SIR2 suppresses replication gaps and genome instability by balancing replication between repetitive and unique sequences

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Replication gaps that persist into mitosis likely represent important threats to genome stability, but experimental identification of these gaps has proved challenging. We have developed a technique that allows us to explore the dynamics by which genome replication is completed before mitosis. Using this approach, we demonstrate that excessive allocation of replication resources to origins within repetitive regions, induced by *SIR2* deletion, leads to persistent replication gaps and genome instability. Conversely, the weakening of replication origins in repetitive regions suppresses these gaps. Given known age- and cancer-associated changes in chromatin accessibility at repetitive sequences, we suggest that replication gaps resulting from misallocation of replication resources underlie age- and disease-associated genome instability.

SIR2 | DNA replication | repetitive sequences | replication gaps | ribosomal DNA

Staggered initiation of DNA replication, which is common across eukaryotes from fungi to humans, means that, at any given time, in S phase, only a fraction of replication origins is activated. In recent years, a model has emerged to explain this pattern of DNA replication (1, 2). This model assumes that the pool of initiation factors required to fire licensed origins in S phase is limited, and therefore sufficient to fire only a subset of licensed origins at any given time. Licensed origins differ in their ability to recruit factors required for firing, so origins with higher affinity or accessibility fire earlier than those with lower affinity or accessibility. After activating the initial set of origins, firing factors are released, enabling the next set of origins to fire. This results in successive waves of origin activation. Genome replication eventually finishes when areas with the least accessible origins are replicated.

In healthy human cells, the least accessible genomic regions consist of repetitive DNA, which represents about half of the genome and tends to be compacted into heterochromatin. However, recent studies suggest widespread opening of hetrochromatin during carcinogenesis and aging (3–5). Such reorganization of chromatin would expose a new suite of origins within repetitive DNA that could potentially compete initiation factors away from unique portions of the genome, thereby disrupting the normal genome-wide hierarchy of replication timing. Increased origin activity within repetitive DNA could thus compromise replication elsewhere in the genome. Given the limited pool of initiation factors, we propose that an increase in density of active origins within repetitive regions could result in a decreased density of such origins in unique regions of the genome, which, when combined with the stochastic nature of origin firing, may occasionally result in replication gaps, i.e., unique regions of the genome that are unable to complete replication before mitosis. This so-called "Random Replication Gap Problem" (RRGP) is the subject of long-standing speculation, but such gaps have never been experimentally demonstrated (6, 7). We have identified a situation in yeast in which repetitive sequences more effectively compete for replication initiation factors, and experimentally limiting this competitive advantage promotes replication of unique regions of the genome and extends lifespan (8).

Repetitive sequences in yeast are limited to the ribosomal DNA (rDNA) and subtelomeric repeats. Yeast rDNA comprises ~150 copies of a tandemly repeated 9.1-kilobase (kb) sequence, and represents 10% of total genomic DNA. As each repeat has its own origin of replication and there are ~300 non-rDNA origins, ribosomal origins represent 1/3 of all replication origins in the yeast genome. In a cross between two genetically diverse strains of yeast, we identified a naturally occurring single nucleotide polymorphism within the rDNA replication origin (ribosomal DNA Autonomously Replicating Sequence, or rARS) that both decreases origin activity and extends lifespan by 40% (8). By limiting replication initiation at repetitive rDNA, this polymorphism promotes replication elsewhere in the genome. Specifically, this polymorphism can (*i*) increase origin firing at other chromosomal origins, (*ii*) promote replication of plasmids with weak origins, and (*iii*) partially suppress the temperature sensitivity of a mutation in *ORC2*, which encodes a protein needed for origin "licensing"—the formation of a prereplicative complex at the origin. Calorie restriction, which is known to extend lifespan in organisms from yeast to mammals (9), mimics the effect of this polymorphism by suppressing rDNA origin activity. Conversely, the absence of the histone deacetylase Sir2 (silent information regulator), which decreases replicative lifespan (10), increases rDNA origin efficiency while decreasing

Significance

Because the factors required to fire origins of DNA replication are less abundant than the origins themselves, during S phase, these factors are recycled from one area of the genome to another, and, consequently, genome replication occurs in waves. Unique DNA sequences, which contain protein-encoding genes, replicate before repetitive "junk" sequences. By modulating competition for replication resources between these types of sequences, we demonstrate that increased allocation of resources to repetitive sequences, which we previously showed to be associated with reduced lifespan, prevents completion of replication in unique portions of the genome. We suggest that, as cells age, repetitive sequences compete more effectively for replication initiation factors and that the resulting replication gaps form the basis of replicative senescence.

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origin efficiency at other loci (11, 12). Overall, these observations suggest that increased replication of repetitive regions of the genome can lead to replication gaps elsewhere, and that the presence of such gaps may constitute the proximal cause of death in cellular aging.

To directly determine whether competition between repetitive and unique regions of the genome leads to random replication gaps (RRGs), we developed a technique that both identifies and quantifies such gaps. Combining this technique with our ability to experimentally manipulate the balance of origin activity in repetitive versus unique regions, we demonstrate here that increased origin firing at repetitive sequences promotes the formation of replication gaps elsewhere.

Results

To identify genomic regions potentially vulnerable to the RRGP, we needed a method to monitor the final stages of genome replication. Deep sequencing has been used effectively to dissect the early stages of DNA replication, as read depths change from 1× to 2× over the course of S phase (13, 14). Because this transition begins at active replication origins, comparing read depths for S-phase-sorted cells with cells in G1 or G2 has been an efficient and sensitive method to elucidate the starting points for genome duplication. We reasoned that, by focusing on read-depth changes over the course of G2, flow sorting followed by deep sequencing should prove similarly powerful in dissecting the dynamics of S phase completion, and should thereby allow us to identify those genomic regions that replicate last. We therefore subdivided G2 into early and late fractions and used S-phase cells to identify early origins, as has been done previously (Fig. 1*A*) (13, 14). All read depths were normalized to those in G1.

We tested our method, which we refer to as G2-seq, using a strain of yeast containing an artificial chromosome. Although the native chromosomes replicate in a timely manner, the artificial chromosome completes replication only very late in the cell cycle. This yeast artificial chromosome (YAC) contains a 240-kb fragment of the human TCR-β (T-cell receptor, beta chain) region deleted for sequences that fortuitously behave as DNA replication origins in yeast (15–17). The replication of this YAC, which now depends on a strong yeast origin (ARS1) at the tip of the left arm, is markedly delayed, making the YAC genetically unstable and prone to large deletions due to incomplete replication (16, 17). We analyzed G2-seq data from a strain containing this YAC using two complementary methods: (*i*) by comparing completion of replication along native chromosomes (e.g., chromosome XV, Fig. 1*B*, *Left*) with that along the YAC (Fig. 1*B*, *Right*) and (*ii*) by comparing global replication of individual chromosomes with the global replication of the YAC (Fig. 1*C*). Patterns of origin firing at native chromosomes revealed by S-phase read depths (Fig. 1*B*, in red) coincided with previous results generated using deep sequencing (13, 14) and density transfer techniques (18, 19). The native chromosomes in this strain completed replication at different times across their length. For example, the region surrounding the strong early origin ARS1511 on chromosome XV completed replication before the end of S phase, the region to its left surrounding ARS1510 completed replication by early G2, and, by late G2, replication of the entire chromosome was complete. In contrast,

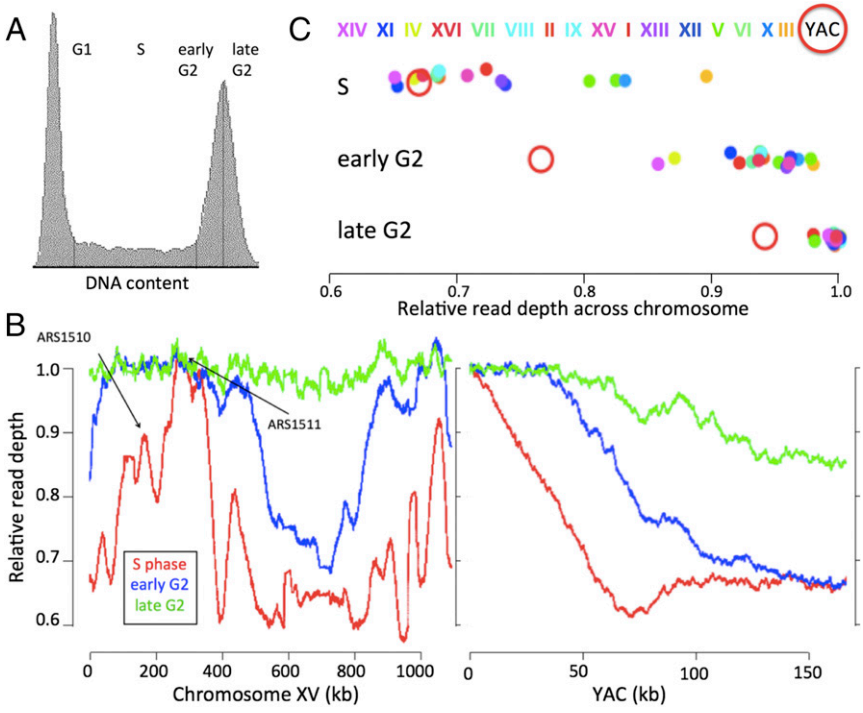

Fig. 1. G2-seq detects late-replicating regions. (*A*) In G2-seq, cells are sorted according to DNA content into G1, S, early G2, and late G2 by flow cytometry, and DNA is isolated and sequenced. (*B*) Replication profiles of a native chromosome (chromosome XV) and a YAC, both in the same strain. [This host strain, which includes the *cdc7-1* mutation, was chosen as a positive control to demonstrate the ability of our technique to identify late-replicating regions because it is identical to the previously published strain in which late replication of this YAC was originally reported (16).] (*Left*) Chromosome XV exhibits known pattern of origin firing in S phase, and its replication is complete by late G2; *x* axis shows positions (kb) on chromosome XV, and *y* axis shows relative read depth in the phase in question divided by read depth in G1, with both read depths normalized to genome-wide maxima. A read depth of 0.5 indicates that the read depth in question is equal to that in G1; a read depth of 1.0 indicates that it is twice that in G1. Two origins described in *Results* are indicated with arrows. (*Right*) YAC origin-free human TCR-β sequence is replicated from yeast origin on *Left*, and late G2 fraction contains cells with incompletely replicated DNA. (*C*) YAC is last chromosome to complete genome duplication; *x* axis shows relative read depth across the chromosome. Sixteen native chromosomes, represented as small filled circles, are color-coded and listed on the top in the order of appearance in WT cells during S phase. YAC is represented as the open red circle.

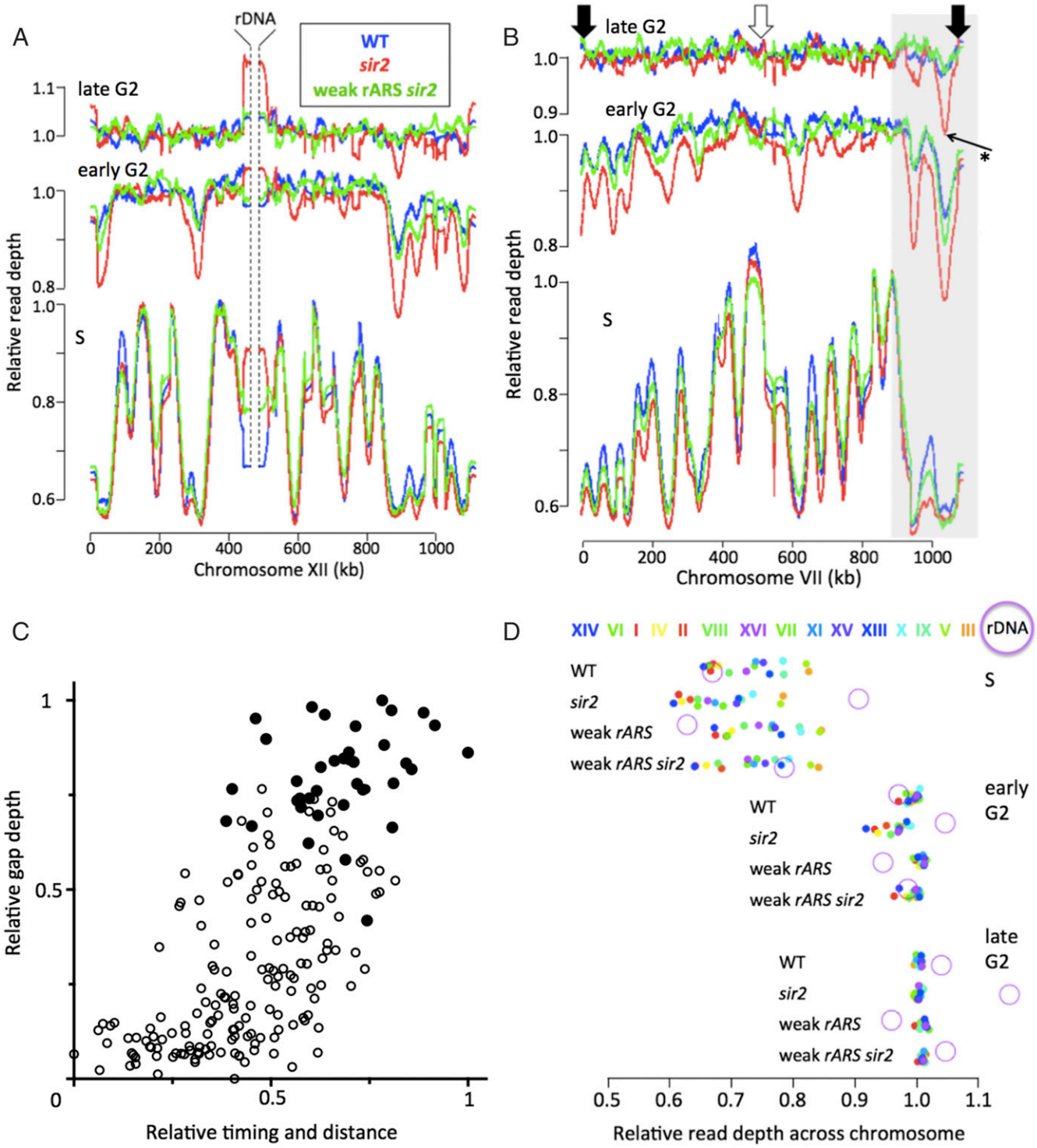

Fig. 2. Replication timing of rDNA is inversely correlated with replication gap formation elsewhere in the genome, which itself is correlated with origin activity and interorigin distance. (*A*) Replication profile of chromosome XII. Advancement of rDNA replication (WT, blue) by deletion of *SIR2* (*sir2*, red) is suppressed by weakening rDNA origin (weak rARS Δ*sir2*, green). Overreplication of rDNA in late G2 likely reflects higher numbers of extrachromosomal rDNA circles in G2 populations, which contain few daughter cells, than in G1 populations, which consist predominantly of virgin daughter cells. (*B*) Replication profile of chromosome VII. G2-seq reveals a replication gap on the right arm of chromosome VII caused by deletion of *SIR2* that is suppressed by the weaker rDNA origin. Filled arrows show locations of *URA3* markers used to measure loss of heterozygosity, and open arrow shows location of centromere, marked with *HIS3*. Arrow with asterisk (*) shows replication gap remaining even in late G2, as described in *Results*. The gray box on the right serves to highlight the region discussed in *Results*. (*C*) Replication gap depth is correlated with interorigin distance and origin activity/timing, with larger interorigin distances and weaker/later origins associated with deeper gaps. Relative gap depth for each pair of origins on the *y* axis is calculated from early G2 read depths (normalized to G1) as the minimum interorigin read depth relative to median read depth. The filled dots correspond to 20% of largest gaps observed in late G2 cells. The parameter on the *x* axis is a linear combination of replication timing/activity of each origin and the distance separating those origins (see *Materials and Methods* for details). (*D*) Deletion of *SIR2* advances replication of the rDNA (large open circles) while delaying replication of other chromosomes, and this effect is suppressed by weakened rDNA origin; *x* axis shows relative read depth across the chromosome. Individual chromosomes, represented as small filled circles, are color-coded and listed on the top in the order of appearance in WT cells during S phase.

replication of the YAC, which initiates from ARS1 at the tip of the left arm, continued to progress rightward late into G2 (Fig. 1*B*). In fact, for 15% of these YACs, the far right end remained unreplicated even in late G2. Use of a different metric—namely, comparison of the global replication progress of individual chromosomes with that of the YAC—yielded similar results: YAC replication lagged behind that of individual chromosomes in both early and late G2 (P = 2.7e-7, and P = 4.0e-17, respectively; Fig. 1*C*). These results indicate that G2-seq is a sensitive and quantitative method useful for identifying replication gaps resulting from sparsely spaced active origins of replication.

We next applied G2-seq to a situation in which our model predicts the existence of replication gaps in native chromosomes, namely *sir2* mutants. Because Sir2 represses origin firing in rDNA (11), G2-seq in *sir2* mutants should detect increased rDNA origin firing and earlier completion of rDNA replication compared with wild-type cells, at the expense of reduced origin firing and the appearance of replication gaps in the unique portions of the genome. Consistent with *SIR2*'s known role in repressing origin activation at the rDNA, we found that rDNA replication initiated and was completed earlier in *sir2* mutants (Fig. 2*A*, red curve) than in wild-type cells (Fig. 2*A*, blue curve). Conversely, replication throughout the rest of the genome was delayed in *sir2* mutants (Fig. 2 *A* and *B* for chromosomes XII and VII, and *SI Appendix*, Fig. S1 for the rest of the genome). Furthermore, certain regions of the genome, for example, an interval near the right end of chromosome VII, remained unreplicated even late in G2 in the absence of *SIR2* (Fig. 2*B*, arrow with asterisk). A focus on this region through S phase and then early and late G2 (Fig. 2*B*, gray box on right) illustrates the strengths of G2-seq in providing insight into the completion of DNA replication: S-phase read depths, although ideal for identifying early origins, provide little insight into which regions will complete DNA replication last. In contrast, those regions of the genome where converging replication forks are last to meet, and therefore where genome replication terminates, are clearly revealed through analysis of read depths in early and late G2 (Fig. 2*B*, gray box on right). This finding demonstrates the long-hypothesized so-called RRGs predicted to occur when adjacent active origins are separated by sufficient distances (6).

Although, because of the stochastic nature of origin firing, the gaps are presumably "random" with regard to the cells in which they occur, they are not random with respect to genomic location or they would not be detectable by our method. We evaluated whether the gap formation at specific locations might be dictated by the distance between adjacent origins and their timing/efficacy. Although neither of these factors alone was strongly correlated with the severity of gaps, their combination, expressed as the sum of the relative lateness with which each origin fires and their relative separation, proved to be an accurate predictive parameter (*Materials and Methods*): The depths of the gaps in early G2 in the *sir2* mutant showed a striking linear dependence on this parameter (P = 2.2e-16, adjusted R^2 = 0.46; Fig. 2*C*, with deepest 20% of late G2 gaps indicated with filled dots). These results demonstrate that a combination of distance and activity of adjacent origin is a strong determinant of gap formation.

If replication gaps emerge due to excessive origin firing at repetitive rDNA, their formation should be suppressed by weakening rDNA origins. To test this prediction, we examined replication gaps in a *sir2* mutant with a weakened rDNA origin previously shown to increase longevity (8), which we refer to as the weak rARS. As expected, *sir2* mutants with the weak rDNA origin (Fig. 2*A*, green line) replicated their rDNA later than those with the wild-type origin (Fig. 2*A*, red line). Furthermore, we found that this weakened origin indeed suppressed gap formation induced by *SIR2* mutation elsewhere in the genome (Fig. 2 *A* and *B* for chromosomes XII and VII, and *SI Appendix*, Fig. S1 for the rest of the genome). Having strong or weak rDNA origins did not grossly alter genome-wide replication profiles in *SIR2* wild-type strains, because rDNA replicated late for both instances (Fig. 2*D* and *SI Appendix*, Fig. S2). Global analysis of individual chromosome replication confirmed that the replication of the rDNA array is advanced in the absence of *SIR2* compared with wild-type cells, and that this advancement is suppressed by the weakened rDNA origins (Fig. 2*D*). At the same time, a reciprocal pattern was seen for the rest of the genome: In *sir2* mutants, the advancement of rDNA replication was accompanied by delayed replication of individual chromosomes, and this delay was suppressed by weakening the rDNA origins (weak rARS *sir2* in Fig. 2*D*), as evident both in S phase and early G2. Global analysis of rDNA and individual chromosome replication revealed subtle differences in replication dynamics between strains with weak versus wild-type rDNA origins in the presence of *SIR2* (Fig. 2*D*). We consistently observed a subtle delay in rDNA replication in the strain with weak rDNA origins (weak rARS) compared with the wild-type strain at all three time points (S and early and late G2) (Fig. 2*D* and *SI Appendix*, Fig. S2*A*), whereas advancement of individual chromosome replication in the weak rARS strain relative to wild type was apparent in S and early G2 (Fig. 2*D*). Together, these results provide further support for our hypothesis that the replication deficiency caused by deletion of *SIR2* results from the shift of replication resources from unique to repetitive regions of the genome.

Allocation of replication resources to the rDNA and away from the unique regions of the genome induced by *SIR2* deletion can be expected to cause a prolongation of S phase and, consequently, an increase in fraction of cells in S phase relative to wild-type cells. Although there was some variability to this phenotype, this is what we observed in flow cytometry profiles of Sir2-deficient cells: Sir2-deficient cells had a higher fraction of cells in S phase (*SI Appendix*, Fig. S3). Furthermore, this increase was suppressed by weak rDNA origins, demonstrating that the slowing of the S phase in Sir2 mutants, like the formation of genome-wide replication gaps, was a result of misallocation of replication resources to the rDNA.

If the replication gaps we identified using G2-seq are biologically significant, regions of the genome with such gaps should be associated with increased genetic instability. As such, one might expect higher rates of marker loss at the end of a chromosome arm with a significant gap compared with a similarly sized chromosome arm without such a gap. Mechanisms that could lead to marker loss include conversion of the gap to a double strand break followed by a loss of sequences centromere-distal to the gap. Chromosome VII, with chromosome arms of approximately equal length, only one of which contains a particularly significant replication gap, provided an ideal opportunity to examine the effect of replication gaps on genome stability. To compare marker loss rate between left and right arms, we generated a diploid strain that is heterozygous for the *URA3* (uracil requiring) marker, whose loss can be selected for on 5-fluoroortic acid (5-FOA), at the right end of chromosome VII (centromere-distal to the replication gap) and, in a separate strain, on the left arm of chromosome VII, which does not exhibit comparable replication gaps (arrows in Fig. 2*B*, *Top*). *HIS3* (Histidine) was inserted at the centromere of the same chromosome that contains *URA3* in both strains, which enabled us to score only loss of *URA3* that resulted from recombination or deletion limited to a chromosome arm (as reflected by growth on medium lacking histidine and containing 5-FOA) but not the loss of the entire chromosome, which would lead to a loss of both *URA3* and *HIS3* markers. As expected, the rate of *URA3* marker loss was approximately 3 times higher on the right arm of chromosome VII compared with the left arm (Table 1). Furthermore, marker loss rates increased approximately threefold to fivefold in the absence of *SIR2*, with a still apparent difference between loss on the right and left arms (Table 1). We conclude

Table 1. Marker loss rate on chromosome VII

Location	LOH rate per 10^5 cells (95% CI)	Relative LOH
SIR2 left arm	1.0 (0.6–1.8)	1.0
SIR2 right arm	3.2 (2.1–6.9)	2.7
sir2 left arm	6.3 (4.9–8.1)	5.3
sir2 right arm	8.9 (7.3–13.3)	7.4

CI, confidence interval; LOH, loss of heterozygosity.

that the replication gaps that we identified using G2-seq have deleterious biological consequences as manifested in an increase in genetic instability.

Discussion

We present evidence that, in yeast, (*i*) there is competition for origin firing between the repetitive rDNA and unique genomic sequences (essentially the rest of the genome), (*ii*) tipping the balance in favor of repetitive DNA by deleting *SIR2* can lead to replication gaps persisting late into the cell cycle, (*iii*) these replication gaps can be suppressed by decreased origin activity within the repetitive rDNA, and (*iv*) chromosomal regions centromere-distal to regions most susceptible to replication gaps are genetically unstable. These results, which constitute a demonstration of these long-postulated gaps (6), both raise and address important questions relevant to completion of genome replication, an area that has thus far been subject to far less scrutiny than replication initiation.

One important open question is what sets apart from others those regions where replication forks are last to converge. Our results demonstrate that a combined effect of large distance between the origins and their late timing strongly predicts the formation of replication gaps in the intervening sequences both in early and late G2. Consistent with our results, a report from Moreno et al. (20) emphasizes the importance of the interorigin spacing in generation of replication gaps: These investigators identified regions of late replication by the presence of ultrafine anaphase bridges. They showed that formation of such bridges was inversely correlated with the density of licensed origins, and, furthermore, that the numbers of bridges increased when the Mcm5 helicase, which is required for origin licensing, was targeted using RNAi (20). More work will be required to make more-specific genome-wide predictions of regions expected to be susceptible to incomplete replication. G2-seq, a technique whose utility is not limited to yeast, should prove valuable in this regard.

Another important open question regarding the completion of genome replication is how common it is that regions remain unreplicated when cells enter mitosis. Moreno et al. (20) observed that, consistent with their theoretical predictions based on origin density, over half of normally cycling human cells exhibit ultrafine anaphase bridges due to incomplete replication. Widrow et al. (21) measured incorporation of bromodeoxyuridine in cells with high levels of cyclin B1, which rises abruptly as cells reach a 4C content of DNA and then falls sharply as they complete mitosis. Widrow et al. (21) estimated that at least 1% of the human genome remains unreplicated very shortly before mitosis, a number they feel could be an underestimate, as sequences they used to assess replication typically replicate early in S phase. The high prevalence of incomplete replication at mitosis in normally cycling cells that is suggested by these studies emphasizes the potential danger to the genome posed by this phenomenon, particularly given our observation that circumstances such as deletion of *SIR2* can significantly exacerbate the problem.

Because replication gaps appear to be common and can lead to chromosome missegregation and nondisjunction in mitosis, several safeguard mechanisms for their resolution before chromosomes are separated have been proposed. During early mitosis in mammalian cells, unreplicated DNA can be processed by the Mus81 endonuclease complex, and this leads to DNA synthesis and promotes completion of replication (22). Another solution has been proposed by Moreno et al. (20), who showed that regions that fail to complete replication by anaphase are associated in the following G1 with stretches of single-stranded DNA but not double-strand breaks, suggesting that a combination of helicases and topoisomerases can resolve incompletely replicated structures in mitosis and allow the resulting single-stranded gaps to be filled in the following cell cycle. However, these proposed safeguards are not failproof. In this report, we demonstrate that sequences centromere-distal to replication gaps are lost at elevated frequency, suggesting that the gaps themselves are prone to breakage. This finding is consistent with the observation of Van Brabant et al. (16, 17) that a marker at the distal tip of the YAC used here was lost at a high frequency after ablation of human sequences serving as yeast origins. Also of interest is whether the consequences of incomplete replication vary across the genome, particularly with regard to repetitive versus nonrepetitive regions. Replication gaps occurring in unique genomic regions are more likely to be lethal than those in repetitive regions, because gaps and subsequent DNA breaks in repetitive regions have mechanisms for repair (e.g., single-strand annealing) that are unavailable in unique regions (23). Therefore, cells may be buffered against the harmful effects of incomplete replication in repetitive regions of the genome like the rDNA. Indeed, we suggest that organizing DNA replication in yeast such that the rDNA replicates last, and perhaps even using completion of rDNA replication as a signal that genome replication is complete, constitutes a reasonable evolutionary strategy for protecting unique regions of the genome.

Finally, we suggest that incomplete genome replication may underlie cellular aging. The rDNA has long been known to be associated with replicative aging, and this has been attributed to toxicity of extrachromosomal rDNA circles or some other aspect of rDNA instability (24, 25). Our results suggest instead that the proximal cause of death may be incomplete replication elsewhere in the genome due to a shift of replication resources to the rDNA. There are two reasons to expect such a shift as yeast cells age. First, Sir2 protein levels decrease with age, with mothers that have arrived at less than a third of their lifespan being devoid of Sir2 (26). Second, daughter cells spend much more time than mothers in G1, as they grow to the cell volume required to enter S phase. Because origin licensing occurs in G1, daughters have more opportunity to license origins throughout the genome compared with mother cells, which have a remarkably short G1. In embryonic stem cells, which also exhibit rapid cycling with short G1, reduced licensing of replication origins creates constitutive replication stress (27). A shift in origin firing toward repetitive sequences likely also occurs during cellular aging in humans, where origins are determined by chromatin accessibility (28, 29), and heterochromatic repetitive sequences open up with age (3, 4). Our model, in which replication gaps increase with age as replication resources shift from unique to repetitive sequences, therefore provides a mechanistic explanation for cellular aging that is relevant to organisms from yeast to humans.

Materials and Methods

Yeast Strains and Media. Yeast strains used are listed in *SI Appendix*, Table S1. Yeast experiments were carried out using standard YPD (yeast peptone dextrose) medium [2% (wt/vol) glucose, 1% yeast extract, 2% (wt/vol) peptone], except for experiments measuring marker loss, which used synthetic complete medium (30). Strain 16528 carrying the YAC was grown at 23 °C, and all other experiments were performed at 30 °C. Oligonucleotides to insert *URA3* on the left arm of chromosome VII are ACTCTAACTATCAATTAAACGACATCTTACATTTCAATTTCATAAagattgtactgagagtgcac and TTTTACGGAAAGCTATATGATATAAACTAGCCAAAAGAATAATTTctgtgcggtatttcacaccg; those to insert URA3 on the right arm of chromosome VII are ACCTCGCCTTGGGCAGGCTCCTCAGTGAAAACCGAAGAAAAAATAagattgtactgagagtgcac and ACCATATATCTAAAACAAGTATCATCGCGCTTCTTTTATTTGTAGctgtgcggtatttcacaccg; those to insert *HIS3* next to *CENVII* are

TCTGCGCGGTTATGGCAAGTTCTCCATCAGTCTCTTATTCCAGCAagattgtactgagagtgcac and TGTACATATGTATTTAGATGCATTGAAGTTCCGTAAAAGAATGCTctgtgcggtatttcacaccg; and those to knock out *SIR2* with *HYG* and *KanMX* are CGGTAGACACATTCAAACCATTTTTCCCTCATCGGCACATTAAAGCTGGagattgtactgagagtgcac and GTAAATTGATATTAATTTGGCACTTTTAAATTATTAAATTGCCTTCTACctgtgcggtatttcacaccg. Plasmid pJH103.1 cut with HindIII was used to knock out *SIR2* with LEU2 (31).

Flow Cytometry. Logarithmically growing cells were fixed using 70% (vol/vol) ethanol, subjected to proteinase K digestion, and their DNA stained with Sytox Green as described (32). Yeast cells were sorted on a BD Biosciences FACSAria II cell sorter, according to DNA content, into G1, S, early G2, and late G2 fractions, with the S-phase fraction being delineated by the G1/S and S/G2 inflection points. These inflection points provided robust landmarks that ensured equivalent progression through S phase in all samples. DNA from a minimum of 1.5e-6 cells from each fraction was isolated using the YeaStar Genomic DNA Kit (catalog number 11-323; Genesee Scientific).

Sequencing Analysis. Fifteen to twenty million 50 base pair single-end reads were generated on an Illumina HiSeq instrument for each sample, aligned to the sacCer3 *Saccharomyces cerevisiae* genome using GSNAP (Genomic Short-read Nucleotide Alignment Program) (33), and read depths were determined using BedTools' genomeCoverageBed (34). The fastq and genomeCoverageBed files for all samples are available under the GEO (Gene Expression Omnibus) accession number GSE90151.

All read depths were processed by first removing spikes, which typically arose at Ty elements, by setting read depths that exceeded 2.5× median read depth to 2.5× median read depth for all regions except the rDNA and the ends of chromosomes, which harbor repetitive sequences; smoothing using a 20-kb sliding window for mean read depth; calculating ratios of read depths to those in G1; and then normalizing those ratios to those at maximally replicated read depths as determined by assessing 12 efficient early-replicating origins in each sample. Smoothing to sliding windows by median rather than mean led to essentially identical results (*SI Appendix*, Fig. S4). Normalizing to completely replicated nonrDNA read depth, instead of total reads, prevents variation in rDNA size and ERC (extra ribosomal circle) copy number from influencing replication profiles. Replication profiles were highly reproducible, as demonstrated by the analysis of biological replicates (*SI Appendix*, Fig. S5).

Calculation of Parameter Predictive for Replication Gap Depth. The parameter to predict the depths of gaps in Fig. 2*C* was calculated according to previously described (19) origin timing and locations (cerevisiae.oridb.org/data_output.php?main=sc_ori_studies&table=Raghu2001_ori&ext_format=&format=tab) as $-(t_{left\_norm} + t_{right\_norm} + d_{norm})$, where t_{left_norm} is the time at which the left origin fires normalized to the time span from the firing of the earliest origin to that of the latest origin, $(t_{latest} - t_x)/(t_{latest} - t_{earliest})$, and normalized interorigin distance is defined analogously. This parameter was then normalized to range from 0 to 1. Replication gap depths, which represent read depth minima for each interorigin region relative to median read depth in that sample, were also normalized to range from 0 to 1.

Marker Loss Measurement. Ten colonies each of diploid strains carrying one copy of chromosome VII marked at the centromere with *HIS3* and marked either on the right (16635 and 16660) or left (16636 and 16662) arms with *URA3*, were inoculated into 2 mL of YPD liquid media, grown overnight at 30 °C, and 5 and 10 μL were plated on 5-FOA-HIS plates. Colonies were counted after 3 d, and marker loss rates were calculated according to the Lea–Coulson method (35).

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