A minimal rDNA array replicates early, delays genome replication, and uncouples anaphase entry from S phase completion

Elizabeth X. Kwan, Gina M. Alvino, Kelsey L. Lynch, Paula F. Levan, Haley M. Amemiya, Xiaobin S. Wang, Sarah A. Johnson, Joseph C. Sanchez, Madison A. Miller, Mackenzie Croy, Seung-been Lee, Maria Naushab, Josh T. Cuperus, Bonita J. Brewer, Christine Queitsch*, and M. K. Raghuraman*

Department of Genome Sciences; University of Washington; Seattle, Washington, 98195; United States of America

*Corresponding authors (queitsch @ u.washington.edu ; raghu @ u.washington.edu)

ABSTRACT:

Ribosomal DNA (rDNA) copy number varies widely among individuals in many species, but the phenotypic consequences of such copy number fluctuations remain largely unexplored. In the yeast *Saccharomyces cerevisiae,* each rDNA repeat contains an origin of replication. Previous studies have demonstrated that the yeast rDNA locus can be a significant competitor for replication resources, suggesting that rDNA copy number variation may affect timely completion of genome-wide replication. We hypothesized that reduction in rDNA copy number and thus rDNA replication origins would reduce competition from the rDNA locus and thereby improve non-rDNA genome replication. To test this hypothesis, we engineered yeast strains with short rDNA arrays of 35 copies, a minimal copy number that still maintains wild type level ribosome function. Contrary to our hypothesis, the minimal rDNA strain displayed classic replication defects: decreased plasmid maintenance, delayed completion of chromosomal replication, and increased sensitivity to replication stress agonists. Although a normal rDNA array replicates late in S phase, the minimal rDNA array initiated replication in early S phase, resulting in delayed replication across the non-rDNA portions of the genome. Moreover, we discovered that absence of the rDNA fork barrier protein Fob1p increased DNA damage sensitivity in strains with early

replicating rDNA. We present evidence that this increased sensitivity may be due to compromised regulation of cyclin phosphatase Cdc14p and premature entry into anaphase. Our results indicate that precocious rDNA replication, rather than total number of rDNA origins, compromises replication of the genome. Taken together, we suggest that the rDNA's large, late-replicating state is evolutionarily conserved to promote genome stability through timely genome replication and coordination of S phase completion with anaphase entry.

Keywords: rDNA, ribosomal DNA, replication, genome, Cdc14, Fob1, replication time, anaphase

INTRODUCTION:

Ribosomal DNA (rDNA) is uniquely situated at the intersection of ribosome biogenesis and genome replication, essential processes required for cell growth and proliferation. The rDNA sequence is comprised of four ribosomal RNA genes, which encode the core components of the ribosome, and multiple rDNA copies are arranged in tandem to form large arrays. Many species have hundreds of rDNA copies per haploid genome: *e.g.,* 90-300 in *Saccharomyces cerevisiae*, 70-400 in *Caenorhabditis elegans*, 80-600 in *Drosophila melanogaster,* 500-2500 in *Arabidopsis thaliana*, and 30-800 in humans (Mohan and Ritossa, 1970; Morton et al., 2020; Parks et al., 2018; Thompson et al., 2013). Curiously, this high rDNA copy number is often in substantial excess of what is required to meet ribosome demands, leading to silencing of the majority of rDNA repeats in a wide variety of species (Conconi et al., 1992, 1989; Dammann et al., 1993; French et al., 2003; McStay and Grummt, 2008; Ye and Eickbush, 2006). Yet, active maintenance of seemingly overabundant rDNA copies is conserved (Nelson et al., 2019), hinting that high rDNA copy number may have roles beyond ribosome production.

Investigations of the phenotypic consequences from rDNA copy number variation have uncovered a broad range of cellular processes in diverse species. While ribosome deficiencies do result when rDNA copy number drops below a threshold (Delany et al., 1994; French et al., 2003; Ritossa and Atwood, 1966; Sanchez et al., 2017), several other phenotypes have been

associated recently with more physiologically relevant rDNA copy number variants that are sufficient for ribosome biogenesis (Gibbons et al., 2014; Ide et al., 2010; Lu et al., 2018; Michel et al., 2005; Paredes et al., 2011; Picart-Picolo et al., 2020; Wang and Lemos, 2017; Xu et al., 2017). Additionally, the plastic nature of the rDNA locus allows copy number fluctuations in the face of stressors such as nutrient availability (Aldrich and Maggert, 2015) or replication defects (Ide et al., 2007; Lynch et al., 2019; Salim et al., 2017; Sanchez et al., 2017). We were intrigued by the rDNA reductions seen in the presence of replication stress, which strongly suggested that rDNA copy number variation might directly influence replication of the genome.

We predicted that reducing the number of rDNA copies would appreciably promote genome replication. Because of the vast length of the highly repetitive rDNA arrays, replication initiation must occur within the rDNA sequence. Replication initiation has been documented in the rDNA of several species (Bénard et al., 1995; Brewer and Fangman, 1988; Coffman et al., 2006; Hyrien and Méchali, 1992; Little et al., 1993; López-estraño et al., 1998), with the most extensive characterization coming from the budding yeast *S. cerevisiae* where each 9.1 kb repeat contains a potential origin of replication (rDNA Autonomously Replicating Sequence or rARS, (Brewer et al., 1992; Miller and Kowalski, 1993)). A wild type yeast rDNA array of 150 copies would therefore contain 150 replication origins, although only 30-40 of these origins are estimated to initiate replication ("fire") in a wild type cell within an S-phase (Brewer and Fangman, 1988; Linskens and Huberman, 1988). Origin firing is thought to be limited by low abundance of initiation factors that promote the temporal staggering of origin activation by shuffling from earlier to later activating origins around the genome (Collart et al., 2013; Lynch et al., 2019; Mantiero et al., 2011; Yoshida et al., 2013). Recent work has demonstrated that hyperactivation of rDNA replication can divert replication factors away from unique regions of the genome (Shyian et al., 2016; Yoshida et al., 2014) and lead to persistent underreplication of certain genomic regions (Foss et al., 2017). We reasoned that reduction of rDNA from the wild type burden of 100-200 copies would alleviate competition for limiting initiation factors at the other 300 unique origins of replication across the rest of the genome (Nieduszynski et al., 2007). We anticipated that this predicted alleviation would manifest as improved plasmid maintenance,

earlier replication of the rest of the genome, and reduced genome instability in the face of replication stress.

Here we assess genome replication in isogenic strains with rDNA arrays that are either wild type in size (100-180 copies) or minimal (35 copies). Contrary to our expectations, we discovered that the minimal rDNA array does not appear to reduce competition with non-rDNA origins, but instead results in drastic advancement of rDNA replication time in conjunction with increasing the density of active rDNA origins. This large burst of early rDNA initiations from the minimal rDNA array causes an overall delay in replication in the rest of the genome. Surprisingly, loss of the replication fork barrier gene *FOB1* further sensitized strains with early replicating rDNA to DNA damage and premature exit into anaphase, suggesting new roles for both Fob1p and rDNA in monitoring S phase progression. These unexpected findings show that the rDNA array is not merely a passive repetitive element requiring replication, but an active force in coordinating genome replication and cell cycle progression.

RESULTS:

Strains with minimal rDNA arrays do not exhibit ribosome defect phenotypes

Our predictions regarding how rDNA copy number variation would affect replication outside the rDNA locus were straightforward: more rDNA copies would correspond to increased replication initiation at the rDNA locus and reduced replication initiation elsewhere the genome (Figure 1A), while reducing rDNA copy number would lead to fewer rDNA origin initiation events and allow increased initiation of non-rDNA origins. This predicted change in replication potential would be visible using testable replication assays such as plasmid maintenance, 2D gel electrophoresis, and density transfer analysis of replication timing.

To examine the effect of rDNA copy number changes on genome replication, we generated isogenic strains with a reduced rDNA array at the endogenous rDNA locus using the pRDN1-Hyg plasmid-based method described previously (Chernoff et al., 1994; Kobayashi et al., 2001; Kwan et al., 2013). We do advise verifying ploidy when the method is employed in

the future as we noticed increased ploidy in 20-25% of the isolates with rDNA reductions (Figure S1A-B). Because maintenance of reduced rDNA requires the deletion of *FOB1* to prevent rDNA recombination and expansion, we included both *FOB1* and *fob1Δ* strains with wild type long rDNA as control strains. We also examined strains with a weakened rDNA origin ($rDNA^{RM}$) resulting from a variation in the rARS sequence (Kwan et al., 2013), which we hypothesized would further reduce rDNA replication initiation events and enhance the benefits to origins outside the rDNA locus. Since we were concerned about possible *de novo* rDNA copy number changes over the course of the study, we froze multiple samples from log-phase cultures that had been CHEF gel verified (Figure 1B), and then utilized these frozen stocks as inoculants for each experiment. We generated strains with 35, 45, and 55 copies of rDNA and decided to focus on strains with minimal rDNA array (35 rDNA *fob1Δ* and 35 $rDNA^{RM}$ *fob1Δ*) in comparison to strains with wild type copy number (170 rDNA, 180 rDNA *fob1Δ*, and 100 $rDNA^{RM}$).

Previous work had demonstrated that 35 rDNA copies are sufficient for wild type levels of rRNA transcription and ribosome biogenesis (Dauban et al., 2019; French et al., 2003; Ide et al., 2010; Kim et al., 2006). Before embarking on replication analyses with the minimal rDNA strains, we wanted to confirm that any phenotypes we observed were not due to ribosome biogenesis defects. We checked the ribosomal sufficiency of the 35-copy rDNA array by assessing phenotypes that should be negatively impacted by a dearth of ribosome biogenesis: growth rate, sensitivity to cycloheximide over a range of concentrations, and relative 25S rRNA abundance in our strains (Abovich et al., 1985; Rosado et al., 2007; Sanchez et al., 2017). We found that the minimal rDNA strains behaved similar to the wild type rDNA controls in all of these assays (Figure 1C-E, S1C), confirming that rDNA reduction to 35 rDNA copies does not generate significant ribosome biogenesis defects.

<u>Minimal rDNA strains show reduced plasmid maintenance and increased sensitivity to MMS and hydroxyurea</u>

We examined the effects of rDNA copy number reduction on plasmid maintenance, a basic assay that identifies mutants with DNA replication defects on the basis of poor plasmid replication when compared to wild type controls (Maine et al., 1984; Shima et al., 2007; Tye, 1999). Replication initiation at a plasmid origin is subject to the same competition for replication factors as genomic origins. Based on our prediction that origins outside the rDNA would benefit from reduced rDNA copy number (and therefore rDNA origins), we anticipated that strains with short rDNA arrays would show improved plasmid maintenance compared to the wild type rDNA controls. We looked at maintenance of a plasmid containing the genomic replication origin *ARS1* (Autonomously Replicating Sequence 1, (Kwan et al., 2013)). To our surprise, both of the minimal rDNA strains displayed the highest loss rates of the *ARS1* test plasmid: 16.3%/generation for minimal rDNA strain and 15.2%/generation for the minimal $rDNA^{RM}$ strain, almost double that of the control *fob1Δ* strain with wild type rDNA (8.5%/generation, $p = 0.03$) and higher than either of the wild type rDNA *FOB1* strains, regardless of the rARS allele (11.8%/generation and 12.2%/generation, Figure 2A). Because the Southern blot method that we used reflects the amount of plasmid relative to genomic DNA sequences in the culture (Brewer and Fangman, 1994), these differences can be ascribed to differences in plasmid replication rather than segregation. These data indicate that strains with a minimal rDNA array have defects in plasmid replication, counter to our initial prediction that rDNA reduction would improve DNA replication outside the rDNA locus.

If strains with minimal rDNA arrays do indeed have general DNA replication defects, they should exhibit increased sensitivity to conditions that induce replication stress and DNA damage (Shimada et al., 2002; Trabold et al., 2005). Hydroxyurea (HU) induces replication stress by inhibiting the production of dNTPs, which does not directly damage DNA (Alvino et al., 2007) but slows cell cycle progression through activation of the replication checkpoint (Santocanale and Diffley, 1998). In contrast MMS is an alkylating agent that induces DNA damage (Paulovich and Hartwell, 1995). Consistent with previous work by (Ide et al., 2010), we found that strains with minimal rDNA had greater sensitivity to DNA damage by MMS (Figure 2B). Furthermore, we found that rDNA reduction also confers greater sensitivity to HU,

suggesting that reducing rDNA copy number could be inducing problems with both DNA replication and repair.

The minimal rDNA strain shows delayed replication completion across multiple chromosomes

To determine if rDNA reduction also leads to chromosomal replication defects, we performed an S phase specific CHEF gel electrophoresis assay on our strains with wild type or minimal rDNA copy number. Previous publications had found strains with reduced rDNA show replication defects for the rDNA-containing Chromosome XII, but did not explore the potential genome-wide replication effects (Ide et al., 2010, 2007). CHEF gel electrophoresis can be used to monitor completion of chromosome replication as chromosomes that are undergoing replication will not migrate from the well, presumably due to branched replication structures (Hennessy et al., 1991; Lynch et al., 2019). Non-replicating chromosomes, either pre-replication or those that have completed replication, migrate into the gel at their expected sizes. To measure replication completion for individual chromosomes, we quantified the relative amount of Southern blot hybridization signal remaining in the well at 20-minute intervals across a synchronous S phase. For this assay, G1 synchronized cells were released into S phase in medium containing 0.008% MMS (Ide et al., 2010) and cell cycle progression was confirmed by flow cytometry (Figure S2A-B).

We examined replication completion for several chromosomes of different lengths as well as the rDNA array itself in *fob1Δ* strains with wild type or minimal rDNA. As expected, for samples collected prior to the onset of S phase, chromosomes migrated at their typical positions in the CHEF gel (0 minute samples, Figure 2C). We found that both strains began showing retention of chromosomes in the wells at the same time after release into S phase, indicating that the minimal rDNA strain does not have a delayed start of chromosomal replication. However, the two strains differed in their time of replication completion: Chromosomes IV (1531 kb) and VII (1090 kb) were delayed by 10-15 minutes in the 35 rDNA *fob1Δ* strain (Figure 2D, S1D). The extent of delay in completion of chromosome replication

appears to be roughly proportional to chromosome length. We observed a smaller delay in replication completion for Chromosome II (813 kb) and barely any delay for chromosome VI (270 kb), the second smallest *S. cerevisiae* chromosome (Figure 2D, S1D-E). We had difficulty quantifying Chromosome XII for the wild type rDNA strain: 56% of chromosome XII did not migrate from the plug for the G1 sample, possibly due to its very large size (Figure S1D,F). We instead quantified replication completion of intact rDNA arrays, using an FspI digest to liberate the rDNA locus from its Chromosome XII context (Figure S1D). We found that a higher fraction of the minimal rDNA array completed replication than the wild type rDNA array (Figure 2D). Our examination of multiple chromosomes indicates that the minimal rDNA strain shows significant genome-wide delays in chromosomal replication but fewer problems with completion of rDNA replication itself. We therefore conclude that dramatic rDNA copy number reduction is associated with general replication defects across the genome and are not specific to the rDNA locus.

<u>Minimal rDNA strains advance rDNA replication and delay late replicating genomic regions</u>

To investigate the full scale of replication defects resulting from reduced rDNA copy number, we performed density transfer experiments to examine genome-wide replication kinetics in depth. The density transfer method exploits the semi-conservative nature of DNA replication to distinguish newly replicated DNA from unreplicated DNA at defined loci in the genome. Cells are grown in isotopically dense ("heavy") medium, synchronized, and then released into isotopically normal ("light") medium such that the newly replicated DNA forms a "heavy/light" hybrid molecule. Hybrid replicated DNA can be resolved from unreplicated dense DNA by cesium chloride gradient fractionation and analyzed using quantitative Southern blotting and microarrays (Alvino et al., 2007; Raghuraman et al., 2001). Although this method cannot distinguish active origin firing from passive DNA replication, it allows us to identify replication differences across the genome. We collected samples across an S phase and assessed replication kinetics both on an individual locus level and a genome-wide scale. We used flow cytometry to confirm that the minimal and wild type rDNA strains had similar G1 arrest and progression through S phase (Figure S2C).

We initially focused on the rDNA locus, which is known to be late-replicating in *S. cerevsiae* and several metazoan eukaryotic species (Coffman et al., 2005; Concia et al., 2018; Foss et al., 2017; Labit et al., 2008; Schübeler et al., 2002). We calculated rDNA T_{rep}, the time at which half-maximal replication was achieved, for each strain and confirmed that rDNA is indeed late replicating in the wild type rDNA strain (T_{rep} =40.0') (Figure 3A-B, S3A). However, it is obvious from the kinetic curves that the minimal rDNA array replicates well ahead of the wild type array with a T_{rep} of 28.9'. In fact, the minimal rDNA becomes one the earliest replication regions rather than one of the latest, as observed with the wild type rDNA (Figure 3B). The minimal $rDNA^{RM}$ array also showed advancement of replication time in comparison to the wild type copy number $rDNA^{RM}$ control (Figure S3B), demonstrating that the minimal rDNA array replicates substantially earlier than the long arrays regardless of the rARS allele. This drastic shift of rDNA replication time of the minimal rDNA array could alter the order in which limited replication factors typically are recruited to different genomic origins rather than simply altering the number of competitors from the rDNA locus.

To evaluate the effects of this early replicating rDNA, we compared the replication kinetics for multiple unique regions across the genome (Figure 3B, S3C-D, Table S2). *ARS305* and *ARS607* are two of the earliest and most efficient replication origins in *S. cerevisiae* (Friedman et al., 1997; Hoang et al., 2007). We found that both *ARS305* and *ARS607* are not greatly altered in minimal and wild type rDNA strains, indicating that these early efficient origins are not affected by advanced timing of the minimal rDNA locus. Regions of the genome that are known to be later replicating, however, appear to incur replication delays in the minimal rDNA strain. We examined *ARS501* (also called *ARS522*) and *ARS735.5* as well as ChrV:534000, a fragment on the right arm of chromosome V that does not contain an origin (previously called "R11"; (Ferguson and Fangman, 1992)). Each late-replicating locus was delayed in the strain with a minimal array but their relative replication order was maintained. Thus, while the replication time of early origins appears largely unaffected by early replication

of the minimal rDNA array, late origins and non-origin regions experience delays in replication time.

Strains with minimal rDNA have altered time of origin initiation

Altered replication kinetics can be the result of changes to the time of origin activation and/or efficiency, defined as a change in the fraction of the population that initiates replication at that given origin during that cell cycle. The initial rise of the rDNA kinetic curves suggests that the minimal rDNA array begins replicating earlier than the wild type array (Figure 3A), but to directly examine replication initiation, we employed 2D gel electrophoresis using synchronized cells sampled across S phase. If the time of replication initiation were changed, we should see a difference in when the replication "bubble" arc is detected at a given origin (Figure S3E). Strong rDNA origin (rARS) initiation in the minimal rDNA locus began robustly at 15 minutes into S phase, whereas the wild type rDNA locus initiated replication later (at 20 minutes) reaching a maximum by 20-25 minutes (Figure 3C). These results confirm that early rDNA origin initiation is responsible for the earlier replication of the minimal rDNA locus seen by density transfer.

The replication time alterations seen by density transfer were confirmed by 2D gels at other genomic origins also. In the minimal rDNA strain, we observed ~5-minute delays in replication initiation at late origins such as *ARS735.5* and *ARS1414* (Figure 3D, S3G) with no apparent change in cumulative origin use. We found no difference in initiation timing at the early, efficient *ARS305* (Figure S3F), which reflects the density transfer data and also supports the idea that both the minimal rDNA and wild type strains entered S phase at the same time. We therefore conclude that the replication time changes in the minimal rDNA strain are due to delayed initiation at late non-rDNA origins of replication.

Delayed replication progression is seen genome-wide in the minimal rDNA strains

Since individual loci exhibited altered replication time, we wanted to examine the genome-wide effects in strains with early-replicating minimal rDNA arrays. To assess

replication kinetics genome-wide, we hybridized our fractionated density transfer samples to microarrays and plotted percent replication against chromosomal coordinates to create chromosomal profiles that describe the landscape of replication over time (Alvino et al., 2007; Raghuraman et al., 2001). We picked the earliest S phase sample in which replication was detected and the three subsequent 5-minute intervals afterwards. In the earliest sample with detectable hybrid density DNA at replication origins, we find only subtle differences between the earliest replication profiles of the minimal rDNA strain and that of the wild type strain. (3.1% vs. 2.7% genome replicated, Figure 3E, S4, S5A-B). However, at the next 5 minute interval, we find that the minimal rDNA strain achieved lower levels of replication completion (7.2% genome replicated) when compared to the wild type strain (15.9%) (Figure S5A). This trend continued for the next two intervals, indicating slower advancement of replication across the genome for the minimal rDNA strain. We did not identify any specific genomic regions being preferentially altered in their replication profile; chromosome-wide replication profiles from minimal rDNA strain and wild type rDNA strain are super-imposable when they achieve similar levels of genome replication (Figure S5B). These data, alongside the single locus data, indicate that there is a genome-wide replication delay in the minimal rDNA strain but that no region of the genome is specifically targeted.

Cyclin regulation reflects DNA replication delays in cells with minimal rDNA

Delayed completion of genome replication should also manifest as delayed appearance of cell cycle landmarks. Cyclin regulatory changes accompany the conclusion of S phase, including the degradation of the CDK activator Clb5p (Schwob and Nasmyth, 1993), the phosphorylation of limiting initiation factor Sld2p (Bloom and Cross, 2007; Lynch et al., 2019; Masumoto et al., 2002), and the reinstatement of the CDK inhibitor Sic1 (Barberis et al., 2012; Khmelinskii et al., 2007; Schwob et al., 1994; Verma et al., 1997). If S phase were extended in the minimal rDNA strain due to lagging genome replication, we would expect to see delayed regulatory changes to Clb5p, Sld2p, and Sic1p. We separately tagged Clb5p, Sld2p, and Sic1p at each endogenous locus using a 3HA tag marked by KanMX6 (Longtine et al., 1998) in both the minimal rDNA and wild type rDNA strains. We then collected synchronized samples of each

strain throughout S phase for analysis via flow cytometry and western blotting. The tagged minimal rDNA and wild type rDNA strains appeared to have similar S phase progression (Figure S2E). However, when we examined the cyclin signaling by western blotting, we found that the minimal rDNA strain showed 10-minute delays in the degradation of Clb5-HA, the phosphorylation of Sld2-HA, and the degradation and reappearance of Sic1-HA (Figure S5E-G). These delays in cyclin signaling in the minimal rDNA strain match the strain's delayed genome-wide replication.

Strains with minimal rDNA retain competent Rad53p activation

The ATM/ATR DNA damage and replication stress response pathways both lead to phosphorylation of Rad53p (Friedel et al., 2009; Segurado and Tercero, 2009; Thaminy et al., 2007). Therefore, we wanted to investigate if the minimal rDNA strain would show earlier and increased Rad53p phosphorylation. To do so, we generated Rad53-3HA tagged strains and examined cellular responses in HU, but found no significant difference in Rad53p phosphorylation between the minimal rDNA strain and the wild type rDNA strain (Figure S5H). This result suggests that the observed genome replication delay and sensitivity to replication stress in the minimal rDNA strain are not due to altered signaling through canonical ATM/ATR pathways.

How many rDNA origins initiate replication in early S phase?

We hypothesized that the replication delays in the minimal rDNA strain arise from early rDNA replication rather than more rDNA initiation events. Approximately one in five of the rDNA origins is thought to serve as an initiation site during S phase in cells with wild type rDNA (Brewer and Fangman, 1988; Linskens and Huberman, 1988). By this estimation, a strain with 180 rDNA copies would have ~36 active origins across the rDNA locus — close to the maximum number of possible rDNA origin initiations in our minimal rDNA strains with 35 rDNA copies. The replication defects we see must therefore come from the shift of numerous late rDNA origins now replicating early. We wondered how many rDNA origins fire in early S

phase and if the increased early firing from the minimal rDNA array would suffice to affect replication time genome-wide.

Digestion of replicating rDNA with the restriction enzyme NheI generates a variety of distinct molecular intermediates/structures such as “bubbles” from active initiation, passively replicated “Y” fragments, and “X” fragments produced by converging replication forks (Figure 4A), all of which can be resolved by 2D gel electrophoresis and Southern blotting (Figure 4B) (Brewer and Fangman, 1988, 1987). However, accurate quantification of early rDNA initiation requires replication in the presence of the ribonucleotide reductase inhibitor hydroxyurea (HU) to slow down the fast-moving replication forks. Because *S. cerevisiae* replication forks travel at a rate of 1.5 kb per minute at 30ºC (Bell and Labib, 2016), forks initiated at an rDNA origin will travel off the 4.7 kb origin-containing fragment (Figure 4A) in under 2 minutes and/or and fuse with an oncoming rDNA fork in ~3 minutes. We therefore released G1-synchronized cultures into S phase in the presence of 200 mM HU, which allowed us to capture more replication intermediates as well as limit initiation to only early S phase (first 60 minutes in HU; (Alvino et al., 2007; Feng et al., 2006). Our rDNA quantification was aided by the fact that the NheI digest generates a 24.4 kb single-copy rARS fragment at the telomere proximal end of the rDNA array in addition to the high-copy 4.7 kb rARS fragment from the rest of the rDNA locus (Figure 4A-B). We used this single-copy rDNA fragment for normalization, allowing us to generate a “per cell” comparison of active rARS initiation in early S phase.

We found that minimal rDNA strains have at least a 10-fold increase in rDNA initiation during early S phase. The minimal rDNA strain showed indisputably greater signal from the bubble arc, which represents active replication initiations, than the wild type during S phase in 200 mM HU (Figure 4C, S5C). To generate more precise measurements of early rDNA replication, we estimated the number of rDNA initiations per cell (Figure 4A-B). At the 30 minute sample time, we estimate that the minimal rDNA strain had 9.1 rDNA initiations per cell whereas the wild type rDNA strain showed less than one rDNA initiation per cell (Figure 4D).

Our quantification of initiation from the bubble arc is likely an underestimate due to the movement of replication forks off the rARS-containing fragment even in 200 mM HU (Figure 4A). We found passively replicating Y-arc and convergent double-Y intermediates (Figure 4B) in the adjacent non-origin 35S fragment (Figure 4E, S5D), which represent replication forks that have traveled from a neighboring rDNA origin. Quantification of these Y and double-Y fragments relative to the linear 1N spot indicate that the wild type rDNA array had an additional 2 initiation events per cell while the minimal rDNA array had an additional 11 origin initiations (Figure 4F). Combining the active and passive replication values, we estimate that the wild type strain contains 2 active rDNA origins while the minimal rDNA strain contains 20 active rDNA origins in early S phase. Considering that the earliest replicating origin subset across the *S. cerevisiae* genome is only 60 origins from a population of cells (Alvino et al., 2007; Yabuki et al., 2002), 18 additional early-replicating rDNA origins in a single cell could easily generate significant competition for limiting replication factors or nucleotides in early S phase (Mantiero et al., 2011; Shyian et al., 2016), causing delays that would cascade throughout the genome replication program.

<u>Strains with early replicating rDNA: increased DNA damage sensitivity uncovers a synthetic interaction with *fob1Δ*</u>

Two other yeast mutants replicate their rDNA precociously: *rif1Δ* and *sir2Δ* (Figure 5). Rif1p inhibits local replication initiation by recruiting protein phosphatase 1 (PP1, Glc7p), which removes premature phosphorylation and activation of replication helicase subunit Mcm4p (Davé et al., 2014, p. 1; Hiraga et al., 2014; Mattarocci et al., 2014). Rif1p binds at the rARS and delays rDNA replication (Hafner et al., 2018; Shyian et al., 2016). Deletion of *SIR2* also advances rDNA replication of a 150-copy rDNA array without reducing the rDNA copy number (Foss et al., 2017; Saka et al., 2013; Yoshida et al., 2014). Sir2p is a histone deacetylase responsible for silencing half of the rDNA repeats. Without *SIR2*, reduced nucleosome occupancy and increased transcription at accessible rDNA repeats facilitate the translocation of MCM helicases away from a repressive chromatin environment (Foss et al., 2019), likely generated by rARS-bound Rif1p. These unregulated MCM helicases can therefore be subject to

early phosphorylation and early replication initiation. We propose that the minimal rDNA array is similar to rDNA in a *sir2Δ* mutant. Both mutants have arrays that are euchromatic and highly transcribed (French et al., 2003; Ide et al., 2010), allowing loaded MCM double hexamers to slide away from the rARS-bound Rif1p. This scenario leads to the premature recruitment of replication factors by the phosphorylated helicases and early replication initiation.

While this model explains the rDNA's shift to early replication in a *sir2Δ* strain, rif1*Δ* strain, or minimal rDNA strain, the model does not account for their differences in sensitivity to DNA damaging agents: the *sir2Δ* mutant does not share the increased DNA damage sensitivity seen among the other strains with early rDNA replication (Figure 6A). This finding was perplexing as the reported genomic replication defects of *sir2Δ* appear more severe than those in strains with minimal rDNA (Foss et al., 2017; Yoshida et al., 2014). We confirmed that loss of *SIR2* results in early replicating rDNA (Figure 6B), though we observed no obvious increase in cumulative rDNA origin initiation across S phase (Figure 6C). Additionally, as a proxy for plasmid maintenance, we examined abundance of the parasitic 2 micron plasmid, which also decreases in the presence of replication defects (Maiti and Sinha, 1992; Storici et al., 1995). Strains with early replicating rDNA (*sir2Δ* or minimal copies) had reduced 2 micron plasmid abundance to approximately 50% of the levels in wild type control strains (Figure 6D). The similarities shared between *sir2Δ* and minimal rDNA strains suggest that these strains should share replication defects, yet their DNA damage sensitivity remained incongruous.

We wondered if the lack of increased DNA damage sensitivity could be the result of other compensatory roles held by Sir2p during S phase. In addition to rDNA-specific regulation, Sir2p is involved in modulating replication across the genome. Sir2p stabilizes nucleosomes and restricts replication initiation, whereas deletion of *SIR2* can rescue defects in MCM helicase loading independent of rDNA effects (Hoggard et al., 2020, 2018). Because Sir2p has roles in restricting genome-wide DNA replication, we hypothesized that deletion of *SIR2* would rescue the minimal rDNA strain's sensitivity to replication stress by alleviating genome-wide replication stress generated by an early-replicating rDNA locus. We therefore examined MMS

sensitivity upon *sir2Δ* deletion in the minimal rDNA strain and the wild type rDNA strain. We found that deletion of *SIR2* failed to rescue the greater sensitivity of the minimal rDNA strain. However, this finding was complicated by our observation that the *sir2Δ fob1Δ* strains were much more sensitive to MMS even with 180 rDNA copies (Figure 6E). This *FOB1*-dependent MMS sensitivity was reproducible among multiple strain isolates, each of which was assessed for rDNA copy number (Figure S6A-B), confirming a synthetic defect in DNA damage sensitivity between *fob1Δ* and *sir2Δ.*

We had not considered that *fob1Δ* might drive sensitivity of strains with early rDNA replication to DNA damage/replication stress. The *fob1Δ* background is required to prevent expansion of 35-copy rDNA arrays (Kobayashi et al., 1998). However, we hoped that the rDNA expansion might be slow enough to capture and assess DNA damage sensitivity of a short rDNA array after *FOB1* restoration. Accordingly, we examined spores from a cross of the minimal rDNA strain (35 rDNA copies; *fob1Δ*) strain with a MATα 150 rDNA *FOB1* strain. Each spore was allowed to form a colony, immediately inoculated into cultures, and both rDNA copy number and MMS sensitivity were assessed from the same culture. As a convenient control, we examined our *fob1Δ* strain with 55 rDNA copies, which exhibited greater sensitivity to MMS than the minimal rDNA strain. We found that our isolated *FOB1* spores with short rDNA had approximately 55 rDNA copies when plated on MMS (Figure S6C). These *FOB1* spores with 55 rDNA copies were more resistant to MMS than either *fob1Δ* strain with 55 rDNA copies or minimal rDNA (Figure S6D). These results suggest that it is the loss of *FOB1* in combination with rDNA reduction that is responsible for the sensitivity to DNA damage. We therefore propose that the presence of Fob1p itself plays a significant role in the mitigation of early rDNA replication effects outside of genome replication delays.

<u>The mitotic exit regulator Cdc14 is mislocalized in the minimal rDNA strain</u>

The replication fork-block protein Fob1p has other roles in addition to its eponymous <u>FO</u>rk-<u>B</u>locking activity: stall-independent DNA recombination (Ward et al., 2000), recruitment of topoisomerase (Krawczyk et al., 2014), and sequestration of the mitotic-exit phosphatase

Cdc14p via recruitment of Net1p binding protein (Huang and Moazed, 2003; Stegmeier et al., 2004). We were intrigued by the latter role of Fob1p since delayed genome replication may alter mitotic exit. Cdc14p and Net1p are recruited to two regions of the rDNA: bound to Fob1p at the Replication Fork Barrier (RFB) and upstream of the 35S transcription start site by a yet unidentified factor (Huang et al., 2006; Huang and Moazed, 2003; Stegmeier et al., 2004). Cdc14p is sequestered in the nucleolus by its inhibitor Net1 until its release to the nucleus during early anaphase and to the cytoplasm during late anaphase and mitotic exit (Shou et al., 1999; Stegmeier et al., 2004; Visintin et al., 1999). Notably, in a *fob1Δ* mutant, the Cdc14p inhibitor Net1p is aberrantly released to the nucleus during anaphase (Dauban et al., 2019). Loss of Fob1p allows bypass of defects in anaphase release and mitotic exit whereas Fob1p overexpression delays mitotic exit (Stegmeier et al., 2004). However, deletion of *FOB1* itself does not increase sensitivity to replication stress (Figure 6A). Furthermore, Cdc14p overexpression leads to defects in cyclin regulation (Visintin et al., 1998), and mislocalization of Cdc14p increases sensitivity to MMS (Breitkreutz et al., 2010; Eissler et al., 2014; Kaneva et al., 2019).

We were curious to determine whether Cdc14 localization itself would be altered by rDNA copy number reduction in a *fob1Δ* mutant background. We examined localization of Cdc14-GFP in comparison to DAPI-stained nuclei in both the minimal rDNA and wild type rDNA strains arrested in G1. For the wild type rDNA strain, we found that 89.6% of cells showed Cdc14-GFP normally sequestered to the nucleolus, which excludes DAPI (Figure 7A-B). The 10.4% of wild type rDNA cells visualized with Cdc14-GFP overlapping the DAPI-stained nucleus are likely due to the chance orientation of the nucleolus rotating above or below the nucleus during microscopy. In the minimal rDNA strain, a mere 32.2% of G1 cells showed nucleolar Cdc14-GFP with 67.8% of cells showing diffuse nuclear Cdc14-GFP.

This aberrant Cdc14p localization is not due to a loss of nucleolar integrity. We examined nucleolar structure in our strains using Utp13-GFP, a nucleolar protein involved in ribosome biogenesis (Huh et al., 2003; Woolford and Baserga, 2013). Utp13-GFP localization

was identical in both the minimal rDNA and the wild type rDNA strains (Figure 7B-C), indicating that nucleolar structure was not altered, consistent with previous findings (Dauban et al., 2019). The minimal rDNA strain's aberrant Cdc14-GFP localization may result in a mitotic exit defect that exacerbates effects from delayed genome replication.

A redundant mitotic exit checkpoint in yeast?

If Cdc14p is involved in coordinating genome replication and mitotic exit, we would expect delayed anaphase entry in response to early rDNA replication to accommodate genome replication defects. Given the substantial delays in DNA replication and cyclin signaling, we anticipated late anaphase entry in all strains with early replicating rDNA: the 35 rDNA *fob1Δ* strain, the *sir2Δ* and *rif1Δ* single mutants. We examined DAPI-stained nuclear morphology across S phase as a proxy for entry into anaphase ((Hartwell et al., 1974; Yellman and Roeder, 2015), Figure 7D). We found that the *sir2Δ* mutant does indeed show later anaphase entry than wild type, indicating that anaphase entry does respond to delayed genome replication in a *sir2Δ* mutant (Figure 7E-F). However, the minimal rDNA strain and the *rif1Δ* strain entered anaphase at the same time or earlier than their wild type control strains, suggesting these mutants have lost the mechanism delaying anaphase in response to delayed genome replication (Figure 7F). The link between genome replication status and anaphase entry appears to be partially dependent on *FOB1*: no difference was observed in anaphase entry between *sir2Δ* and *SIR2* strains in the absence of *FOB1* (Figure 7G). The observed disconnect between S phase completion and anaphase entry mirrors our earlier results with the MMS sensitivity assay: the strains showing anaphase progression in spite of replication delays are also the strains with increased MMS sensitivity (Figure 6A,E). We therefore suggest that the observed DNA damage sensitivity arises from the disruption of FOB1-dependent coordination between anaphase entry and genome replication completion, suggesting Fob1p as a redundant, yeast-specific checkpoint for S phase completion functioning in concert with Cdc14p sequestration (Figure S7).

DISCUSSION:

Minimal rDNA arrays replicate early and delay genome replication

Here, we characterized the replication consequences of reducing the *S. cerevisiae* rDNA locus from its wild type 180 copies to a minimal array of 35 copies. We identified alterations in replication timing not just at the rDNA locus but also across the genome, pointing to the interplay between rDNA biology and the rest of the genome. We found that the minimal rDNA array replicates at the very earliest part of S-phase whereas the isogenic long rDNA is among the latest replicating regions of its genome. We quantified the number of active rDNA origins and found that the minimal array has approximately 20 additional rDNA initiations in early S- phase. Although the total number of genomic origins active in a single cell is not known, the earliest replicating subset consists of only 60 single-copy genomic origins (Alvino et al., 2007; Yabuki et al., 2002). We propose that an additional 18 early-replicating rDNA origins will divert away the limiting factors that are required to activate non-rDNA origins, thereby creating delays in origin activation and subsequent delays in replication at unique regions of the genome.

Our findings contradict previous interpretations of similar data, whose authors conclude that rDNA copy number reduction leads to replication defects that affect only the rDNA locus and do not extend to the rest of the genome (Ide et al., 2010). Those authors did not examine the rDNA array independent of its large chromosomal context. Their conclusions were extrapolated from delayed replication completion of Chromosome XII which contains the rDNA. In addition, replication of other large chromosomes also appears visibly impaired in their publication (Ide *et al.* 2010, Figure 2D), consistent with our findings and interpretation.

The dramatic shift of rDNA replication timing to the early part of S-phase in the minimal rDNA strains is not without cost. In addition to the timing changes, we observed evidence of replication stress by impaired plasmid maintenance, heightened sensitivity to HU and MMS, and delayed completion of chromosome duplication. It appears paradoxical that reducing rDNA copy number generates replication stress given that the full-length wild type rDNA array is dramatically shortened in many conditions of imposed replication stress, either in the presence of DNA replication mutants or limiting conditions like hydroxyurea (Ide et al., 2007; Lynch et al.,

2019; Salim et al., 2017; Sanchez et al., 2017). Indeed, rDNA copy number reduction has been proposed to be a compensatory mechanism for replication stress (Ide et al., 2007; Kwan et al., 2013; Salim et al., 2017). We propose that rDNA copy number reduction will affect genome replication only if the rDNA replication timing is affected. This proposal is supported by our earlier studies describing a mutant that induces replication stress and reduces rDNA copy number (250 to 90) (*SLD3-AID*, (Lynch et al., 2019)). The reduced rDNA array remained late replicating, possibly because its copy number reduction did not suffice to alter replication timing. Further work is necessary to determine the copy number reduction leading to early rDNA replication and if this shift in replication time is gradual or precipitous.

Notably, not all replication mutants respond to rDNA copy number reduction. Hoggard *et al.* 2018 reported that the replication licensing mutant *cdc6-4* is not rescued by reducing the rDNA array to 35 copies, nor did the rDNA reduction increase the *cdc6-4* temperature sensitivity. Competition for limiting initiation factors differs from competition for origin licensing factors; therefore, the discrepancy in replication stress mechanisms reveal distinct phenotypes in these mutants. Precise quantification of both rDNA replication time and number of rDNA initiations will be needed to understand the role of rDNA replication for each replication stress of interest.

<u>The synthetic interaction between early rDNA replication and *FOB1* uncovers a putative cell cycle checkpoint in yeast</u>

Early replicating rDNA is primarily responsible for genome replication defects (this study, (Foss et al., 2017; Ide et al., 2010; Yoshida et al., 2014)), yet the increased DNA-damage sensitivity in strains with early-replicating rDNA is not solely a response to delayed genome-wide replication. As we show, *sir2Δ* single mutants possess wild type rDNA array, early replicating rDNA arrays and suffer genome-wide replication defects (Foss et al., 2017) but these strains are not sensitive to DNA damage. Comparing the *sir2Δ fob1Δ* and *sir2Δ* strains, we unexpectedly found a synthetic interaction between *fob1Δ* and early rDNA replication, which results in increased sensitivity to the DNA-alkylating agent MMS.

How does Fob1p contribute to MMS sensitivity and other agents increasing replication stress in strains with early replicating rDNA? We considered Fob1p's namesake FOrk Blocking role (Brewer and Fangman, 1988; Kobayashi, 2003) and also its more obscure molecular functions. The most obvious hypothesis would be that loss of Fob1p may increase collisions between replication and transcription machinery. However, loss of Fob1p alone does not increase sensitivity to replication stress despite the loss of the replication fork barrier and the putative increase in replication-transcription conflicts (Figure 6A).

Previous studies proposed that rDNA copy number reduction sensitizes strains to DNA damage because there is less opportunity for repair in a short array in which all copies are transcribed (Ide et al., 2010). This interpretation was based on the observation that a deletion of the PolI-subunit *RPA135*, and consequently cessation of endogenous rDNA transcription, erased the difference in MMS sensitivity between a 20-copy rDNA strain and a strain purportedly carrying 110 copies. However, deletions of rDNA transcription machinery, including *RPA135*, are known to result in an 80% reduction in rDNA copy number (Brewer et al., 1992; Kobayashi et al., 1998). Therefore, the rDNA copy number in the aforementioned two strains may not have been very different — the similarity in MMS sensitivity could simply stem from similarity in rDNA copy number between the two strains compared. Notably, rDNA copy number in these strains was not verified in this study (Ide et al., 2010). The MMS-sensitivity phenotype of the *sir2Δ fob1Δ* strain, which contains over 150 rDNA copies, also does not fit the model proposed by Ide *et al.* This strain should have the capacity to repair DNA lesions since there are likely enough repeats with little or no RNA PolI transcription. Indeed, loss of *SIR2* causes only moderate changes in the number of accessible, presumably actively transcribed rDNA copies (*i.e.* 40% accessible vs 60% non-accessible in wild type yeast; 50% accessible vs 50% non-accessible in *sir2Δ*, (Smith and Boeke, 1997)). In our hands, the *sir2Δ fob1Δ* strain shows possibly even greater MMS sensitivity than the minimal rDNA *fob1Δ* strain (Figure 6E). Taken together, our results demonstrate that the capacity for DNA damage repair provided by

additional, silenced rDNA copies does not explain the sensitivity of minimal rDNA strains to DNA damage. However, our results point to an unexpected involvement of Fob1p.

Fob1p also recruits topoisomerase to the rDNA locus (Egidi et al., 2020; Krawczyk et al., 2014), which may be particularly important as DNA unwinding by concurrent transcription and replication generates significant torsional stress. Fob1p-dependent single stranded nicks at the rDNA are present throughout the cell cycle, including G1 (Burkhalter and Sogo, 2004), and may contribute to stall-independent Fob1-mediated recombination (Ward et al., 2000). Recent work has documented that regulation of torsional force is important for the survival of strains undergoing replication stress (Minchell et al., 2020). Fob1p's recruitment of topoisomerase might be of even greater importance in *sir2Δ* and *rif1Δ* backgrounds, in which loss of rDNA silencing might yield more actively transcribed repeats and increased torsional stress. We might therefore predict that this torsional stress impairs rDNA replication, but our findings indicate that rDNA replication progresses early and undisturbed in *sir2Δ fob1Δ* cells and cells with reduced rDNA (Figure 3, 6B).

We further considered Fob1's role in tethering the cell cycle regulatory protein phosphatase Cdc14p, involved in early anaphase release (FEAR) and mitotic exit networks (MEN) (D'Amours and Amon, 2004; Shou et al., 1999; Stegmeier et al., 2004; Visintin et al., 1998). Curiously, Cdc14p is sequestered in the nucleolus until anaphase, and misregulation of Cdc14 wreaks havoc on anaphase progression and increases sensitivity to DNA damage/replication stress (Villoria et al., 2017; Yellman and Roeder, 2015). Because strains with precocious rDNA replication time showed a synthetic interaction with Fob1p-dependent DNA damage sensitivity, we propose a model that is consistent with published data, adds to previously defined Cdc14 regulation, and explains our findings. Specifically, this model explains 1) how rDNA replication might coordinate whole genome replication and anaphase entry and 2) how Fob1p might function as a redundant checkpoint in Cdc14 sequestration and mitotic exit.

In a wild type yeast cell (Figure S7A), ~2000 Cdc14p molecules (Cherry et al., 2012; Ho et al., 2018; Kulak et al., 2014) are sequestered at two sites within an rDNA repeat: at the transcription start site (TSS) and the replication fork barrier (RFB) bound by Fob1p. Cdc14p remains bound at all 180 rDNA repeats during G1 and early S phase. In late S phase, 36 rDNA origins fire and replication forks begin to dislodge Cdc14p from the nearby TSS, but Cdc14p remains bound to Fob1p at the 180 RFB sites. Fob1p, which enforces unidirectional replication (Brewer and Fangman, 1988; Linskens and Huberman, 1988), acts as second, more stable tether for Cdc14p because the RFB can only be replicated by an oncoming replication fork from an adjacent active origin.

Given that a 1 in 5 rDNA origins initiate in a wild type cell (Brewer and Fangman, 1988; Linskens and Huberman, 1988), the nearest active origin is ~45 kb (5 rDNA arrays) away on average, and it will take the replication machinery ~30 minutes to reach the stalled fork. Hence, rDNA will complete replication 30 minutes after rDNA origins fire in S phase, which is consistent with the observation that the rDNA locus is the very last region to complete replication. Thus, Cdc14p is sequestered in the very last region of the genome to replicate and will be fully dislodged by replication at the very end of S phase. In other words, full replication of the rDNA signals the completion of the entire genome and enables subsequent anaphase entry through Cdc14p release.

In a *fob1Δ* mutant with wild type rDNA copy number (Figure S7B), Cdc14p is not bound to the RFB, but it remains associated with the rDNA TSS until rDNA replication completion in late S/early anaphase (Stegmeier et al., 2004) . Cdc14p's binding protein Net1p is prematurely released during anaphase in *fob1Δ* cells (Dauban et al., 2019). However, the wild type-length rDNA is still replicated in late S phase and the genome finishes replication by the time of complete Cdc14p release, maintaining coordination of both replication processes. This *fob1Δ* strain retains wild type sensitivity to DNA damage and replication stress agents.

In a *fob1Δ* mutant with minimal rDNA copy number (Figure S7C), Cdc14p nucleolar localization is severely disrupted even prior to S phase. The 35 rDNA copies may not provide sufficient binding sites for Cdc14p, in particular because Fob1p is missing at the RFB. Further studies are needed to determine if the presence of Fob1p will suffice to restore Cdc14p nucleolar sequestration. Importantly, the minimal rDNA array replicates early, which delays genome replication and dislodges the rest of Cdc14p in early to mid S phase. Therefore, free, unsequestered Cdc14p l can induce anaphase entry while the genome has not completed replication yet. This disconnect between completion of genome replication and anaphase entry may lead to premature cell cycle progression without allowing sufficient time for DNA repair, resulting in increased sensitivity to DNA damage and replication stress agents (Figure 2B, 6A, (Ide et al., 2010))

In a *sir2Δ* mutant with wild type rDNA copy number (Figure S7D), the rDNA replicates early while replication delays are observed elsewhere. This strain shows wild type sensitivity to DNA damage (MMS, Figure 6A). The presence of Fob1p promotes the retention of Cdc14p at the RFB and also enforces slower, unidirectional rDNA replication. Although the rDNA array replicates early, it would still take 30 minutes to for rDNA replication to be complete. During this time, Cdc14p will remain bound to the RFB via Fob1p while the bulk of genome will complete replication, apparently even with rDNA-induced delays. In this way, anaphase entry remains coordinated with genome replication completion. Consistent with this interpretation, we observed delayed anaphase in *sir2Δ* single mutant strains, which suggest anaphase entry remains coordinated with genome replication.

In a *sir2Δ fob1Δ* mutant with wild type rDNA copy number (Figure S7E), Cdc14p is sufficiently bound to the rDNA array in G1, but is missing at RFBs because of the absence of Fob1p. Fob1p absence also leads to more rapid, bidirectional replication of the rDNA array, which is completed in 15 minutes in this strain. This early and rapid replication of the rDNA array will dislodge Cdc14p from the nucleolus while the rest of the genome is in the midst of replicating. Therefore, the *sir2Δ fob1Δ* strain loses coordination between completion of genome

replication and anaphase entry, resulting in increased sensitivity to DNA damage (Figure 6E, 7G).

It is more challenging to fully explain the phenotypes of the *rif1Δ* mutant with wild type-length rDNA arrays. This strain shows early rDNA replication, delayed genome replication, and uncoupled anaphase entry. Unexpectedly though, premature anaphase in *rif1Δ* mutants is independent of *FOB1* and *rif1Δ* single mutants enter anaphase even before wild type (Figure 7F). It has been reported that Rif1p interacts with Cdc14p (Breitkreutz et al., 2010; Xue et al., 2011); however, more experiments are needed to determine the significance of this interaction for cell cycle progression.

Taken together, we propose that the evolutionarily conserved excess of rDNA copies in concert with their late replication in eukaryotes act as checkpoint for whole genome replication via Cdc14p sequestration in the nucleolus. Cdc14p sequestration redundantly requires the presence of the yeast-specific Fob1p at RFBs. DNA damage sensitivity in strains with minimal rDNA arrays arises from the absence of Fob1p.

<u>rDNA replication: implications for genome stability and the cell cycle</u>

As we have shown, rDNA copy number can affect the essential processes of whole genome replication and subsequent cell cycle progression, extending the phenotypic impact of this genomic element far beyond ribosome biogenesis. Although *S. cerevisiae* populations typically maintain strain-specific rDNA copy number (Kwan et al., 2016), the repetitive nature of rDNA arrays can allow for rare array contraction below the range of natural variation. Such cells would exhibit the phenotypes observed in the minimal rDNA strain. At the very least, rDNA copy number should be taken into consideration as a background variable when interpreting the consequences of other genetic perturbations. In fact, rDNA copy number changes are frequently observed after standard *S. cerevisiae* genetic manipulation practices (Kwan et al., 2016). Moreover, we observed that the rDNA reduction method used here generated polyploidy in over 20% of isolates examined. This observation suggests that genome

stability and nuclear separation might be impaired upon first loss of rDNA repeats, possibly due to miscommunication between genome replication and mitotic exit machinery.

For metazoans, rDNA copy number reduction may have pathological implications for health outcomes. A recent study reports that rDNA copy number reduction precedes pathogenesis in an mTOR-activated cancer mouse model, suggesting that rDNA reductions may act as driver mutations in certain cancers (Wang and Lemos, 2017; Xu et al., 2017). Early rDNA replication may also be associated with premature aging. Hutchinson-Gilford progeroid cell lines show bloated nucleoli (Buchwalter and Hetzer, 2017) and increased DNA damage that appears late in S phase (Chojnowski et al., 2020), echoing phenotypes we observed in strains with early replicating rDNA. Although the replication fork barrier protein Fob1p itself is not conserved, some Cdc14p homologs are known to localize to the nucleolus in metazoans (Berdougo et al., 2008; Kaiser et al., 2004; Manzano-López and Monje-Casas, 2020; Saito et al., 2004; Wu et al., 2008). We speculate that Cdc14p regulation in metazoans many be more sensitive to early rDNA replication without the redundant Fob1p tether. However, since metazoans tend to have multiple Cdc14p paralogs, not all of which localize to the nucleolus (Berdougo et al., 2008), these may contribute together to ensure proper mitotic exit.

Finally, a more philosophical note: how plausible is DNA replication as a gauge of whole genome replication status? While the nucleolus was originally deemed an oddly mundane location for such an exciting molecule, Cdc14p sequestration in the nucleolus is now recognized as an important hallmark of cell cycle regulation (Amon, 2008). We argue that this mundane organelle, and Cdc14p's localization within in it, are ideal for cell cycle control. Every cell contains nucleoli, membrane-free organelles that form around the rDNA arrays. Nucleoli and rDNA transcription are highly responsive to cell and organismal physiology, including nutritional status, stress, and aging. The rDNA is late replicating and associated with Cdc14p whose release into the nucleus coincides with anaphase entry (Figure 7H). We end with the speculation that late-replication of the rDNA locus may exist across a wide variety of species to

both mitigate replication competition and coordinate replication status with cell cycle progression.

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Author contributions:

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Declaration of interests:

The authors declare no competing interests.

FIGURE LEGENDS:

Figure 1: Strains with minimal rDNA maintain wild type growth phenotypes. We detected no significant difference in growth rate between strains with minimal or wild type rDNA copy numbers. (A) Cartoon depicting rDNA arrays in strains used in this study and prediction of genome replication effects resulting from competition for replication factors. Strains differed at the rDNA locus in copy number and/or presence of a weak rDNA origin ($rDNA^{RM}$). (B) CHEF gel confirmation of rDNA copy number. Ethidium bromide stained gel (left) and the resulting Southern blot (right) hybridized with a single copy Chr. XII probe (*CDC45*). (C) Growth rate measurements of the five strains. Doubling times (min) indicated in parentheses. (D) Strain sensitivity to the ribosomal translation inhibitor cycloheximide was assessed using a dose response curve in medium containing 0-200 ng/mL cycloheximide. (E) Quantification of rRNA abundance relative to 170 rDNA strain (wild type). Nucleic acid (genomic DNA and total RNA) from each strain was run as a single sample on a gel, which was then split for both northern blotting for 25S rRNA and Southern blotting for a single copy gene (*ACT1*). 25S rRNA abundance was quantified and normalized to the *ACT1* Southern blot loading control.

Figure 2. Strains with minimal rDNA copy number exhibit non-rDNA replication defects. (A) Loss of maintenance of an *ARS1*-containing plasmid was assessed in exponentially grown cultures. *ARS1* plasmid abundance was assessed through Southern blotting and normalized to the endogenous *ARS1* locus. (*) indicates significant difference in plasmid loss rate calculated from slope variance ($p = 0.03$) (B) Spot assays for sensitivity to 200 mM HU and 0.016% MMS. (C) Migration assay of chromosomes from cells released into S phase in the presence of 0.08% MMS using CHEF gel electrophoresis and Southern blotting. Samples were collected in 20 minute intervals and prepared for CHEF gel electrophoresis to resolve intact replicating vs. non-replicating (fully replicated and unreplicated) chromosomes. Chromosomes are unable to migrate out of the plug/well while replicating (Hennessy & Botstein 1991), allowing for quantification of replicating vs. non-replicating chromosomes by comparing the signal in the gel

body vs. plug/well. Hybridization to specific sequences allows examination of individual chromosomes, IV and VI shown. (D-G) Quantification of the percentage of chromosomes undergoing replication in 0.08% MMS: chromosomes VI, IV, and VII as well as the isolated rDNA array. Completion of chromosome replication is reflected in the decrease of signal in the CHEF gel well.

Figure 3: A minimal rDNA array replicates early and delays genome replication. We calculated rDNA T_{rep}, the time at which half-maximal replication was achieved, for several loci. (A) The minimal rDNA array replicates 11 minutes earlier than the 180-copy rDNA array. Replication kinetic curves were generated from density transfer slot blot analysis. (B) Index of T_{rep} for different replication origins and genomic region Chr. V: 534. (C,D) 2D gel analysis of the rDNA origin of replication (rARS) and *ARS1414* throughout S phase. (E) Replicating DNA from multiple density transfer samples representing different time points were hybridized to microarrays and used to generate genome-wide replication profiles. Chromosome VII replication profiles across S phase for the 180 rDNA *fob1Δ* strain (top) and the 35 rDNA *fob1Δ* strain (bottom). Total genomic replication levels and time interval are indicated on the right. Locations of centromere and origins of replication are noted by yellow circles or origin lines. 50% replication threshold is denoted by pink horizontal line.

Figure 4: rDNA copy number reduction drives a 10-fold increase in rDNA replication initiation during early S phase. (A) Schematic of rDNA locus organization, relevant NheI-digest fragments, Southern blot probe locations, and replication intermediates predicted throughout S phase. The 24.4 kb fragment on the telomere proximal edge of the rDNA array serves as an internal reference for quantification as it is present in a single copy per cell and hybridizes to the rARS probe. (B) Representation of different replication intermediates on a 2D gel. (C,E) Timed 2D gels of cells released into S phase in the presence of 200 mM HU. 2D gels were probed with both (C) the rARS probe fragment and then (E) the 35S probe. Converging replication fork intermediates indicate initiation from adjacent rDNA repeats and are seen in the

strain with 35 rDNA copies but not the strain with 180 rDNA copies. (D) Replication bubbles created by rARS initiations were quantified and normalized to the signal in the single copy 24.4 kb linear spot. (F) Estimation of replication fork intermediates present in the 4.4kb NheI fragment without the rARS. Total replication fork signal was normalized to the 4.4 kb 1N spot and adjusted for rDNA copy number.

Figure 5: Proposed mechanisms leading to early replicating rDNA.
Top panel, late replication at the rDNA is enforced by Rif1p bound to the rDNA origin of replication (rARS), which promotes the dephosphorylation of MCM via recruitment of PP1 protein phosphatase. Middle panel, without Rif1p, aberrant MCM phosphorylation allows early initiation at rDNA origins (Shyian 2016). Bottom panel, early rDNA initiation also occurs in strains lacking Sir2p, in which reduced silencing results in reduced nucleosome occupancy and MCM movement away from the rDNA origin and Rif1p repression. Minimal rDNA arrays likely resemble a *sir2Δ* mutant as they are highly transcribed, lack silencing, and therefore replicate early.

Figure 6: Sensitivity to MMS is due synthetic interaction between Fob1p with early rDNA replication. (A) The 35 rDNA *fob1Δ* strain displays increased sensitivity to 0.016% MMS whereas the *sir2Δ* strain and *fob1Δ* strain show wild type sensitivity. (B) Timed 2D gels of rDNA replication initiation in a 170 rDNA *sir2Δ fob1Δ* strain. (C) Quantification of rDNA initiation signal per cell (relative to the 24.4 kb single copy rDNA spot) over the course of S phase for 180 rDNA *fob1Δ,* 35 rDNA *fob1Δ*, and 180 rDNA *sir2Δ fob1Δ* strains. (D) Abundance of the 2 micron plasmid, a parasitic element partially dependent on the *S. cerevisiae* replication machinery, was assessed in logarithmically growing cells using quantitative Southern blotting and normalized to a single copy genomic probe (ChrXV:810). (E) Deletion of *FOB1* increases MMS sensitivity of strains with early replicating rDNA.

Figure 7: Minimal rDNA strains show poor nucleolar sequestration of Cdc14p and premature anaphase entry.

(A) C-terminally tagged Cdc14-GFP was visualized in 180 rDNA *fob1Δ* and 35 rDNA *fob1Δ* cells. (B) Quantification of G1 cells with nucleolar localization of Cdc14-GFP or Utp13-GFP. (C) Utp13-GFP, a nucleolar protein that is involved in rRNA processing, was used to ascertain nucleolar structure (Woolford and Baserga 2013). Both 35 rDNA *fob1Δ* and 180 rDNA *fob1Δ* cells show normal nucleolar structure. (D) Examples of DAPI-stained nuclei representing cells scored as either being "before anaphase" or "after anaphase entry." (E-G) Quantification of percentage of culture that had entered anaphase for each strain over time. (H) Proposed model regarding consequences of rDNA copy number reduction and early rDNA replication.

METHODS:

Yeast strains, probe fragments, plasmids, and media:

Yeast strains used are listed in Supplementary Table 1. Yeast strains were grown, unless noted otherwise, in synthetic complete media buffered with 1% succinic acid (per liter: 1.45g yeast nitrogen base, 20 g glucose, 10 g succinic acid, 6 g NaOH, 5 g $(NH_4)_2SO_4$, 2.8 g amino acid powder mix with pH adjusted to 5.8). In cases when YPD medium is used, per liter: 20 g bacto peptone, 10 g yeast extract, and 20 g glucose.

rDNA reduction:

S288c *fob1Δ* strains transformed with the pRDN1-Hyg plasmid, were first isolated by selection for uracil prototrophy, then plated onto medium containing hygromycin B to select for rDNA copy number reduction (Chernoff et al., 1994; Kobayashi et al., 2001; Kwan et al., 2013). Individual colonies were picked for screening by CHEF gel electrophoresis to measure rDNA copy number (Figure S1). We identified and isolated strains with 35, 45, and 55 copies of rDNA and decided to focus on strains with 35 rDNA copies ("35 rDNA *fob1Δ*" and "35 rDNA^{RM} *fob1Δ"*), restoring endogenous *URA3* to facilitate downstream replication assays. Genetic crosses were used generate prototrophic strains and GFP-tagged strains.

During the isolation of strains with reduced rDNA copy number by this pRDN1-HYG plasmid method, we noticed that 20-25% of the isolates with rDNA reductions had either diploidized or

tetraploidized (Figure S1), something we had not previously observed when constructing strains by transformation. Subsequently, we verified ploidy of each strain for each experiment by flow cytometry and used only confirmed haploid strains for each experiment. This frequent increase in ploidy may be related to rDNA reduction by this pRDN1-Hyg method. While interesting, we have not identified the biological mechanism involved and strongly suggest verifying ploidy when this rDNA reduction method is employed in the future.

Preparation of DNA in agarose plugs:

DNA was isolated in agarose plugs according to previously published protocols (Tsuchiyama et al., 2013). Each 90 µL plug contained either ~10^8 stationary phase cells for CHEF gels, ~5 x 10^7 log phase cells for rDNA 2D gels, or ~10^8 log phase cells for single-copy origin 2D gels. Collected cells were washed with 50 mM EDTA, resuspended in 90 µL 0.5% SeaPlaque GTG agarose in 50 mM EDTA, and transferred into plug molds. Once solidified, plugs were incubated in 1 mL spheroplasting solution (1 M sorbitol, 20 mM EDTA, 10 mM Tris-HCl pH7.5, 14 mM β-mercaptoethanol, 0.5 mg/mL Zymolyase-20T (Amsbio)) for 2-5 hours at 37ºC. Plugs were washed once with LDS (1 % lithium dodecyl sulfate, 100 mM EDTA, 10 mM Tris–HCl pH 8.0) and incubated overnight at 37ºC in LDS overnight with gentle shaking. Plugs were then washed 3 x 30 minutes in 0.2X NDS (1X NDS pH 9.5: 0.5 M EDTA, 10 mM Tris base, 1% Sarkosyl) and 5 x 30 minutes in TE pH 8.0. Processed plugs were stored at 4ºC in TE pH 8.0 until use.

CHEF gel analysis:

We used contour-clamped homogeneous electric field (CHEF) gel electrophoresis to resolve intact *S. cerevisiae* chromosomes. A slice of each genomic DNA agarose plug was embedded in a 0.8% agarose gel (0.5X TBE) and each gel contained one wildtype sample as reference. For most CHEF gels, we ran the samples in 2.3L of 0.5X TBE using a Bio-Rad CHEF-DRII electrophoresis cell at 100V for 66 hours (switch time = 300 to 900 seconds). The gels were then stained with ethidium bromide to visualize all chromosomes, including the rDNA-containing chromosome XII. To examine the size of the excised rDNA array, genomic DNA

samples in plugs were digested with BamHI or FspI and then run on a 0.8% CHEF gel at 165 V for 64 hours (switch time = 47 to 170 seconds). Chromosome XII size and rDNA copy number were further examined via Southern blotting. For size comparison, known standards (*H. wingei* and/or Yeast Ladder from New England BioLabs) were included in each CHEF gel run.

Sample collection for chromosome replication completion assay:

Cells were grown to mid-logarithmic phase (2.5 x 10^6 cell/mL) , arrested in G1 with 3 μM α-factor, and released into S phase (by the addition of 0.15 mg/mL Pronase (EMD Millipore)) in the presence of 0.008% MMS. Samples were collected every 20 minutes for CHEF gel electrophoresis and prepared as described above in agarose plugs for CHEF gel electrophoresis. The same Southern blot membrane was probed for all measured chromosomes except for the FspI-excised rDNA.

Southern blotting:

Each gel was transferred to a GeneScreen Hybridization membrane using standard Southern blotting protocols (Tsuchiyama et al., 2013) We then hybridized each sequence 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. Phosphor screens were scanned using a Bio-Rad Personal Molecular Imaging scanner and analyzed using Bio-Rad's Quantity One software. Southern blots were often stripped and re-probed with a different sequence of interest (CHEF gel blots, 2D gel blots, density transfer blots). To strip a Southern blot, blots were subjected to two washes of 20 minutes each in 500 mL stripping buffer (0.1% SSC; 1% SDS) that had been heated to 100°C. Blot stripping efficacy was gauged by exposure and quantification of phosphor screens before the next probe hybridization.

rRNA quantification:

rRNA quantification was performed as described (Sanchez et al., 2017). Asynchronous logarithmic phase cells were collected and nucleic acids (RNA and DNA) were isolated using a "Smash & Grab" phenol:chloroform extraction protocol (Radford, 1991). The RNA northern

948 blot was hybridized to a ^{32}P-labeled probe for the 25S rRNA sequence. To assess loading
949 normalization, the DNA Southern blot portion was hybridized to a ^{32}P-labeled probe for *ACT1*, a
950 single copy gene. The rRNA and *ACT1* blots were separately exposed to S Bio-Rad phosphor
951 screens and *25S* rRNA and *ACT1* DNA intensity was quantified using a Bio-Rad Personal
952 Molecular Imager and Bio-Rad Quantity One software.
953
954 Density transfer:
955 The density transfer protocol was adapted from (Alvino et al., 2007) Dense medium
956 composition was 0.5% ^{13}C-labeled glucose, 0.5% $^{15}(NH_4)_2SO_4$, 0.00145% yeast nitrogen base
957 (YNB), and 1% succinic acid (isotopically-light medium was the same composition with normal
958 glucose and $(NH_4)_2SO_4$). Cells were cultured in logarithmic phase for at least 10 generations in
959 dense medium with the growth rate assessed for abnormalities. To collect synchronous S-phase
960 cell samples, cultures of ~2.5 x 10^8 cell/mL were arrested with 3 μM α-factor for 1.25 population
961 doublings (approximately 2 hours). Once the cell culture achieved >95% G1 arrest, cells were
962 collected and washed 3 times with isotopically light medium containing α-factor. Cells were
963 resuspended in the original volume of isotopically light medium containing 3 μM α-factor and a
964 100 mL G1 sample was taken for flow cytometry and DNA analysis. Cells were released from
965 G1 into S phase by the addition of 0.15 mg/mL Pronase (EMD Millipore). 100 mL samples
966 were collected and immediately transferred into vessels containing frozen pellets of 40 mL of
967 0.1% sodium azide in 0.2 M EDTA. The entire set of timed samples was collected before
968 pelleting cells, taking a small aliquot for flow cytometry, and transferring the rest of the dry
969 pellet to -20°C for storage until DNA isolation. DNA was extracted using a phenol:chloroform
970 "Smash & Grab" protocol (see above) with an additional chloroform cleanup. Isolated DNA
971 was digested overnight with EcoRI and then centrifuged in CsCl to separate replicated from
972 unreplicated DNA. Cesium chloride gradients were drip-fractionated and the collected samples
973 were analyzed using slot blots and hybridization to microarrays.
974
975 Flow cytometry:

Cells for flow cytometry were fixed in 70% ethanol before processing for flow cytometry. Fixed cells were washed with 50 mM sodium citrate, sonicated, and resuspended in 500 µL 50 mM sodium citrate. RNase A was added to a concentration of 2.5 mg/mL and the samples were incubated for 1 hour at 50°C. Proteinase K (50 µL of 20 mg/mL) was then added and cells were incubated another hour at 50°C before staining with 1X Sytox Green. Cells were analyzed on a BD Canto II flow cytometer and flow cytometry data was analyzed using FlowJo software.

<u>2D gel electrophoresis:</u>

Cells from the 180 rDNA *fob1Δ* strain, the 35 rDNA *fob1Δ* strain, and the 180 rDNA *sir2Δ fob1Δ* strain were grown in logarithmic phase to a culture density of ~2.5 x 10^6 cells/mL. Cultures were then arrested in α-factor for 1.25 doublings before being released into S phase by addition of Pronase (0.15 mg/mL). Samples were collected every 5 minutes: 100 mL for analysis of single-copy genomic origins or 30 mL for analysis of rDNA origins. Collection vessels contained frozen pellets of 0.1% sodium azide in 0.2 M EDTA to halt growth. Cells were washed once with 50 mM EDTA, a small sample taken for flow cytometry, and the remaining dry cell pellets were stored at -20°C until preparation for 2D gel electrophoresis. To extract DNA for 2D gels, cells were embedded in three 90 µL 0.5% SeaPlaque agarose plugs and prepared as CHEF gel plugs. For each 2D gel, each plug was washed 3 x 20 minutes in the appropriate restriction buffer with 1X BSA (100 µg/mL). The solution was then removed and the DNA was digested for 5 hours by addition of 3 µL restriction enzyme directly onto each plug, and then subjected to standard 2D gel electrophoresis methods (Brewer and Fangman, 1987), Southern blotted and hybridized for the sequence of interest. Cumulative origin initiation was estimated by integrating the area under the curve generated from plotting "rDNA initiations per cell" across time.

For 2D gels of cells in hydroxyurea (HU), 20 mL of the culture was transferred to another flask for the "no HU" control to check that cells would have had normal release into S phase. Hydroxyurea was added to the remaining culture to a final concentration of 200 mM and 10

minutes later, 0.15 mg/mL Pronase was added to both cultures to release the cells into S phase. Samples were collected every 30 minutes and prepared as above.

Plasmid maintenance assay:

Cells that contained plasmids were grown to logarithmic phase in selective medium (YPD + 200 µg/mL G418) and then released into non-selective medium (YPD) for the plasmid maintenance assay. Cells were kept in logarithmic phase growth and samples were collected approximately every 4 hours over the course of 48 hours. The growth rate was monitored to ascertain the number of generations/divisions between samples. DNA was extracted from cells using the "Smash & grab" protocol, digested with XmnI, and run on an agarose gel to resolve the 5.4 kb plasmid *ARS1* fragment from the 3.4 kb genomic *ARS1* fragment (used as a "per cell" loading control). The gel was then Southern blotted and the membrane hybridized to a ^{32}P-labeled *ARS1* fragment. We quantified the amount of signal from plasmid *ARS1* and genomic *ARS1* for each sample and generated a plasmid maintenance curve for each strain, from which we were able to calculate the rate of plasmid loss per generation and estimate significance using linear regression.

Spot assays:

Cells were grown to log-phase, diluted in sterile water in 3-fold dilutions, and 2.5 µL was spotted onto YPD plates containing either no drug (control), 0.016% methyl methanesulfonate (MMS), or 200 mM hydroxyurea (HU). Plates were scanned after 40-48 hours of growth at 30ºC.

Cycloheximide sensitivity assay:

Cells were grown to log phase, upon which 3 x 10^4 log-phase cells were transferred to each well in a 96-well plate containing 150 µL medium per well and the appropriate concentration of cycloheximide (0-200 ng/mL). Each condition was performed in triplicate and optical densities were measured at 30°C for 48 hours using a Bio-Tek reader. The maximum log-phase growth rate was manually calculated for each well.

Western blotting:

Cells were grown in log phase to ~2.5 x 10^8 cell/mL before α-factor arrest and release. For each strain, 1.5 mL was collected for protein extraction and 1 mL was collected for flow cytometry. Collected cell pellets were resuspended in 200 µL SUMEB buffer (1% SDS, 8 M urea, 10 mM MOPS pH 6.8, 10 mM EDTA, 0.01% bromophenol blue) supplemented with protease inhibitors and 5% β-mercaptoethanol. Glass beads (~100 µL 0.5 mm acid-washed) were added and cells were vortexed for 3 minutes. Lysates were incubated at 65°C for 10 minutes with intermittent shaking and then centrifuged for 5 minutes at 4°C at 20,000 x g. The clarified supernatant was transferred to a new tube and protein concentration was assessed using a Qubit (Thermo Fisher). For each sample, 15 µg of protein was run on a Novex Tris-acetate SDS-PAGE gel and transferred to a nitrocellulose membrane for immunoblotting. HRP-conjugated antibodies against HA (Sigma Aldrich #12013819001) and Pgk1 (Abcam #ab197960) were used in this work.

Microscopy:

Cells were fixed according the protocol described on the Koshland lab web site (http://mcb.berkeley.edu/labs/koshland/Protocols/MICROSCOPY/gfpfix.html): collected cell pellets were resuspended in paraformaldehyde solution (4% paraformaldehyde, 3.4% sucrose) and incubated at room temperature for 15 minutes. Cells were then washed once with KPO_4/sorbitol solution and resuspended in 50 µL KPO4/sorbitol solution (0.1 M KPO_4 pH 7.5, 1.2 M sorbitol) and stored at 4°C until visualization. Before fluorescence microscopy, cells were sonicated and incubated with 0.5 µg/ml DAPI for at least an hour.

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Figure 1: Strains with minimal rDNA maintain wild type growth phenotypes.

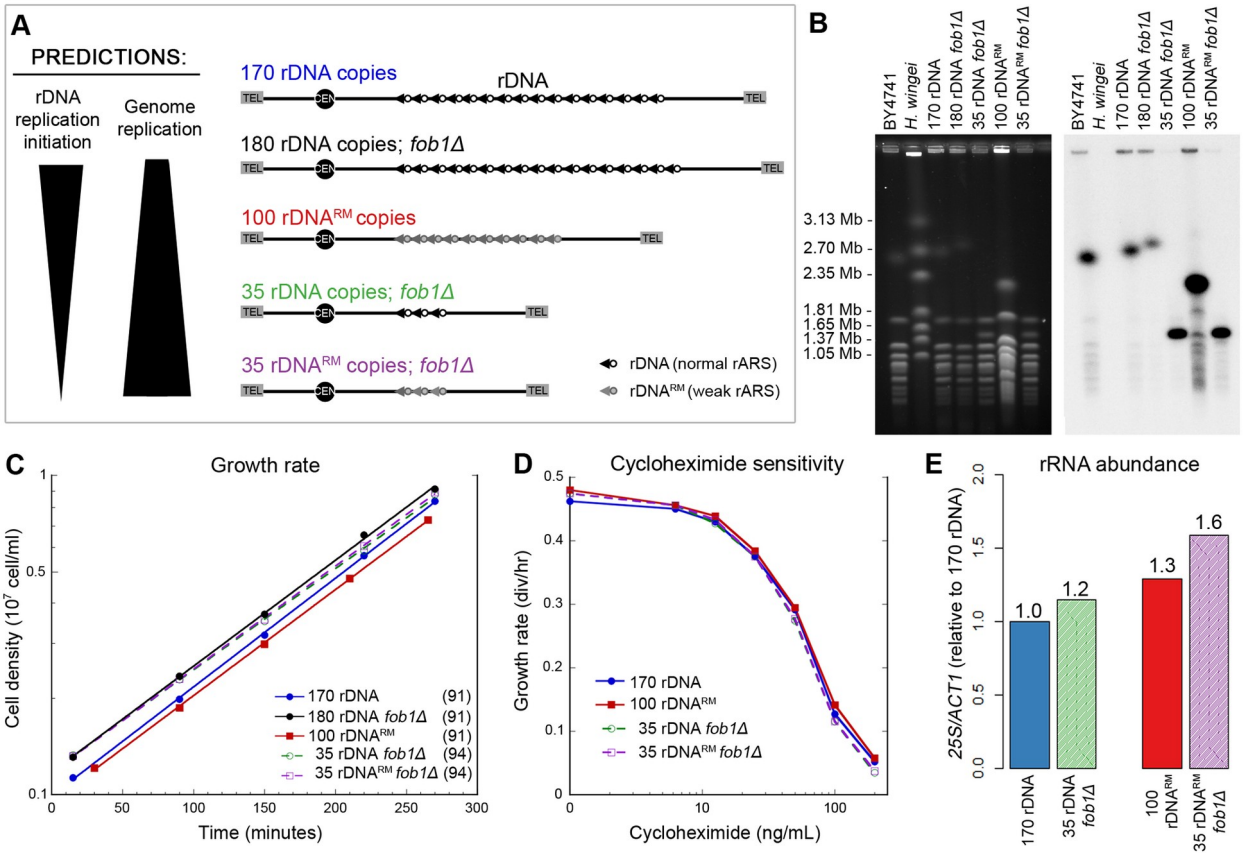


Figure 2. Strains with minimal rDNA copy number exhibit non-rDNA replication defects.

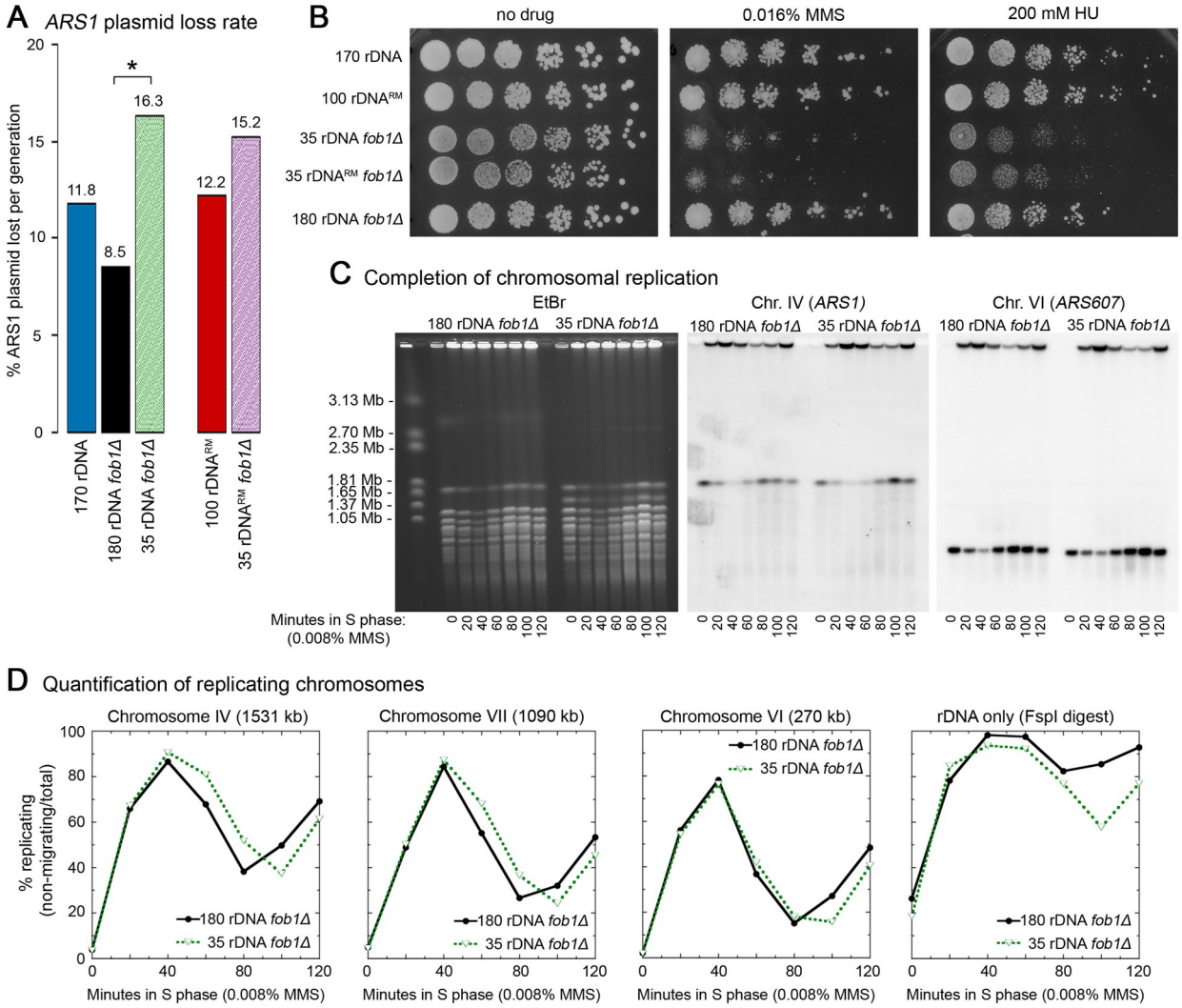


Figure 3: A minimal rDNA array replicates early and delays genome replication.

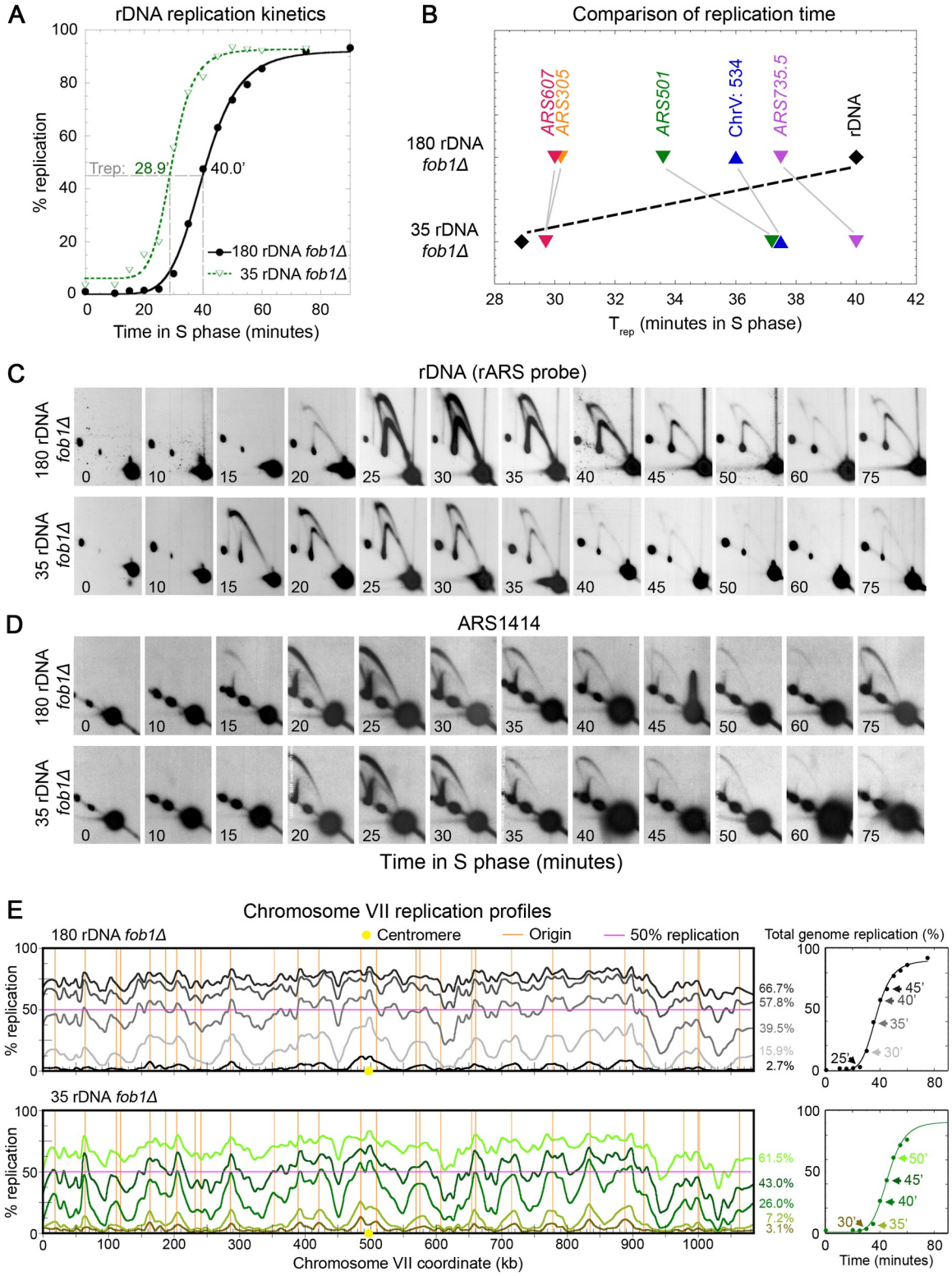
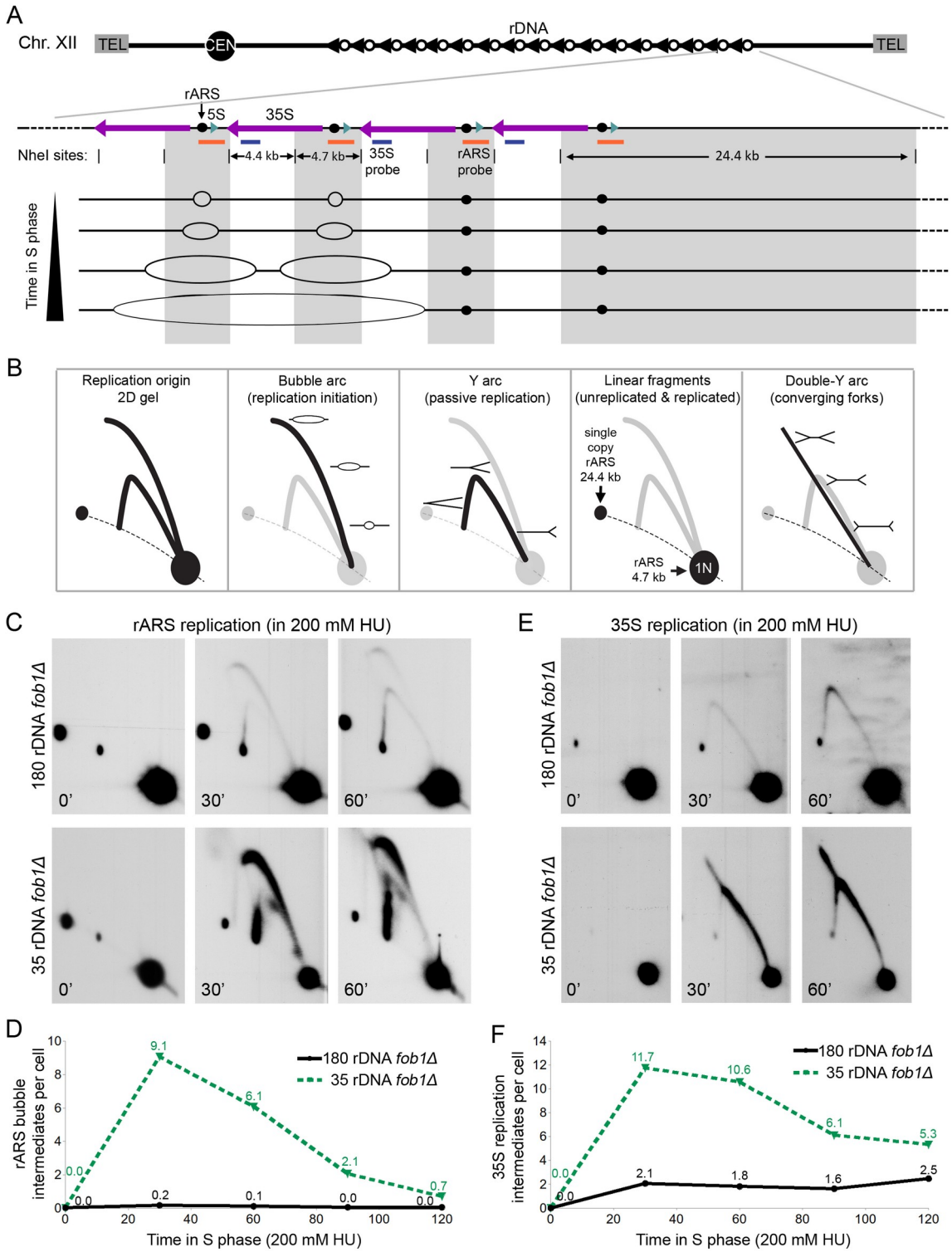


Figure 4: rDNA copy number reduction drives a 10-fold increase in rDNA replication initiation during early S phase.



1073 **Figure 5: Proposed mechanisms leading to early replicating rDNA.**

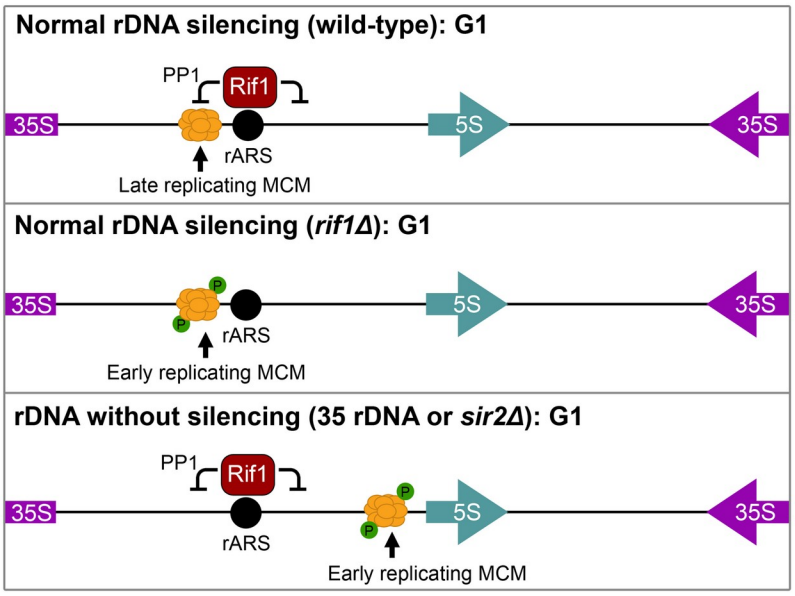


Figure 6: Sensitivity to MMS is due synthetic interaction between Fob1p with early rDNA replication.

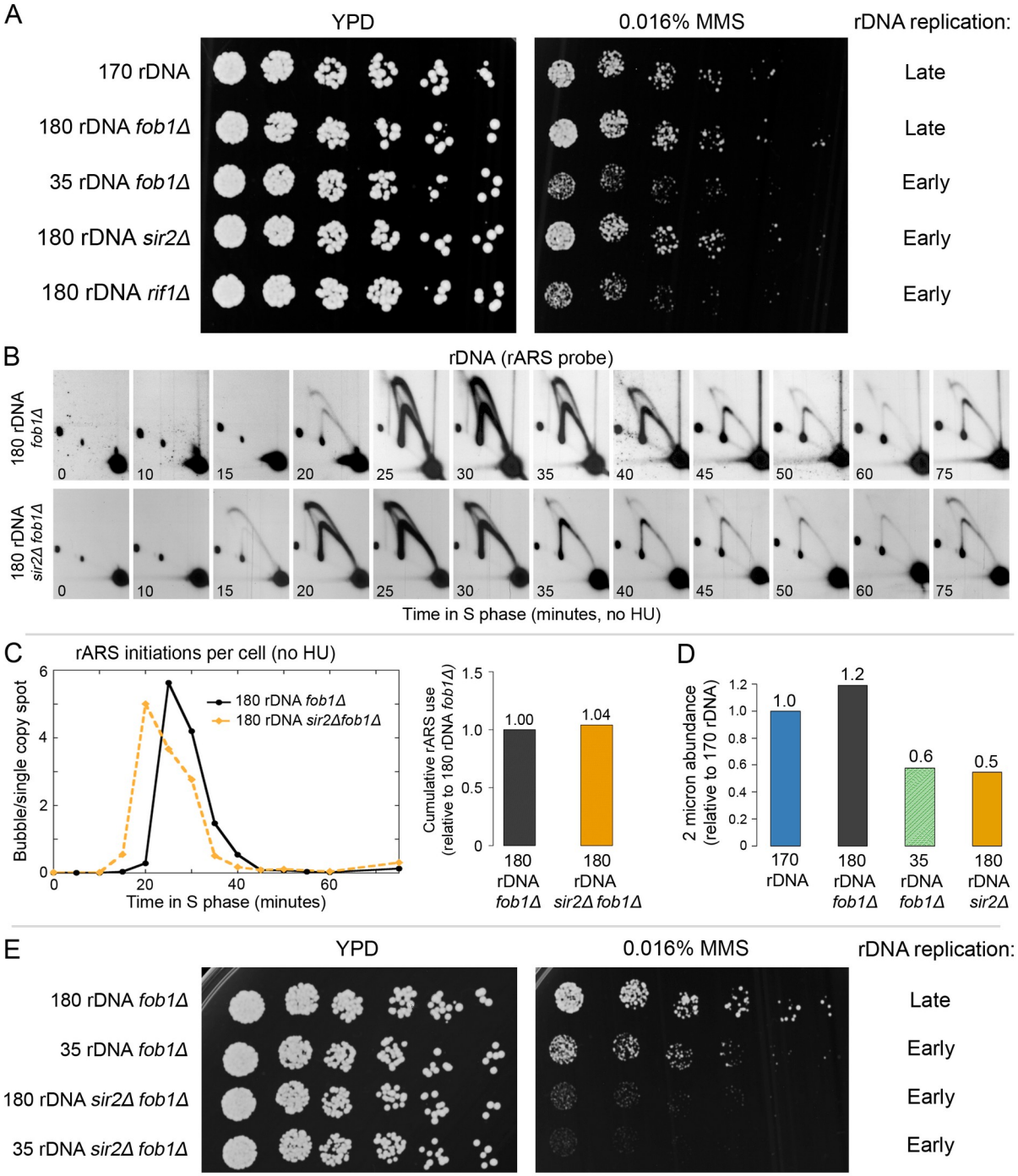
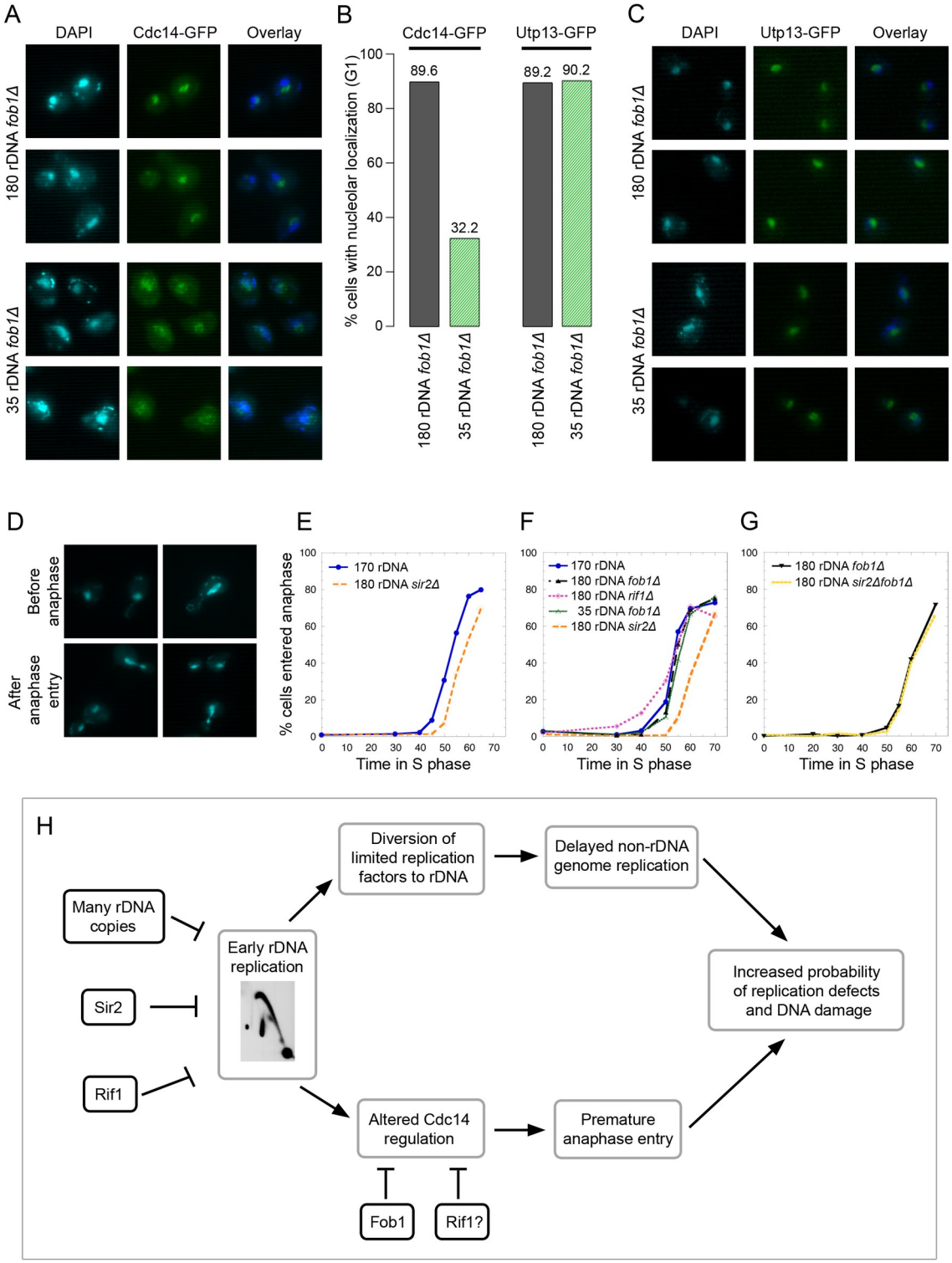



Figure 7: Minimal rDNA strains show poor nucleolar sequestration of Cdc14p and premature anaphase entry.