-copy rDNA strain in asynchronous culture is consistent with previous observations that strains with these rDNA copy numbers do not differ significantly in growth rates, rRNA expression, or ribosome function (French et al., 2003; Ide et al., 2010; Kim et al., 2006; Kwan et al., 2023; Mohan and Ritossa, 1970).

However, in late S-phase, genes upregulated in the 35 rDNA copy number strain were enriched for GO terms related to ribosomal structure and assembly. For example, 115 of the 420 upregulated genes in the 35 rDNA copy number strain belonged to the 'structural constituent of ribosome' category (GO:0003735). In contrast, the genes downregulated in the 35 rDNA copy strain (n=288) were enriched for GO categories related to protein degradation (*e.g.,* GO: 0051603 and GO:0000502).

To gain further insight in the significance and meaning of these S-phase-specific expression changes, we focused on genes with at least a 1.5-fold change in expression. We found 88 genes that were upregulated in the short rDNA strain (adj-pval<1e-6), with GO term enrichments for structural constituent of chromatin, protein heterodimerization activity, and nucleosome. The top ten most upregulated genes were *HSP12*; five small nucleolar RNAs involved in rDNA processing; *PCNA*, a sliding replication clamp; *DDR2*, a multi-stress response gene named for its function in the DNA damage response; *SOE1*, a tRNA gene and suppressor of

CDC8, a kinase functioning in the biosynthesis of deoxyribonucleotides; and *SIP18*, a gene involved in salt stress.

There were 83 downregulated genes in the short rDNA strain (adj-pval<1e-7), with GO term enrichments for protein folding, response to stress, and MCM helicase complex. The latter enrichment could be consistent with the genome replication defects in this strain; the MCM helicase is essential for DNA replication initiation and elongation, and is recruited to the origins of DNA replication as part of the pre-replicative complex (Bell and Labib, 2016). The top ten most strongly downregulated genes included both HSP70s, (*SSA1* and *SSA2)*, *SIS1*, a co-chaperone of HSP40, *STI1*, a co-chaperone of HSP90, and *HSP104*, a disaggregase functioning with HSP70 and HSP40 required for the acquisition of thermotolerance (Lindquist and Kim, 1996). The other genes induced as part of the canonical heat stress response, the two HSP90s HSC82 and HSP82 were the 13th and 15th most strongly downregulated genes, respectively. While some of observed expression genes and GO annotations were consistent with prior studies (Ide et al., 2010, 2007; Kwan et al., 2023; Salim et al., 2017), the unexpected S-phase-specific finding of altered chaperone expression warranted further investigation as to whether rDNA copy number variation might play a role in response to stress or altered growth conditions.

rDNA copy number variants show altered fitness in different growth conditions

To characterize possible stress response phenotypes associated with rDNA copy number variation, we performed spot assays to assess heat stress, osmotic stress, ethanol stress, and thermotolerance acquisition in our strains. rDNA copy number variation did not affect the

response to the examined stresses or the acquisition of thermotolerance in these assays (**Figure S2**).

To determine whether rDNA copy number variation may cause more subtle phenotypic effects in response to heat stress, we performed competition experiments at 37°C, as described before. Our results differed markedly from those observed in standard growth conditions: (1) the gradual fitness increase with increasing rDNA copy number gave way to a pronounced fitness threshold coinciding with ~98 copies; and (2) the fitness plateau shifted to include copy numbers up to 200 (**Figure 4A, Figure S3A**). Although the 35 rDNA copy number strain still showed by far the greatest fitness defect among tested rDNA copy number variants, compared to its fitness in standard laboratory conditions, it was little affected by heat stress (**Figure 4B, FigureS3B**). Strains with higher copy numbers from 80 to 95 copies showed sharply decreased fitness in response to heat stress compared to their performance in standard growth conditions (**Figure 4B, FigureS3B**), resulting in the observed fitness threshold. In contrast, the strain with the longest rDNA array of 200 copies showed greater fitness in response to heat stress than in standard growth conditions (**Figure 4B, FigureS3B**).

To rule out the possibility that heat stress altered rDNA copy numbers during the course of these experiments, we examined CHEF gels and Southern blots of all strains at the start of competitions (day 0) and at the end of competitions (day 5). We found that rDNA copy numbers did not change during the heat stress competitions (**Figure 4C,D, FigureS3C,D**).

We next examined if shifts in fitness among the rDNA copy number variants were specific to heat stress or common across other non-standard growth conditions, we performed competition experiments with the nonfermentable carbon source glycerol at 30°C, as described. Our results from glycerol media competitions differed from those obtained in standard laboratory condition and in response to heat stress (**Figure 5A,B, FigureS4A,B**). Remarkably, the strain with the fewest rDNA copies (35) was no longer the strain with the worst fitness. In replicate experiments, this strain showed fitness comparable to or better than the strain with 55 copies; strains carrying 80 and 100 copies performed only slightly better. As for heat stress, we observed a sharp fitness threshold; however, in the presence of glycerol, this threshold shifted from ~98 to 140 copies, with the 100 rDNA copy number strain showing strong fitness defects. The strains with the longer rDNA arrays of 160 to 200 copies performed better compared to their performance in standard growth conditions.

Again, to rule the possibility that glycerol exposure altered rDNA copy numbers during the course of these experiments, we examined CHEF gels and Southern blots of strains at the start and at the end of competitions. rDNA copy numbers did not change during the competitions (**Figure 5C, D, FigureS4C, D**). We conclude that rDNA copy number variation outside and within the naturally occurring range contributes to a strain's fitness in response to different environments. Yeast cells face variable environments throughout their culturing in the laboratory (exponential growth, stationary phase, different carbon sources, freeze-thaw, etc.) and certainly outside of it. Thus, the excess of rDNA copies not required for ribosome biogenesis in standard growth conditions may buffer condition-specific requirements for ribosome biogenesis

and/or genome replication, the two processes already identified to be affected by rDNA copy number variation.

A strain with substantially reduced rDNA copy number generates fewer petites than a wild-type strain

Intrigued by our finding of the profound shift in fitness in response to glycerol, a nonfermentable carbon source, we wanted to examine mitochondrial function in the 35 rDNA copy number strain compared to our wild-type control strain (180 copies). To examine mitochondrial function and mitochondrial genome stability, we measured the frequency of *petite* formation (Dimitrov et al., 2009). *Petites* are yeast colonies that have lost mitochondrial respiration function, and therefore *petite* colonies are much smaller than respiration-competent, *grande* colonies on specialized glycerol media (**Figure 6A**). We performed *petite* frequency assays by selecting 15 medium sized colonies from both strain backgrounds (35 rDNA copies, 180 rDNA copies), diluting them and plating approximately 350 cells onto glycerol-containing plates. After incubation for 5 days at 30°C, we scored all *petite* and *grande* colonies to determine the *petite* percentage for both strains.

To our surprise, the 35 rDNA copy number strain produced significantly fewer *petite* colonies (48.2%) than the 180 rDNA copy number strain (57.5%) (**Figure 6B**). In a replicate plating experiment, we found 43.1% of petites in the 35 rDNA coy number strain versus 57.8% of petites for the 180 rDNA copy number strain, confirming that the former consistently produces fewer *petite* colonies than the latter. At a first glance, this result appears inconsistent with the fact that the 35 rDNA copy number strain shows lower fitness than a 180 rDNA copy

number variant in the glycerol competition experiments. While growth in glycerol and *petite* formation are indeed both associated with mitochondrial function, it is entirely possible that rDNA copy number variation affects these two traits in different ways.

Discussion

The phenotypic consequences of naturally occurring rDNA copy number variation have only recently been investigated in a systematic manner (Ide et al., 2010; Kwan et al., 2023; Morton et al., 2023; Paredes et al., 2011). Here, we generated a set of rDNA copy number variants in otherwise isogenic *S. cerevisiae* strains in order to study the fitness consequences of rDNA copy number variation below and within the naturally occurring range of variation. In standard laboratory conditions, the strains with 98 to 160 rDNA copies showed the highest fitness. This copy number range overlaps surprisingly well with the rDNA copy number distribution found in wild yeast isolates (Hall et al., 2022; Morton et al., 2020). Yeast strains with rDNA copy numbers outside this range, both higher and lower, showed reduced fitness with the extreme variants, especially in the lower values, exhibiting more severe fitness defects. Finally, rDNA copy number-dependent fitness is not static: the fitness of rDNA copy variants shifted profoundly in response to different growth conditions, including environmental stress. Our results demonstrate that rDNA copy number variation plays a causal role in the response of yeast cells to different environments.

rDNA copy number variants modulate response to environmental change

In standard laboratory conditions, increasing rDNA copy numbers yielded a gradual increase effect in fitness until reaching the fitness plateau of 98 to 160 rDNA copies. Although the full spectrum of mechanisms behind rDNA copy number-driven fitness remains unknown, the selective advantage conferred by rDNA copy numbers in this range reflects the distribution of copy numbers across diverse *S. cerevisiae* strains. All short rDNA strains with re-introduced *FOB1* rapidly expanded their rDNA arrays to ~100 rDNA copies but further expansion stalled,

consistent with a lack of fitness benefits with further expansion. Of the haploid wild yeast strains previously examined, 75% show rDNA copy numbers within this newly defined fitness plateau (Hall et al., 2022; Morton et al., 2020). The remaining 25% that show higher rDNA copy numbers may experience more diverse environmental conditions than the other strains or carry genetic variants that are associated with rDNA copy expansion.

Previous studies hint at an example that might connect rDNA copy number variation and genetic background. Kobayashi *et al.* report that a short rDNA strain fully expands its array to 150 copies after 150 generations (Kobayashi et al., 1998), which is far higher than the ~100 rDNA copies we observed even after 300 generations. A major difference between the two studies is the strain background: the strains in the earlier study were derived from the W303 background (Nogi et al., 1991; Yano and Nomura, 1991) while our strains were derived from the S288c background. Despite being closely related laboratory strains, W303 and S288c differ in ~7000 non-synonymous gene polymorphisms (Liti et al., 2009; Matheson et al., 2017; Peter et al., 2018) and multiple studies have reported that W303 strains show higher rDNA copy numbers (250-300 copies) than S288c (140-150 copies) (Kwan et al., 2016; Lynch et al., 2019; Michel et al., 2005; Morton et al., 2020). The W303 strain likely harbors genetic variants that facilitate rDNA expansion to higher copy numbers than in S288c strains, such as non-synonymous *SIR2* variants (Jack et al., 2015). Identifying these variants would further our understanding of the regulation of rDNA copy number across all *S. cerevisiae* strains and possibly in metazoans.

We show that environmental stresses alter the relationship between rDNA copy number and fitness. Firstly, the fitness plateau shifts to include higher rDNA copy numbers, and in

glycerol, it excludes the strains with 98 to100 copies that showed high fitness in standard growth conditions. Secondly, we observe a marked copy number threshold in both the heat stress and glycerol competitions as opposed to the gradual fitness change found in standard laboratory conditions. This difference might reflect that there are different molecular underpinnings that drive rDNA copy number-dependent fitness in these different conditions. This threshold result also suggests that rDNA copy number variants below the naturally occurring range, even by only a few copies, exhibit particularly strong deleterious effects in adverse conditions. In other words, the higher naturally occurring rDNA copy number range appears to buffer yeast cells against fluctuations in environment.

This interpretation is consistent with our observation that the S288c background slows rDNA array expansion at ~100 copies and shows high fitness at this copy number, yet the S288c strain typically contains ~150 rDNA copies (Ide et al., 2013; Kwan et al., 2016; Morton et al., 2023). Since the fitness plateau was shifted to higher copy numbers in response to stress, the strain may have been selected for 150 rDNA copies rather than ~100 copies through exposure to a myriad of intermittent laboratory conditions and stresses: cold/freezing temperatures, heat shock, reduced nutrient availability, etc. (Kwan et al., 2016). We speculate that in fluctuating environments the laboratory strain S288c might expand its rDNA array to ~150 copies or even higher copy numbers; however, it is non-trivial to design such an experiment to be informative and artifact-free. At the very least, our results demonstrate that rDNA copy number variation should be taken into account when comparing yeast strains for their response to different experimental growth conditions.

More rDNA is not always better

Our results show that fitness can decrease with higher rDNA copy numbers such as 180 and 200 in standard growth conditions, which may contribute to more sparse representation of wild yeast isolates with higher rDNA copy numbers. The fitness reduction in the 180 and 200 rDNA copy number strains seems inconsistent with a previous model that implicated the excess of rDNA copies over the number required for ribosome biogenesis in maintaining rDNA and genome stability (Ide et al., 2010). If this were the case, additional rDNA copies would be expected to increase fitness rather than reduce it.

A possible mechanism explaining the reduced fitness in strains with increased rDNA copy number is the increased cost of maintaining the additional copies, especially since an extra 25 rDNA copies (~227kb) represent a similar DNA amount as the smaller *S. cerevisiae* chromosomes I (230 kb) and III (316 kb) (Cherry et al., 2012). Given that GFP expression suffices to reduce cell fitness, dedicating resources to maintaining a chromosome's worth of repetitive, presumably silenced DNA could plausibly do the same (Sunshine et al., 2015). Further studies with strains containing a wider range of rDNA copy numbers above the natural range are needed to fully establish the resulting fitness values and distinguish among possible mechanisms. Such strains are not easy to construct; our strain with 200 copies arose by chance.

rDNA copy number matters in metazoans

Although the accurate assessment of rDNA copy number has been a major technical challenge, the phenotypic consequences of rDNA copy number variation has been increasingly studied in multicellular animals and plants (Hall et al., 2022; Kasselimi et al., 2022; Morton et

al., 2023, 2020; Xu et al., 2017). In the nematode *C. elegans*, individual animals with rDNA copy numbers below the naturally occurring range display a gradient of developmental defects, ranging from subtle developmental delays to developmental arrest and strikingly variable morphological defects in postembryonic development (Cenik et al., 2019; Morton et al., 2023). These observations strongly suggest that particular stages of development and particular tissues have specific rDNA copy number requirements, perhaps because each stage and tissue represent different cellular environments. Our finding that rDNA copy number-dependent fitness shifts with environmental conditions is analogous. Although yeast cells do not form specific tissues, they certainly experience a wide range of environmental conditions.

The shifts in environmental response of cells with different rDNA copy number are particularly intriguing in the context of cancer. Reduction of rDNA copy number has been reported in mTOR-related tumors compared to healthy, non-cancerous tissue (Kasselimi et al., 2022; Xu et al., 2017). Because rDNA copy number reduction causes defects in genome replication and cell cycle control in yeast (Ide et al., 2010; Kwan et al., 2023), rDNA copy number reduction in human cells may contribute to the characteristic loss of genome stability that precedes cancer pathogenesis (Hanahan and Weinberg, 2011). Moreover, the reduction in rDNA copy number might shift the fitness of pre-cancerous and/or cancer cells in response to the stressful host environment or treatment conditions, analogous to our observations in yeast.

Acknowledgements

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Figure legends

Figure 1: rDNA copy number affects fitness in standard growth conditions

CHEF gel electrophoresis was performed to verify rDNA copy number in the triplicate clones used to inoculate fitness competitions under standard growth conditions. (A) Ethidium bromide-stained gel and (B) the resulting Southern blot hybridized with a single copy Chr. XII probe (*CDC45*). (C) Graph of fitness values calculated by the rate of population change for each rDNA copy number test strain against the GFP competitor strain (180 rDNA copies). Grey bars indicate the average fitness value for each strain, dots indicate individual fitness calculated for each strain replicate in this batch experiment. Inset: distribution of rDNA copy genotypes in wild *S. cerevisiae* isolates.

Figure 2: rDNA expansion decelerates upon reaching the fitness plateau of ~100 rDNA copies

Examination of chromosome XII size by (A) CHEF gel electrophoresis and (B) Southern blotting (*CDC45* probe). (C) rDNA copy numbers from each sample were estimated from the above CHEF gel and plotted against generation number.

Figure 3: Transcriptome analysis reveals expression changes specific to late S phase

Genes with significantly different expression (padj<0.01) between the 35 rDNA copy number strain and the 180 rDNA copy number strain in late S phase. The three genes that were also differentially expressed in asynchronous growth conditions are represented by black circles, two genes are labeled in black. HSP12 is labeled in red because of its function in the stress response. Genes within select GO categories are indicated with colored dots: blue for cytosolic ribosome (GO:0022626), yellow for proteolysis involved in protein catabolic process (GO:0051603), and red for response to stress (GO:0006950). Many canonical heat stress proteins and their co-chaperones are significantly downregulated in the 35 rDNA copy number strain in late S phase (labeled in red).

Figure 4: rDNA copy number variants show altered fitness during heat stress

(A) Fitness values were calculated as in Figure 1. (B) Fitness differentials from calculating the change in fitness between 37ºC and standard growth conditions. Strains with 80 to 100 rDNA copies performed more poorly at 37ºC than under standard growth conditions. Samples from the last day of the competition were examined for possible rDNA copy number

change using (C) CHEF gel electrophoresis and (D) Southern blotting using an rDNA probe (*NTS2*). The two chromosome XII bands from both rDNA copy number variant test strain and GFP competitor are both visible in the last day competition culture.

Figure 5: rDNA copy number variants show altered fitness in glycerol media

(A) Fitness values were calculated as in Figure 1. A strong fitness threshold is visible between 100 and 140 rDNA copies with strains that had 140 to 200 rDNA copies showing the highest fitness. (B) The fitness change in glycerol differs across rDNA copy number variants. Fitness differentials from calculating the change in fitness between glycerol media and standard growth conditions. Strains with higher rDNA copy numbers (140-200) performed better in glycerol media than in standard growth conditions. Samples from the last day of the competition were examined for possible rDNA copy number change using (C) CHEF gel electrophoresis and (D) Southern blotting using an rDNA probe (*NTS2*). The two chromosome XII bands from both rDNA copy number variant test strain and GFP competitor are both visible in the last day competition culture.

Figure 6: A strain with substantially reduced rDNA copy number generates fewer *petites* than a wild-type strain

(A) Approximately 350 cells from a single colony were plated onto YEPDG plates (0.1% glucose and 3% glycerol) to enhance *petite* vs. *grande* colony phenotypes (Dimitrov *et al.* 2009). For each strain, 15 plates were scored for total number of colonies and percentage of *petite*

colonies. (B) The strain with 35 rDNA copies generated fewer *petite* colonies than the wild-type strain ($p = 0.00039$). (C) Biological replicate of *petite* frequency assay ($p = 0.00017$).

Figure S1: Fitness trends from rDNA copy number variation are highly reproducible

(A) Growth rates for subset of rDNA copy number variant strains used for fitness competition experiments. Doubling time is indicated in parentheses. (B, C) Replicate competition experiments in standard growth conditions (referring to Figure 1).

Figure S2: Examination of rDNA copy number variants by spot assays for response to stress

(A) Thermotolerance on both normal YEPD and glycerol media or (B) salt stress and ethanol stress. (C) Acquisition of thermotolerance was examined by survival of strains pre-treated with a mild heat shock at 37ºC for 60 minutes. Both pre-treated and untreated cells were then exposed to extreme heat shock (50ºC) for 4, 8, and 12 minutes before spotting onto plates. Plates were grown at 30ºC for two days.

Figure S3: Heat stress fitness trends due to rDNA copy number variation are reproducible

(A) Fitness values and (B) fitness differentials for 37ºC competition experiment replicate (referring to Figure 4). (C) CHEF gel and (D) Southern blot (*NTS2*) of competition culture sample on the last day. (E) Growth rates for subset of rDNA copy number variant strains used for fitness competition experiments. Doubling time is indicated in parentheses.

Figure S4: Glycerol fitness trends due rDNA copy number variation are reproducible

(A) Fitness values and (B) fitness differentials for glycerol competition experiment replicate (referring to Figure 5). (C) CHEF gel and (D) Southern blot (*NTS2*) of competition culture sample on the first day.

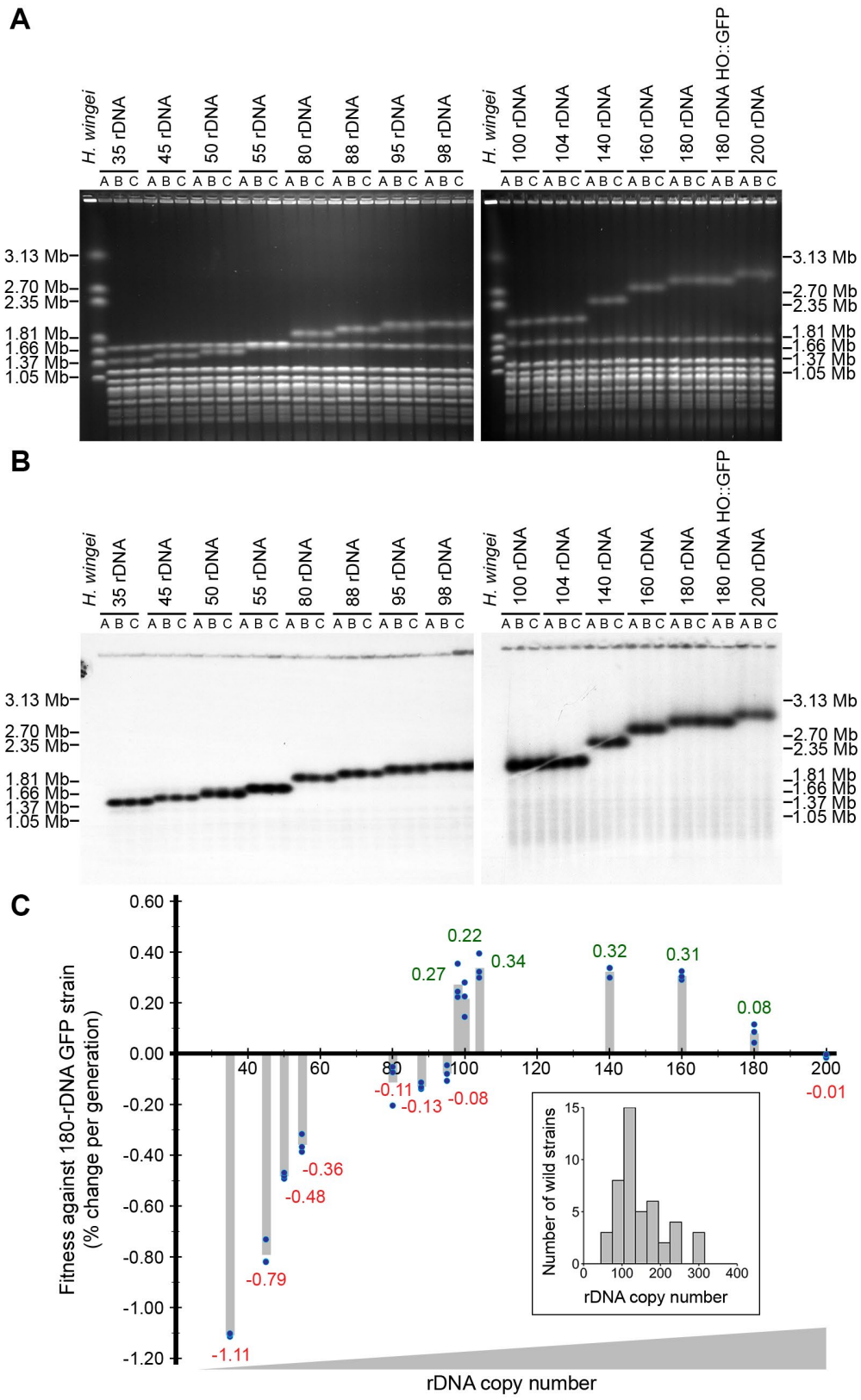

Figure 1: rDNA copy number affects fitness in standard growth conditions.

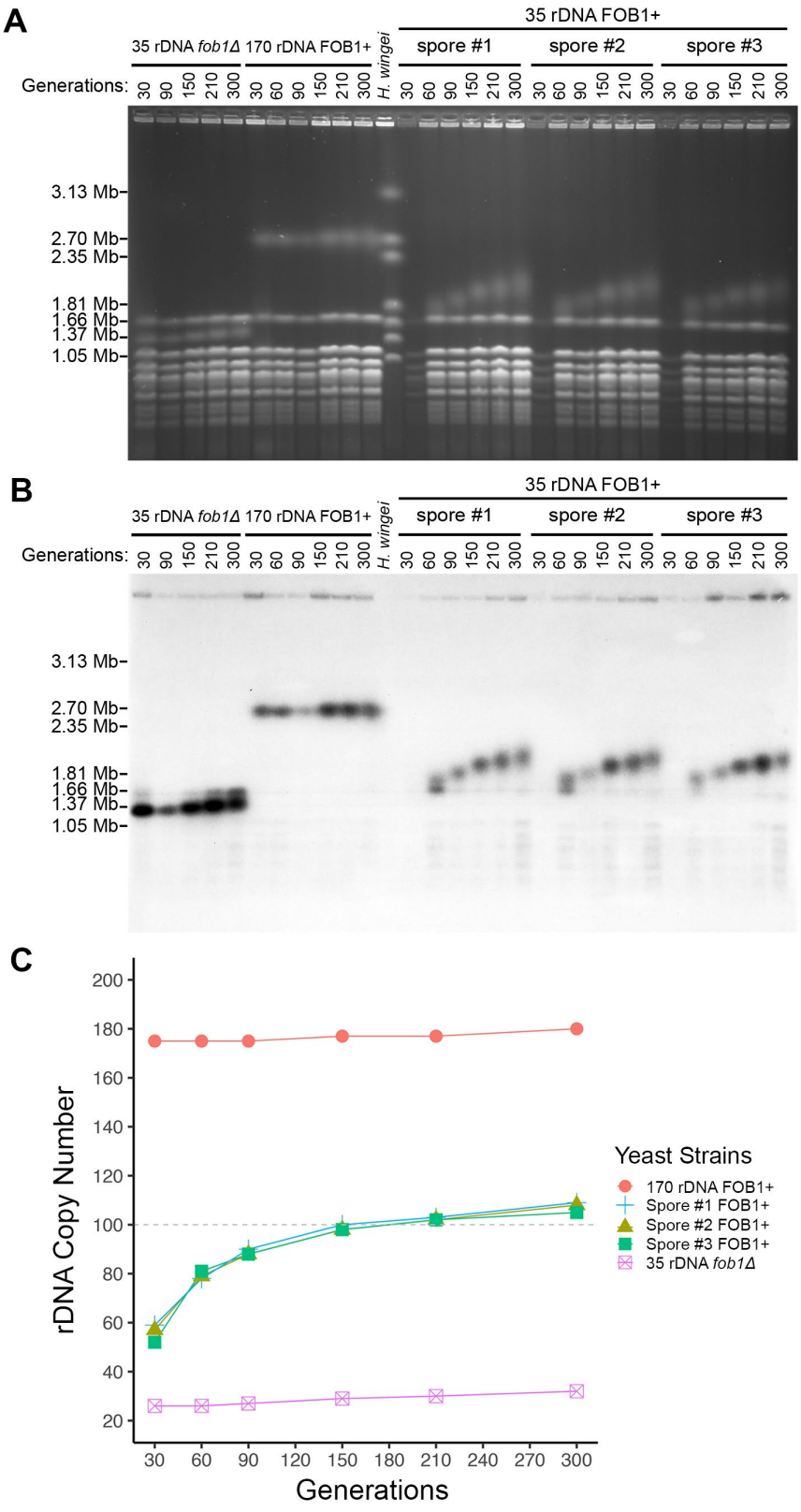

Figure 2: rDNA expansion decelerates upon reaching the fitness plateau of ~100 rDNA copies

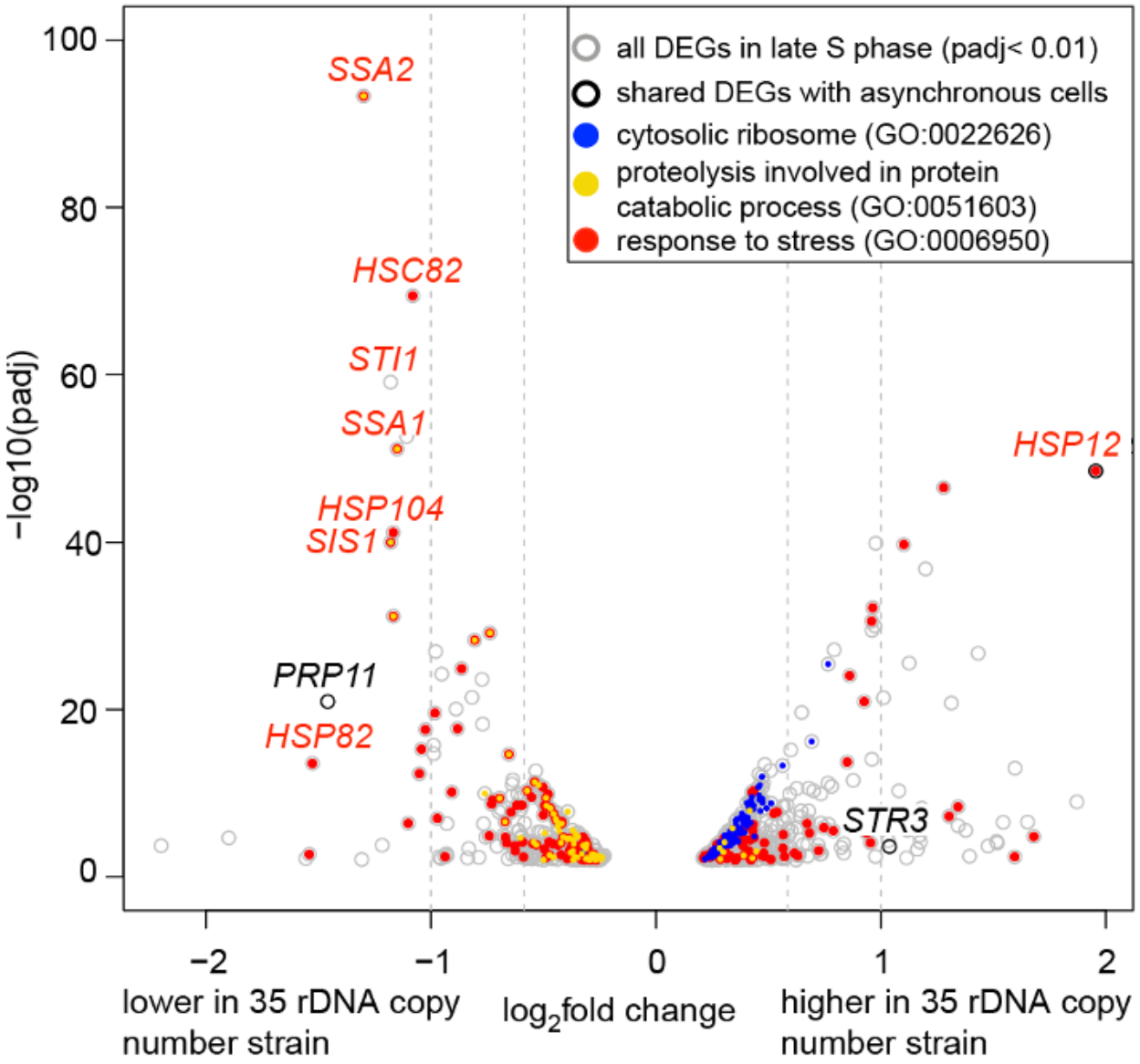

Figure 3: Transcriptome analysis reveals expression changes specific to late S phase

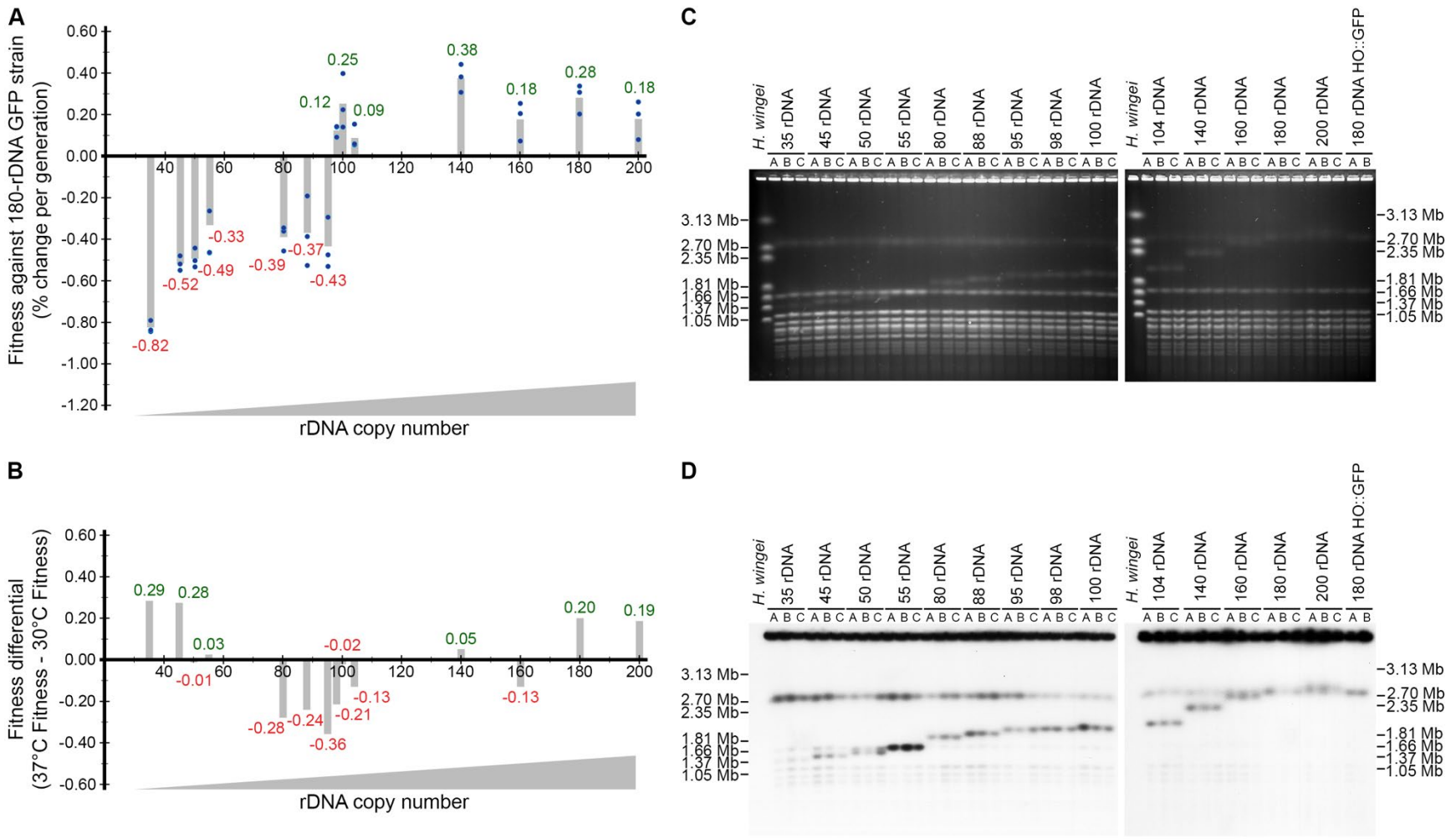

Figure 4: rDNA copy number variants show altered fitness during heat stress

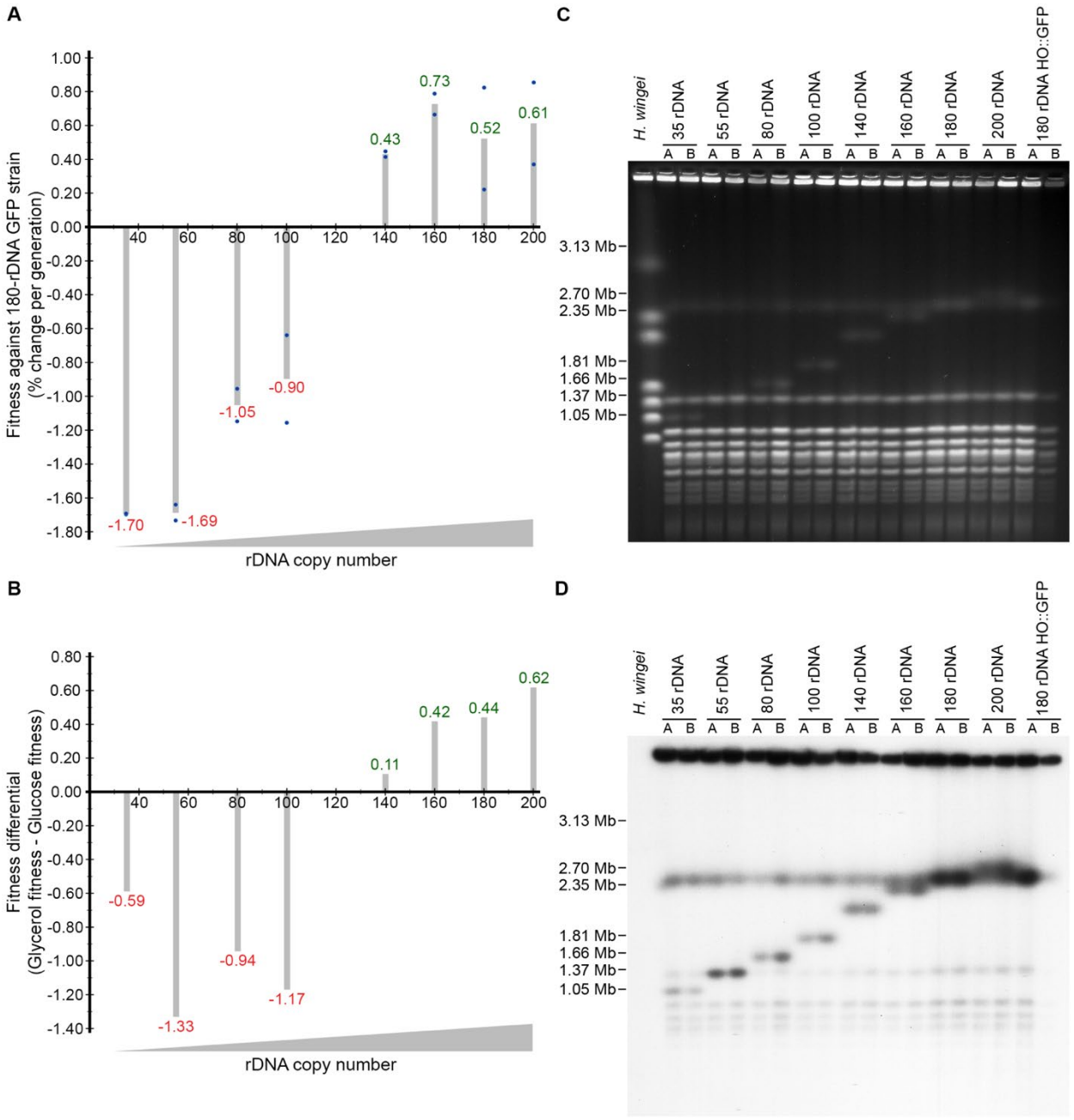

Figure 5: rDNA copy number variants show altered fitness in glycerol media

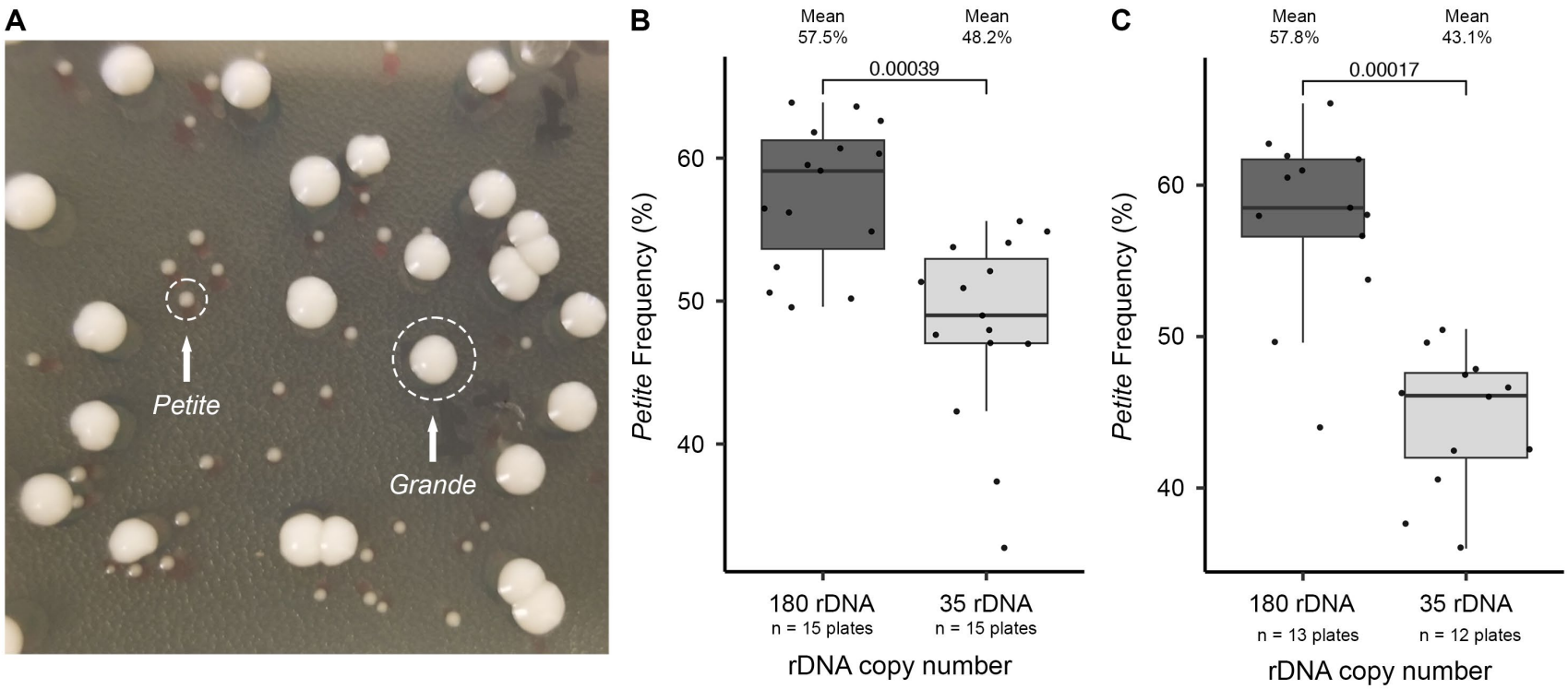

Figure 6: A strain with substantially reduced rDNA copy number generates fewer petites than a wild-type strain

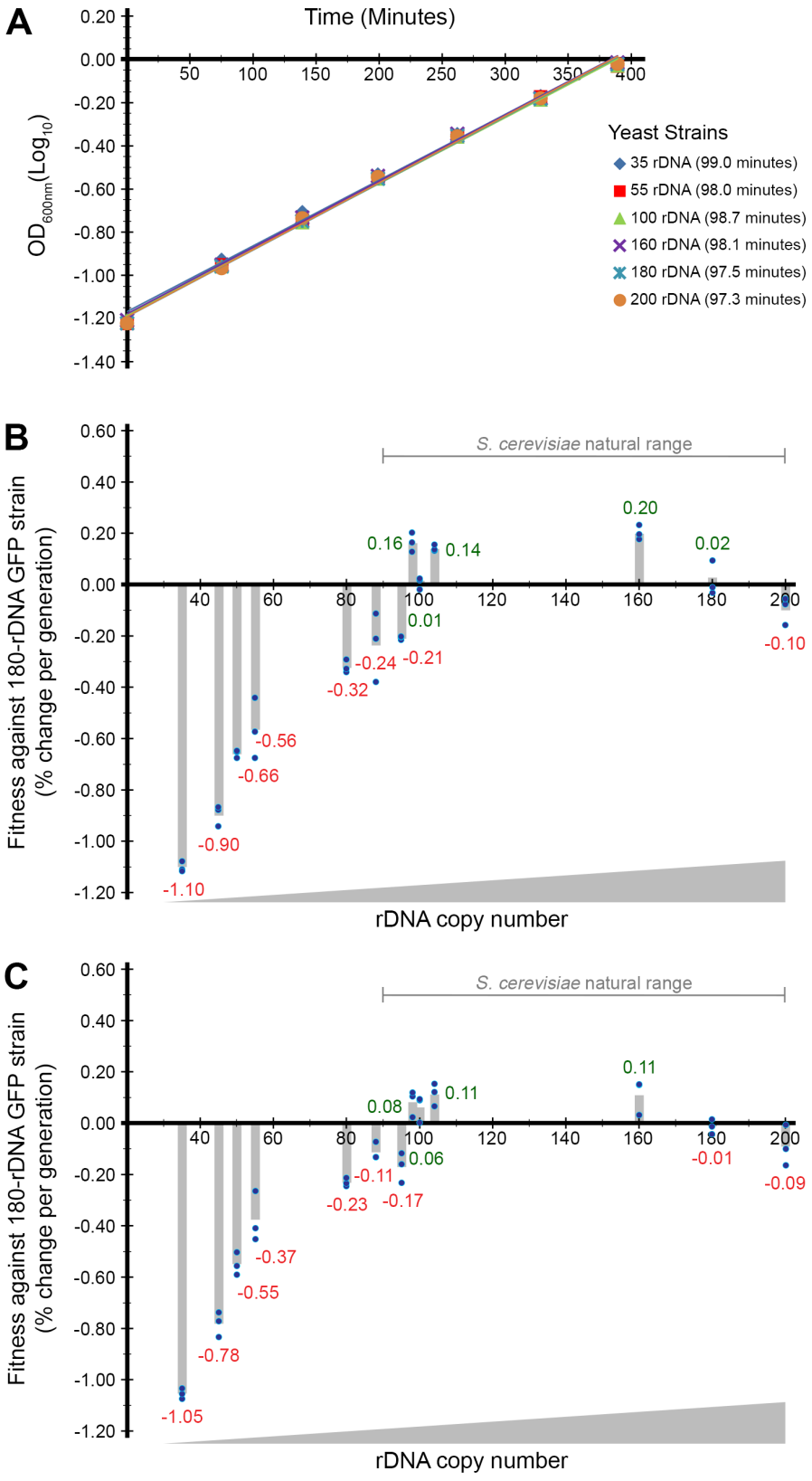

Figure S1: Fitness trends from rDNA copy number variation are highly reproducible

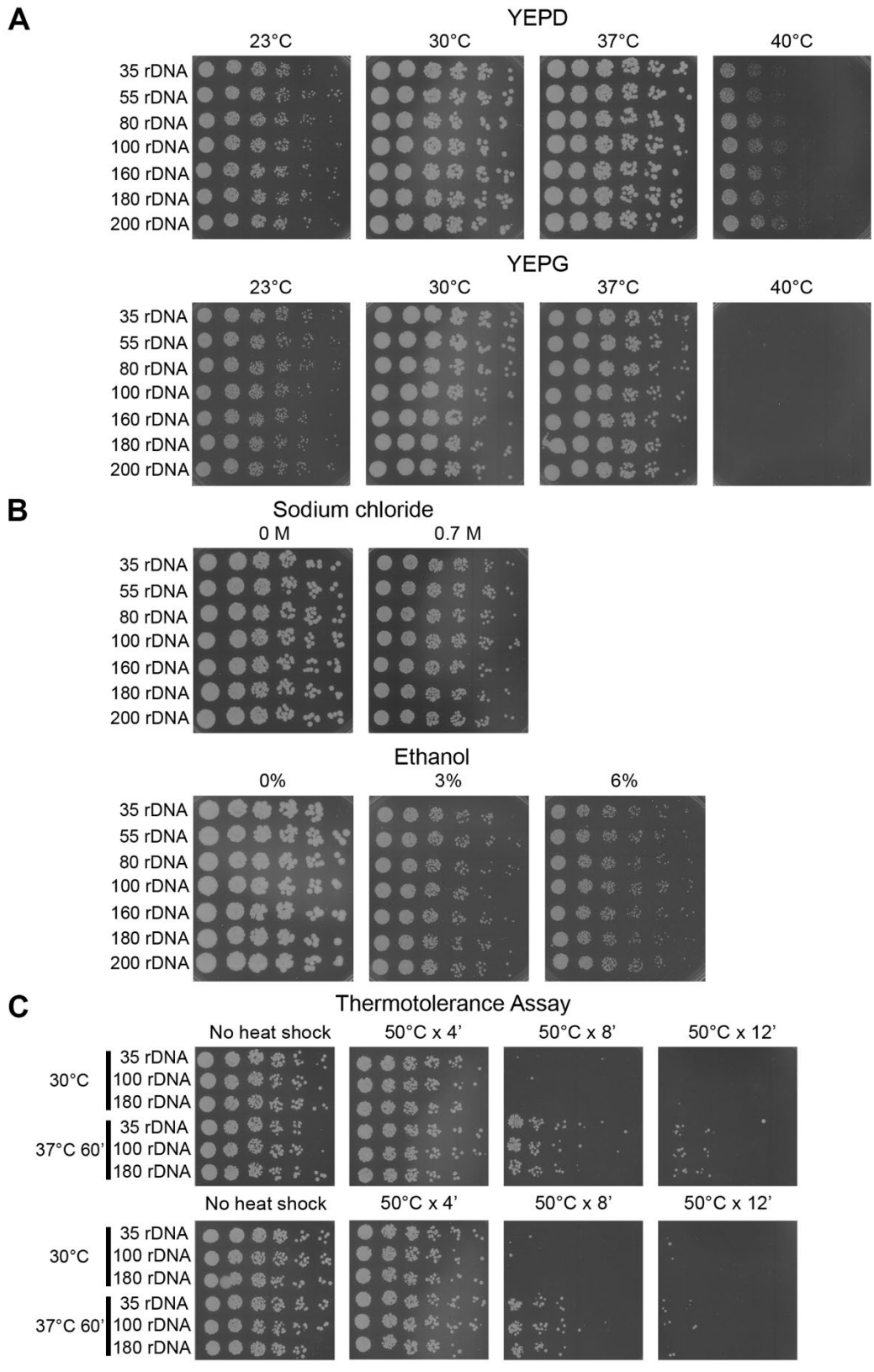

Figure S2: Examination of rDNA copy number variants by spot assays for response to stress

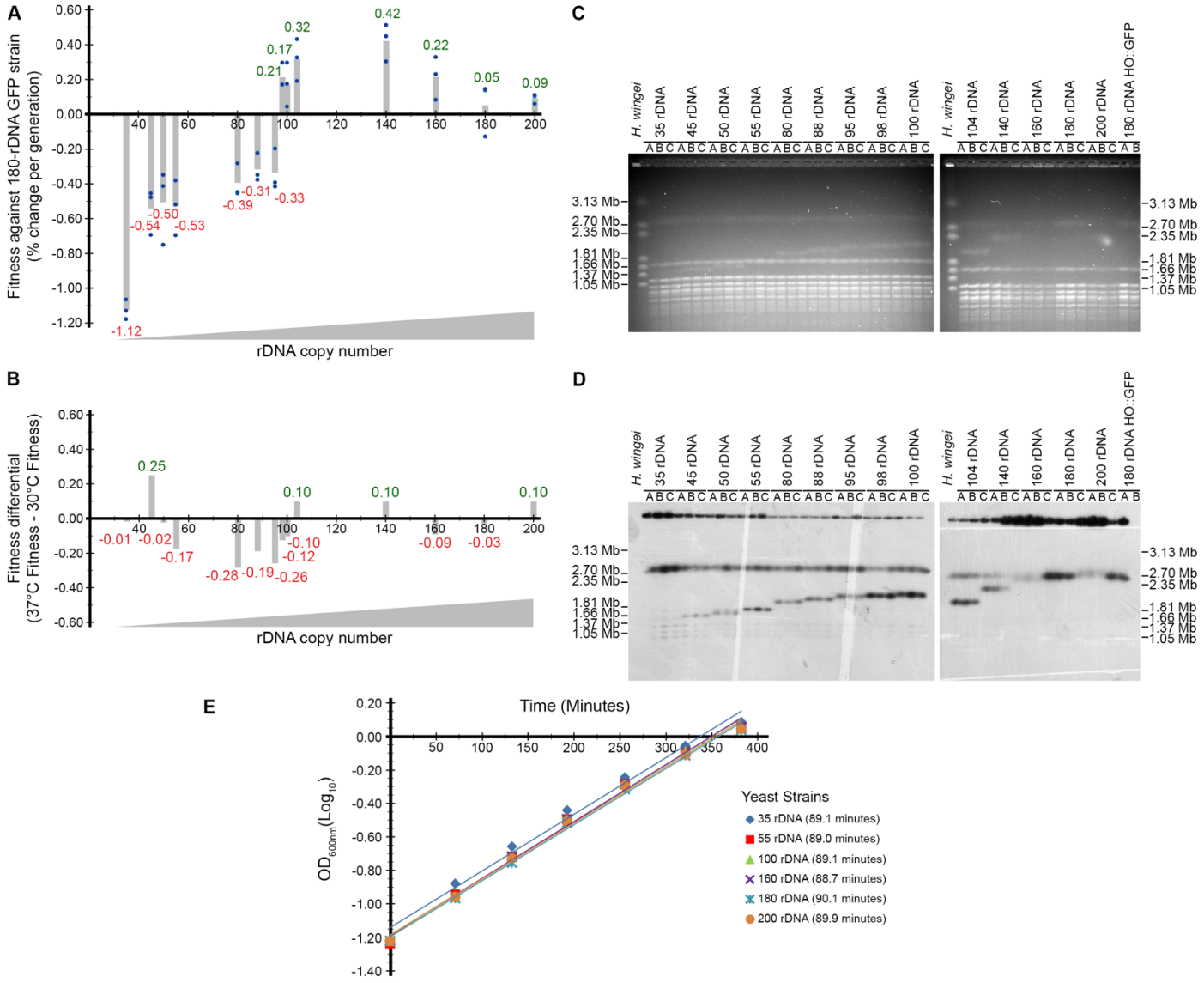

Figure S3: Heat stress fitness trends due to rDNA copy number variation are reproducible

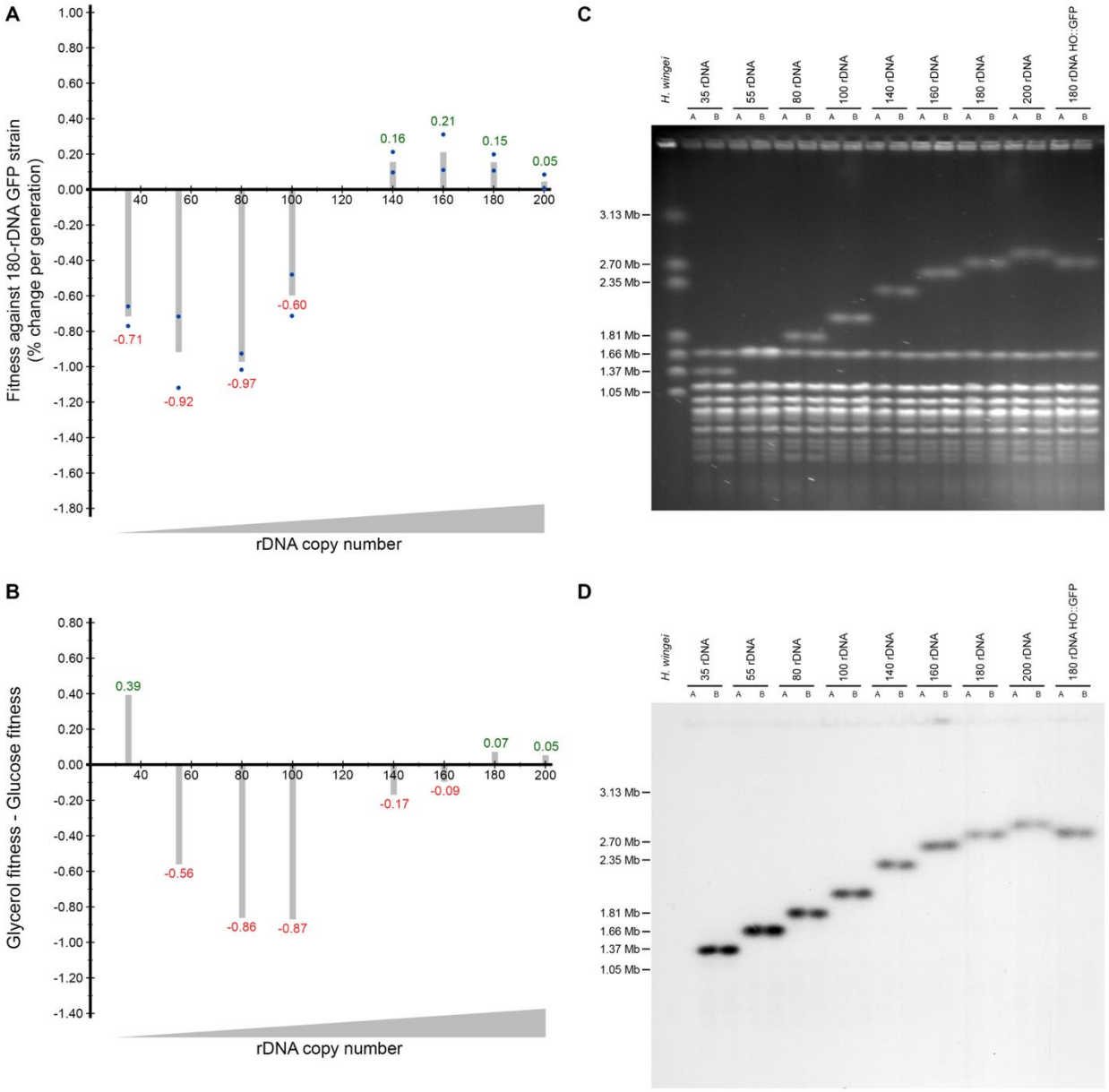

Figure S4: Glycerol fitness trends due rDNA copy number variation are reproducible

Table 1: Yeast strains

Strain	Source	Identifier
S288c MATa fob1::cloNAT; BY rDNA (35 copies)	This study	KT003
S288c MATa fob1::cloNAT; BY rDNA (45 copies)	This study	KT088
S288c MATa fob1::cloNAT; BY rDNA (50 copies)	This study	KT095
S288c MATa fob1::cloNAT; BY rDNA (55 copies)	This study	KT004
S288c MATa fob1::cloNAT; BY rDNA (80 copies)	This study	KT005
S288c MATa fob1::cloNAT; BY rDNA (88 copies)	This study	KT006
S288c MATa fob1::cloNAT; BY rDNA (95 copies)	This study	KT007
S288c MATa fob1::cloNAT; BY rDNA (98 copies)	This study	KT102
S288c w/ RM11-1a MATa fob1::cloNAT; BY rDNA (100 copies)	This study	KT121
S288c MATa fob1::cloNAT; BY rDNA (104 copies)	This study	KT097
S288c MATa fob1::cloNAT; BY rDNA (140 copies)	This study	KT132
S288c MATa fob1::cloNAT; BY rDNA (160 copies)	This study	KT125
S288c MATa fob1::cloNAT; BY rDNA (180 copies)	This study	KT001
S288c MATa fob1::cloNAT; BY rDNA (200 copies)	This study	KT128
S288c MATa fob1::cloNAT; HO::GFP-KanMX; BY rDNA (180 copies)	This study	KT013
S288c MATa fob1::cloNAT; HO::GFP-KanMX; BY rDNA (180 copies)	This study	KT014
S288c MATα fob1::cloNAT; BY rDNA (35 copies)	This study	KT015
S288c MATa FOB1; BY rDNA (170 copies)	This study	KT055
S288c MATa FOB1; BY rDNA (59 copies)	This study	S35-1 G30
S288c MATa FOB1; BY rDNA (57 copies)	This study	S35-2 G30
S288c MATa FOB1; BY rDNA (52 copies)	This study	S35-3 G30
S288c MATa FOB1; BY rDNA (170 copies)	This study	S170-1 G30
S288c MATa FOB1; BY rDNA (170 copies)	This study	S170-2 G30
S288c MATa FOB1; BY rDNA (170 copies)	This study	S170-3 G30

Methods

Yeast strains

All strains used in this study are listed in Table 1 and are derived from the S288c background. Many of the *fob1Δ* strains with rDNA copy number variants were generated by transient reintroduction of *FOB1* through crossing the 35 rDNA *fob1Δ* strain with a 170 rDNA *FOB1* strain (Kwan et al., 2023). Selected *FOB1* spores with reduced rDNA were then passaged through multiple generations. *FOB1* was then deleted via *cloNAT* gene replacement transformation at various passaging stages and rDNA copy number was measured in the resulting strains by CHEF gel electrophoresis. The GFP-tagged competitor strain was generated by replacing the *HO* locus with *eGFP* in the 180 rDNA *fob1Δ* strain (Payen et al., 2014).

Yeast media

Yeast strains used in 30°C and 37°C yeast competition assays were grown in synthetic complete media buffered with 1% succinic acid (per liter: 1.45 g yeast nitrogen base, 20 g glucose, 10 g succinic acid, 6 g NaOH, 5 g $(NH4)_2SO_4$, 2.8 g amino acid powder mix with pH adjusted to 5.8).

Yeast strains used in glycerol yeast competition assays were grown in synthetic complete media buffered with 1% succinic acid (per liter: 1.45 g yeast nitrogen base, 30 g glycerol, 10 g succinic acid, 6 g NaOH, 5 g $(NH4)_2SO_4$, 2.8 g amino acid powder mix with pH adjusted to 5.8).

Preparation of DNA embedded in agarose

Cells were inoculated into 2 mL synthetic complete media buffered with 1% succinic acid and allowed to grow overnight to stationary phase (1.5 – 3 x 10^8 cells/mL). 200 µL of cells were pelleted for 1 minute at 15,000 rcf and the supernatant was discarded. Cells were washed with 85 µL 50 mM EDTA, resuspended in 90 µL 1% SeaPlaque™ GTG™ agarose in 50 mM EDTA then transferred into plug molds. Plugs were allowed to solidify for 15 minutes at 4ºC then incubated in 1 mL spheroplasting solution (1.0 M sorbitol, 20 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.4, 14.3 mM β-mercaptoethanol, 0.5 mg/mL Zymolyase-20T [Amsbio]) for 2 – 4 hours at 37ºC with gentle shaking. Plugs were washed once with LDS (1 % lithium dodecyl sulfate, 100 mM EDTA pH 8.0, 10 mM Tris–HCl pH 8.0) and incubated overnight at 37ºC in LDS. Plugs were then washed 3 x 20 minutes in 0.2X NDS (1X NDS pH 9.5: 0.5 M EDTA, 10 mM Tris base, 1% Sarkosyl) and 5 x 20 minutes in TE pH 8.0. All processed plugs were stored at 4ºC in TE pH 8.0 until use.

CHEF gel analysis

Intact chromosomes were resolved by utilizing contour-clamped homogeneous electric field (CHEF) gel electrophoresis. A small slice (5 mm x 2 mm x 3 mm) of all genomic DNA agarose plugs were embedded in a 0.8% low electroendosmosis (LE) agarose gel containing filtered 0.5X TBE. CHEF gels were run in 2.3 L of 0.5X TBE using a Bio-Rad CHEF-DRII electrophoresis cell at 100V for 68 hours (switch time = 300 to 900 seconds). All gels were stained with ethidium bromide visualize chromosome XII, which contains the rDNA, and all other chromosomes. *Hansenula wingei* (*H. wingei*) chromosomal DNA size marker standards were included in each CHEF gel electrophoresis run for size comparison.

Southern blotting

All CHEF gels were transferred to Genescreen Hybridization membrane using standard Southern blotting protocols (Tsuchiyama et al., 2013). We then hybridized the sequences of interest using a ^{32}P-labeled probe. The blots were exposed to X-ray film and to Bio-Rad Molecular Imaging FX phosphor screens for visualization and quantification of signal intensity.

Yeast competitions

Cells were streaked out on YEPD plates and allowed to incubate overnight at 30°C. Cells were inoculated into 2 mL synthetic complete media with 2% glucose buffered with 1% succinic acid and allowed to grow overnight to stationary phase (1.5–3 x 10^8 cells/mL). 100 µL of overnight cultures was transferred to 5 mL synthetic complete media (1:50 dilution) and allowed to grow at 30°C for ~5 hours. After the 5 hour incubation period, 500 µL of test strain cultures were mixed with 500 µL of 180 rDNA GFP competitor strain culture and vortexed thoroughly. 500 µL of each test strain and GFP competitor strain culture mix were transferred to 1.5 mL sterile water (1:4 dilution) to record cell number of all strains with the Gilford Stasar Spectrophotometer at a wavelength 600. 5 µL of each test strain and GFP competitor strain culture mix was transferred to 5 mL synthetic complete media (1:1000 dilution) and incubated at 30°C overnight. For fitness competitions in glycerol media, cultures were maintained in asynchronous, logarithmic-phase growth instead of letting them go to saturation. Cell fitness was determined by measuring the change in GFP population of cells over time with flow cytometry of cell culture aliquots from day zero and day five. Rate of change over generations

was calculated from the slope of plotting points on a log scale. All fitness defects and advantages seen in strains used in these experiments were reproducible across several competition experiments.

Flow cytometry

Approximately 350 µL of day zero yeast competition experiment cultures were suspended to 1 mL 50 mM sodium citrate and sonicated. For day 1 to 5 competition cultures, 100 µL of experiment overnight cultures were suspended in 1 mL 50 mM sodium citrate and sonicated in preparation of flow cytometry. GFP and non-GFP cell populations were analyzed on a BD Canto II flow cytometer and flow cytometry data was analyzed using FlowJo software.

Preparation of RNA for RNA-seq

Asynchronous and late S phase logarithmic phase cells were collected, and genomic DNA was isolated using an acid phenol: chloroform extraction protocol by resuspending frozen pellets in 200 µL lysis buffer (10 mM Tris, pH 8.0, 10 mM EDTA, 5% SDS), and 200 µL acid phenol and vortexed for 2 min. Cells were resuspended and incubated at 65°C for 1 hour and vortexed occasionally. Incubated samples were kept on ice for 10 minutes before centrifuging samples at 4°C at 15,000 rcf for 10 minutes. After transferring the aqueous layer to new 1.5 microcentrifuge tubes, we added equal volumes of chloroform, and vortexed vigorously before centrifuging samples at 15,000 rcf for 10 minutes. To remove bulk of ribosomal RNA (rRNA) before sequencing library prep, DNA oligos complimentary to rRNA sequences were incubated with RNA samples and then treated with RNase H to degrade RNA:DNA hybrids. RNA was

then purified again using phenol:chloroform and ethanol precipitated. rRNA-depleted RNA was purified with a 2.2X RNAClean SPRI bead treatment (Beckman Coulter). Purified, rRNA-depleted RNA was treated with Turbo DNase (Invitrogen) to remove the rRNA-hybridizing DNA oligos: 0.1 volumes (2 µL) of 10X TURBO DNase Buffer was added to the RNA, followed by 2 µL TURBO DNase. The mixture was incubated at 37°C for 45 minutes. The DNase-treated RNA was then cleaned with 2.2X RNA SPRI beads and a total volume of 12 µL was eluted. 9 µL of the eluted volume was used immediately for poly-A mRNA capture.

9 µL of final DNase-treated RNA and 16 µL nuclease-free ultrapure water were added to 25 µL RNA Purification Beads, mixed and incubated at 65°C for 5 minutes then at 4°C for 30 seconds and finally at 23°C for 5 minutes. Samples were placed on a magnetic stand, supernatant was discarded, and 100 µL Bead Washing Buffer was added. After removing all residual supernatant, 25 µL Elution Buffer was added to samples and were incubated at 80°C for 2 minutes. 25 µL Bead Binding Buffer to samples and then washed with 100 µL Bead Washing Buffer. After removing supernatant, 19 µL ice cold Fragmentation Master Mix (10.5 µL Nuclease-free ultrapure water and 10.5 µL Elute, Prime, Fragment High Mix per sample) was added to each sample and incubated at 94°C for 8 minutes. 17 µL of mRNA PCR product was added to new PCR tubes on ice in preparation of First strand cDNA synthesis.

First strand cDNA synthesis was performed by adding 8 µL First Strand Synthesis Master (9 µL First Strand Synthesis Act D Mix and 1 µL Reverse Transcriptase per sample) to 17 µL of each mRNA PCR product and incubated at 25°C for 10 minutes then at 42°C for 15 minutes and finally at 70°C for 15 minutes. Second strand cDNA synthesis was performed by adding 25 µL

of the first cDNA strand reaction products with 25 µL Second Strand Marking Master Mix and incubated at 16°C for 1 hour. After the incubation period, second cDNA strand reaction products were captured with 90 µL AMPure XP beads, supernatant was discarded, and AMPure XP beads were cleaned up twice with 175 µL fresh 80% ethanol on a magnetic stand. After removing all residual ethanol, AMPure XP beads were resuspended in 19.5 µL Resuspension Buffer and 17.5 µL supernatant was transferred into new PCR tubes. The first and second cDNA products were used immediately or stored at -20°C overnight for use the next day.

In preparation for adenylating 3' ends, 12.5 µL A-Tailing Mix was added to the first and second cDNA products and incubated at 37°C for 30 minutes then at 70°C for 5 minutes. Anchors were ligated to samples by adding 2.5 µL Resuspension Buffer, 2.5 µL RNA Index Anchors (Illumina), 2.5 µL Ligation Mix and incubated at 30°C for 10 minutes followed by adding 5 µL Stop Ligation Buffer. 34 µL AMPure XP beads were added to samples, supernatant was discarded, and AMPure XP beads were cleaned up twice with 175 µL fresh 80% ethanol on a magnetic stand. After removing all residual ethanol, AMPure XP beads were resuspended in 22 µL Resuspension Buffer and 20 µL supernatant was transferred into new PCR tubes. The ligated cDNA products were used immediately or stored at -20°C overnight for use the next day.

Dual-indexed libraries were generated by adding 10 µL index adapters (Illumina) and 20 µL Enhanced PCR Mix to the ligated cDNA products. The mixture was mixed and incubated for 13 cycles at 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds followed by an incubation at 72°C for 5 minutes. 50 µL AMPure XP beads were added to samples, supernatant was discarded, and AMPure XP beads were cleaned up twice with 175 µL fresh 80% ethanol on

a magnetic stand. After removing all residual ethanol, AMPure XP beads were resuspended in 17 µL Resuspension Buffer and 15 µL supernatant was transferred into new PCR tubes. The concentrations and quality of all libraries were examined for quality using TapeStation 2200 (Agilent Technologies) and Qubit (Thermo Fisher Scientific) then subsequently diluted to the appropriate starting concentrations for sequencing.

Libraries were sequenced using a NextSeq 550 with a 75 Hi kit (Illumina). Read lengths used were: Index 1: 8bp, Index 2: 8bp, Read 1: 38bp, Read 2: 38bp.

Transcriptome analysis

We then aligned to the S288C reference genome version R64.1.1 and obtained gene counts using hisat2 version 2.2.1 (Kim et al., 2019). DESeq2 was used to identify differentially expressed genes (Love et al., 2014). See supplemental tables (https://docs.google.com/spreadsheets/d/1cSzxdsBcZTj2jXocglgN5I9OEI1NrgwjIy-h7gyoIZY/edit?usp=sharing) for counts and full DESeq2 results. Genes with an adjusted p-value of less than 0.01 were further analyzed: six that differed between the 35- and 180-rDNA copy number in asynchronous culture (*PRP11*, *HSP12*, *STR3*, *NCE103*, *YGP1*, and *CIN5*) and 708 that differed between the 35- and 180-rDNA copy number in synchronous culture during late S phase, with three genes in common between these two sets (*PRP11*, *STR3*, and *HSP12*).

We performed gene ontology enrichment analysis for the following four sets of genes, each of which consists of only genes determined to be differentially expressed in late S phase between the 35- and 180- rDNA copy number strains: (1) genes that were more expressed in the

35-rDNA copy number strain than the 180-rDNA copy number strain; (2) genes that were more expressed in the 180-rDNA copy number strain than the 35-rDNA copy number strain; (3) genes that were at least 1.5x more expressed in the 35-rDNA copy number strain than the 180-rDNA copy number strain; (4) genes that were at least 1.5x more expressed in the 180-rDNA copy number strain than the 35-rDNA copy number strain. Set (1) was enriched for ribosomal components, for example 110 of the 420 genes in this set were in the "cytosolic ribosome" category (GO:0022626; enrichment padj<1e-95). Set (2) was enriched for proteolysis-related genes, for example 60 of the 288 genes in this set were in the "proteolysis involved in protein catabolic process" category (GO:0051603; enrichment padj<1e-22). Set (3) was enriched for chromatin-related genes, for example 8 of the 88 genes in this set were in the "structural constituent of chromatin" category (GO:0030527; enrichment padj<1e-10). Set (4) was enriched for stress-related genes, for example 36 of the 83 genes in this set were in the "response to stress" category (GO:0006950; enrichment padj<1e-7). We colored each differentially expressed gene that was a member of any of these four enriched GO categories, regardless of whether the gene is in the set enriched for that GO category.

Spot assays

Cells were grown to log-phase, diluted in sterile water in 3-fold dilutions starting with a cell concentration of 4 x 10^5 cells/mL. 2.5 µL was spotted onto YEPD (1% yeast extract, 2% peptone, and 2% glucose), YEPD + 0.7 M Sodium chloride, YEPD + 3% ethanol, YEPD + 6% ethanol, and YEPG plates. All plates were scanned after 48 hours of growth at 30°C. Thermotolerance assays were adapted from Lindquist & Kim 1996.

Petite frequency assays

The procedure adapted from Dimitrov *et al.* 2009 was used. Medium sized colonies from on direct-from-freezer-stock streakouts on YEPD plates were inoculated 2 mL cultures tubes filled with WF-N media. A portion of 2 mL culture was used to determine cell densities of cultures and diluted to 1 x 10^5 cells/mL. Cultures were diluted again to a cell concertation of 2.5 x 10^3 cells/mL. Diluted cultures were sonicated and 150 µL was plated onto YEPDG (1% yeast extract, 2% peptone, 0.1% glucose, and 3% glycerol) plates and allowed to grow at 30°C for 5 days. After the incubation period, all plates were scored for *petite* and *grande* colonies.

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Future directions for these studies

Differential stress responses and fitness under multiple stress conditions

My work describes the impact of rDNA copy number variation on fitness in yeast competition assays conducted under standard laboratory conditions, heat stress, and glycerol stress conditions. However, given more time, I would like to extend my experiments to capture fitness changes under osmotic, oxidative, ethanol, heavy metal, and fluctuating environmental stress conditions. Although the effects of osmotic, oxidative, and ethanol stress on rDNA copy number and fitness have not been evaluated, there is evidence that various heavy metals can affect rDNA copy number. It has been shown that heavy metals such as hexavalent chromium and cadmium cause transient and temporary rDNA copy number expansion in human lymphoblastoid cell lines, primary mononuclear blood cells, and flies (Lou et al., 2021). Additionally, there is evidence that yeast rDNA copy number randomly changes in response to lithium acetate exposure from transformation procedures (Kwan et al., 2016).

Identifying additional compounds and conditions that can affect rDNA copy number is important because rDNA copy number can affect how cells respond to changing environments. For instance, the chemotherapeutic actinomycin D causes rDNA copy number expansion in human lymphoblastoid cells (Lou et al., 2021). It has been shown that replication stress selects for shorter rDNA strains despite increased sensitivity to DNA damaging agents such as methyl methanesulfonate (Ide et al., 2007; Lynch et al., 2019; Salim et al., 2017; Sanchez et al., 2017).

For instance, hydroxyurea causes rDNA copy number reduction in wild-type yeast after 50 to 75 generations (Salim et al., 2017). While rDNA copy number variation and fitness can be evaluated in stress competition assays, the use of passaging experiments allows for examination of rDNA copy number responses in these stress conditions without much manipulation. Using both passaging experiments for optimal rDNA copy number and fitness competition experiments, we may be able to identify additional compounds and conditions that increase, decrease, or maintain rDNA copy number.

Although my yeast competition assays highlighted the impact of a single stress condition on cell fitness related to rDNA copy number change, organisms are subject to a multitude of stressors at a given time. I therefore would like to examine combinations of stress conditions to further characterize the role rDNA copy number plays in cell fitness. Multiple biochemical pathways feed into adaptive mechanisms that yeast employ for survival, such as the nutrient-responsive TOR pathway. Yeast competition assays utilizing multiple stresses can be implemented to characterize fitness changes. For instance, the glycerol stress competitions examined the impact of rDNA copy number on fitness in the context of nonfermentable carbon source; however, these experiments were all conducted at 30°C. Temperature fluctuates in wild environments, therefore performing glycerol stress competitions at higher and lower temperatures may provide more insight into complex adaptive mechanisms that impact fitness. Temperature and carbon source are but a fraction of combined stressors that can be tested to further explore the relationship between differential stress responses and rDNA copy number in yeast.

Fitness consequences of rDNA copy number variation in diploid yeast

Ploidy has an important impact on adaptation in organisms. In yeast, diploids generally adapt slower compared to haploids; however, the nature of mutations between diploids and haploids differ. For instance, evolved haploids have more beneficial recessive mutations while evolved diploids have more heterozygous mutations (Marad et al., 2018). All our experiments used prototrophic S288c haploid yeast strains with varying rDNA copy numbers; however, there may be fitness differences related to maintaining an additional rDNA array. I would like to propose diploid yeast competition assays using prototrophic S288c laboratory yeast strains to get a better understanding of fitness effects. Additionally, we can explore the fitness of diploid yeast with varying rDNA copy number under different environmental conditions such as heat, osmotic, oxidative, ethanol, and glycerol stress. A study examining the fitness effects of mutations in sexual and asexual diploids in a fluctuating environment found increased fitness in asexual diploid yeast (Leu et al., 2020). We can examine fitness in asexual and sexual diploid yeast cells to examine if there are differences due to mating potential.

Although our lab has not investigated rDNA copy number variation and fitness in diploid yeast, we have studied rDNA copy number variation in *C. elegans*, a multicellular diploid species (Morton et al., 2023). The extent of developmental delays and morphological abnormalities in *C. elegans* is inversely proportional to rDNA copy number. The fewer rDNA copies worms have, the more severe are the observed developmental delays and morphological defects. These findings point to tissue specific requirements for rDNA copy number in a multicellular diploid species.

Fitness consequences of rDNA copy number variation in different strain backgrounds

We did not evaluate rDNA copy number variation and fitness in different *S. cerevisiae* strain backgrounds, which may have different fitness profiles; therefore, additional competition experiments will need to be conducted to characterize these. Some general trends will likely recapitulate in different strain backgrounds such as the reduced fitness of short rDNA strains. This assumption is supported by the increased sensitivity to DNA damaging agents of the short rDNA strains in both the S288c and the W303 background (Ide et al., 2010; Kwan et al., 2023; Salim et al., 2017). What will likely differ is the number of rDNA copies associated with improved fitness as indicated by the rDNA expansion differences observed between the short rDNA S288c and W303 strains in passaging experiments (Kobayashi et al., 1998). Evaluating rDNA copy number and fitness in multiple *S. cerevisiae* strain backgrounds will provide an excellent opportunity to build on our current knowledge.

Gene expression changes in different rDNA copy number strains

Our RNA-seq analysis was limited to the shortest rDNA strain with 35 copies, but this analysis can be expanded to other strains below, within, and above the natural rDNA range of *S. cerevisiae* such as the strains with 55, 80, 100, and 140, and 200 rDNA copies. Since fitness increases with increasing rDNA copy number between 35 and 95 rDNA copies in standard growth conditions, we suspect that there will be fewer differentially expressed genes in late S phase in strains with increasing rDNA copy numbers compared to the control strain with 180 rDNA copies.

Although my RNA-Seq data showed downregulation of canonical heat shock proteins, transcriptomic changes do not necessarily reflect protein changes. Examining protein levels of these various heat shock proteins might reveal if there is a direct physiological difference in heat shock protein levels in short versus long rDNA strains. Another approach to address the importance of these cell-cycle-specific expression changes, might be the analysis of chaperone hypomorphs in short rDNA strains.

Given the observed association with the stress response both in late S phase and asynchronous cell (*i.e.* altered regulation of *HSP12*, *DDR2*), it might be informative to assess gene expression in response to the tested stresses. Such experiments might provide insights as to whether the molecular underpinnings of the altered fitness values for our rDNA variants are mechanistically related or not.

In summary, my initial RNA-seq analysis finds transcriptome differences in 35 rDNA copy number strains that likely reflect adaptive mechanisms to maintain homeostasis despite inherent fitness defects.

Characterizing chromatin accessibility of short and long rDNA strains

Most rDNA loci are repetitive in nature. Even though there are multiple copies, only a portion of the rDNA repeats are transcriptionally active at any given time. The proportion of active rDNA repeats generally increase as rDNA copy number decreases. For example, virtually all rDNA repeats are active in yeast with about 42 rDNA copies (French et al., 2003). This result was verified through the Miller chromatin spreading method, and electron microscopy was used

to count the total number of Pol I enzymes per rDNA repeat to calculate elongation rate in yeast strains with 42 rDNA copies and control strains with about 143 rDNA copies (French et al., 2003). It is not known how exactly the proportion of active rDNA repeats scales with total rDNA copy number in different growth phases and cell cycle states. Furthermore, other regions within the rDNA array are subject to post-translational modifications that impact rRNA transcription (Briggs et al., 2001).

Fiber-seq (Stergachis et al., 2020) allows us to examine the accessibility and sequence variation of rDNA arrays of variable sizes: 35, 55, 80, 100, and 140, 160, 180, and 200 rDNA copies. We will be able to obtain chromatin accessibility and DNA sequence of individual rDNA repeats within a given yeast strain. Additionally, we will be able to characterize the distribution of active and inactive rDNA repeats within the rDNA array, based on gene body accessibility. Initial Fiber-seq experiments will involve collecting asynchronous yeast cultures to determine accessibility differences under standard laboratory conditions, which can provide information about the average number of active repeats for short and long rDNA strains. Our lab has already utilized Fiber-seq to examine genomic regions, including the rDNA, in the plant *A. thaliana*, which make implementing Fiber-seq in yeast tractable. Additionally, accessibility of rDNA may differ based on environmental conditions, which may help explain fitness differences observed in yeast competition assays. Fiber-seq may also capture other genomic regions that might facilitate adaptive cellular changes mediated by rDNA copy number and further explain fitness differences.

Characterizing rDNA copy number variation and biomarker detection in human cancers

Biomarkers can provide evidence for cancer initiation and development. For instance, alpha-fetoprotein is a common cancer biomarker used to diagnose liver cancer and germ cell tumors, and to assess stage, prognosis, and response to treatment (Sarhadi and Armengol, 2022). While there are a handful of useful biomarkers in medicine, they are not all equal. Some biomarkers provide information on treatment effectiveness but they are not prognostic. Measurement of rDNA copy number variation might offer us a way to determine cancer risk, cancer classification, and cancer treatment. Xu *et al.* observed rDNA contraction in human cancer genomes with mutations in the PI3K-AKT-mTOR pathway. I would be interested to examine rDNA copy number variation in combination with commonly used biomarkers. Common biomarkers to be evaluated along with rDNA copy number should be within the PI3K-AKT-mTOR pathway and other biochemical systems that feed into this pathway such as the insulin-glucagon axis. Additionally, we know that rDNA copy number also impacts the epigenetic state of genomes, therefore the characterization of epigenetic biomarkers in conjunction with rDNA copy number may allow us to distinguish rDNA-specific effects in these cancer types. rDNA copy number may also play a role in cancer recurrence and should be evaluated in human cancers compared to normal tissues.

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