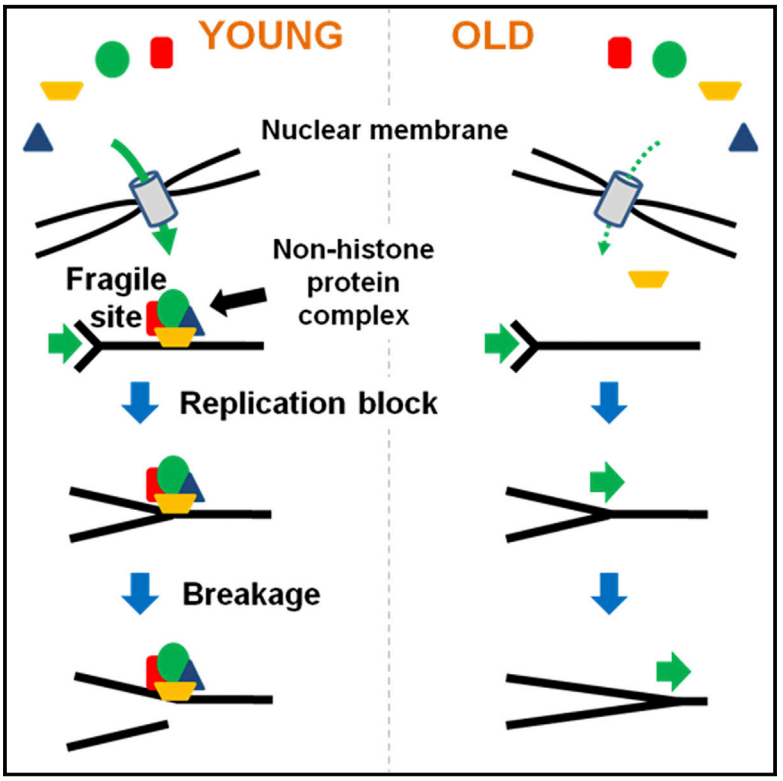
Report

Cell Reports

Absence of Non-histone Protein Complexes at Natural Chromosomal Pause Sites Results in Reduced Replication Pausing in Aging Yeast Cells

Graphical Abstract



Authors

Marleny Cabral, Xin Cheng, Sukhwinder Singh, Andreas S. Ivessa

Correspondence

ivessaan@njms.rutgers.edu

In Brief

Because genomic instability increases during aging, replication pausing/stalling at fragile sites is expected to increase. Cabral et al. now find that pausing/stalling decreases in aging and caloric-restricted budding yeast cells. The authors find that reduced pausing results from the absence of non-histone protein complexes that bind to these sites.

Highlights

- *Reduced* replication pausing at fragile sites in aging and caloric-restricted cells
- Age-dependent reduction in pausing is independent of Rrm3 and Sir2
- Fragile sites-bound proteins are cytosolic in aged and caloric-restricted cells
- Findings may have implications to understand chromosome rearrangements during aging

Cabral et al., 2016, Cell Reports *17*, 1747–1754
November 8, 2016
http://dx.doi.org/10.1016/j.celrep.2016.10.050

Cell Reports
Report

Absence of Non-histone Protein Complexes at Natural Chromosomal Pause Sites Results in Reduced Replication Pausing in Aging Yeast Cells

Marleny Cabral,[1] Xin Cheng,[1] Sukhwinder Singh,[2] and Andreas S. Ivessa[1,3,*]
[1]Department of Cell Biology and Molecular Medicine
[2]Pathology and Laboratory Medicine/Flow Cytometry and Immunology Core Laboratory
Rutgers New Jersey Medical School, Rutgers Biomedical and Health Sciences, 185 South Orange Avenue, Newark, NJ 07101-1709, USA
[3]Lead Contact
*Correspondence: ivessaan@njms.rutgers.edu
http://dx.doi.org/10.1016/j.celrep.2016.10.050

SUMMARY

There is substantial evidence that genomic instability increases during aging. Replication pausing (and stalling) at difficult-to-replicate chromosomal sites may induce genomic instability. Interestingly, in aging yeast cells, we observed *reduced* replication pausing at various natural replication pause sites (RPSs) in ribosomal DNA (rDNA) and non-rDNA locations (e.g., silent replication origins and tRNA genes). The reduced pausing occurs independent of the DNA helicase Rrm3p, which facilitates replication past these non-histone protein-complex-bound RPSs, and is independent of the deacetylase Sir2p. Conditions of caloric restriction (CR), which extend life span, also cause reduced replication pausing at the 5S rDNA and at tRNA genes. In aged and CR cells, the RPSs are less occupied by their specific non-histone protein complexes (e.g., the preinitiation complex TFIIIC), likely because members of these complexes have primarily cytosolic localization. These conditions may lead to reduced replication pausing and may lower replication stress at these sites during aging.

INTRODUCTION

In dividing cells, DNA replication stress might be initiated by the uncontrolled activation of replication origins, impediments to replication movement (e.g., tightly bound DNA-protein complexes and secondary DNA structures), collisions of replication and transcription complexes, various forms of uncontrolled metabolic conditions (e.g., insufficient deoxynucleotide triphosphate [dNTP] pools or histone supplies), and DNA damage (e.g., inter-strand crosslinks) (Branzei and Foiani, 2010; Magdalou et al., 2014). In budding yeast, replication may slow or even stall at up to 1,400 chromosomal sites, which include centromeres, tRNA genes, Ty elements, long terminal repeats (LTRs), subtelomeric and telomeric regions, silent replication origins, and highly transcribed genes (Azvolinsky et al., 2009; Deshpande and Newlon, 1996; Greenfeder and Newlon, 1992; Ivessa et al., 2000, 2002, 2003; Lemoine et al., 2005; Makovets et al., 2004; Szilard et al., 2010). Many of these sites were identified in chromosome translocations, because these sites may break more often and exhibit the characteristic features of "fragile sites" (Di Rienzi et al., 2009; Hu et al., 2014; Ivessa et al., 2000, 2002, 2003; Lemoine et al., 2005; Lindstrom et al., 2011; Raveendranathan et al., 2006; Song et al., 2014; Szilard et al., 2010). Usually non-histone protein complexes occupy these sites, thereby causing replication slowing/stalling (Deshpande and Newlon, 1996; Ivessa et al., 2003).

In various species, including yeast, and tissues mutation rates, DNA damage including broken DNA and mitotic recombination rates, chromosome translocations, deletions, and duplications are increasing during aging, which may impact aging (Hu et al., 2014; Lindstrom et al., 2011; López-Otín et al., 2013; Maslov et al., 2013; McMurray and Gottschling, 2003). Aging also alters chromatin. For example, loss of the core histones H3 and H4 leads to shortening of the yeast replicative life span (RLS), whereas overexpression of H3 and H4 extends life span (Feser et al., 2010; Hu et al., 2014; Pal and Tyler, 2016).

Because numerous forms of DNA damage increase during aging and replication stress might be the result of slowing and/or stalling of replication, we wanted to investigate whether replication pausing/stalling increases at known natural replication pause sites (RPSs) (e.g., tRNA genes) when yeast cells age.

RESULTS

In Replicative Aging Cells, Replication Pauses/Stalls with Lower Rates at the Inactive Replication Fork Barrier, 5S rDNA Genes, and Silent Replication Origins of rDNA

Because we needed large numbers of aged wild-type (WT) cells (8 and 20 cell divisions) to analyze replication intermediates by DNA two-dimensional (2D) gels, we used the mother enrichment program (MEP) (Figures 1A and S1A) (Brewer and Fangman, 1988; Ivessa et al., 2003; Lindstrom and Gottschling, 2009). It is induced by estradiol with the purpose to activate

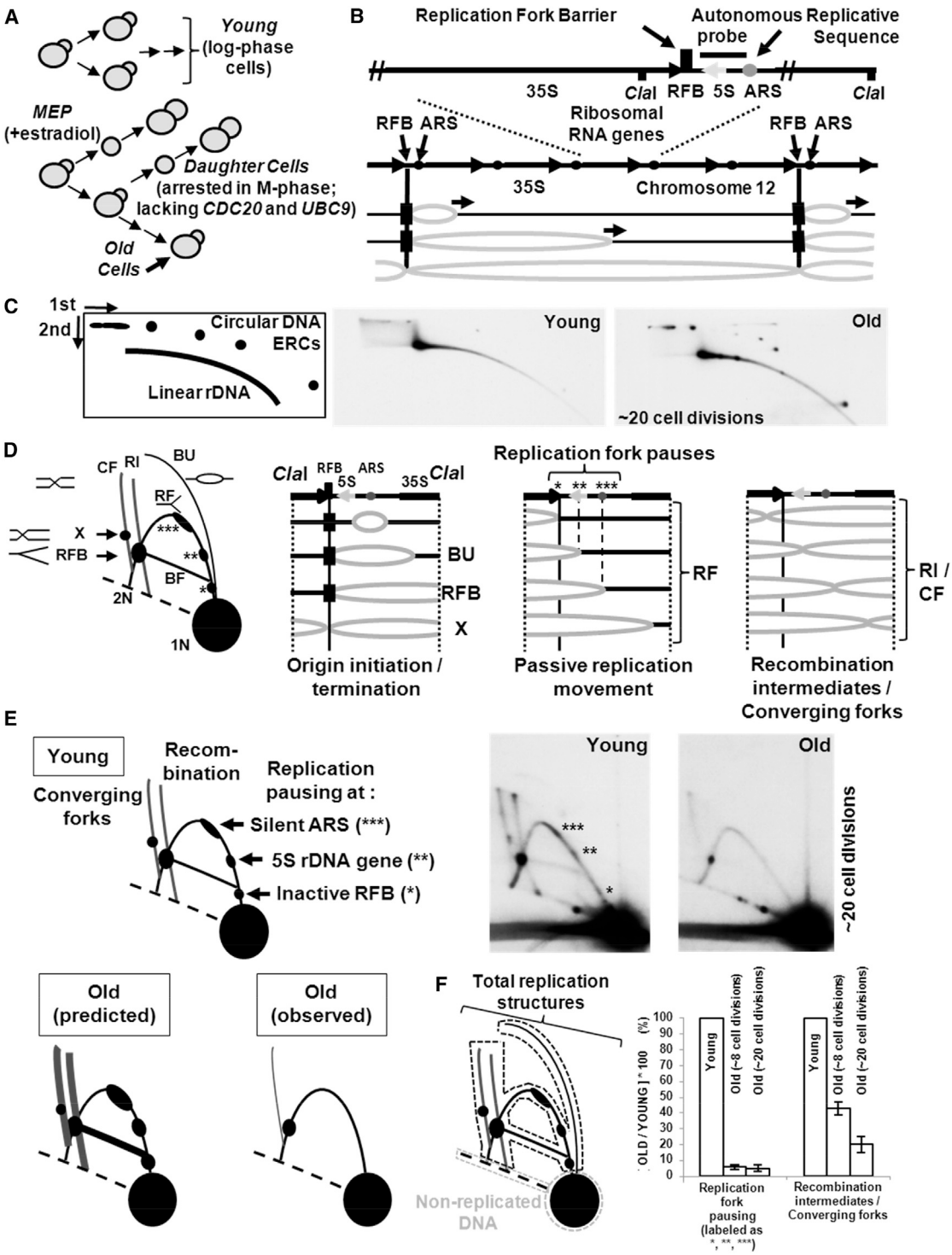


Figure 1. Reduced Pausing at Natural RPSs in rDNA of Aging Yeast Cells

(A) The mother enrichment program (MEP) to purify old yeast cells (Figure S1A).

(B) The regions of the analyzed *Cla*I-rDNA fragment and rDNA probe (*Stu*I-*Bgl*II) are indicated.

(C) Determining the amount of extrachromosomal rDNA circles (ERCs) by DNA 2D gels in young and old WT cells.

(D) Schema of rDNA replication intermediates (*Cla*I DNA fragment): BU, bubble arc (i.e., replication initiation); RFB, replication fork barrier; X, termination of rDNA replication; RF, replication forks; RI, recombination intermediates; CF, converging forks. Putative broken replication forks (BF) are indicated (Martín-Parras et al., 1992). Pauses of replication forks at the natural RPSs in rDNA are indicated (asterisks).

(legend continued on next page)

the splice-out of two essential genes (*UBC9*, *CDC20*), specifically in the daughter cells, to prevent their propagation. Mother cells undergoing approximately eight cell divisions are in the first quarter of their life span, and cells that have divided ~20 times are close to middle age. As a control ("young" cells), we grew this strain for the same amount of time in the absence of estradiol (not expressing the MEP).

We analyzed replication intermediates in the repeated rDNA array, which consists of 100–150 identical 9.1-kbp repeats (Figure 1B) (Brewer and Fangman, 1988; Ivessa, 2013; Ivessa et al., 2000). Extrachromosomal rDNA circles (ERCs) can excise from the array and accumulate in mother cells causing aging (Sinclair and Guarente, 1997). Aged mother cells undergoing ~8 and ~20 cell divisions had a ~8-fold and ~26-fold enrichment of ERCs, respectively, compared to young cells (Figures 1C and S1B) (Ivessa et al., 2000). To analyze replication intermediates by 2D gels, we digested the DNA with the restriction enzyme *Cla*I, which generates mainly a ~6-kb fragment from the 9.1-kb rDNA repeat unit (Figures 1B and 1D) (Ivessa, 2013; Ivessa et al., 2000). rDNA replication is initiated in a subset of the rDNA repeats. The pattern of replication intermediates for any rDNA fragment containing a replication origin (*ARS* [autonomous replicative sequence]) is a composite of intermediates generated from repeats with an active *ARS* and those without an active *ARS* (Figure 1B) (Brewer and Fangman, 1988; Ivessa et al., 2000). For repeats with an active *ARS*, initiation begins and proceeds bi-directionally from the *ARS* (bubble structures, Figure 1D, BU). They are converted to simple Y structures when the rightward-moving fork reaches the end of the fragment. The leftward-moving fork stops at the site-specific replication fork barrier (RFB), creating increased hybridization on the arc of simple Y-shaped intermediates (Figure 1D, RFB). The remaining part of *Cla*I fragments with active *ARS*s is replicated by forks converging on the fork stalled at the RFB. These forks generate intermediates that emanate from the RFB and terminate with a mass of close to 2N (Figure 1D, X). Repeats without an active origin are replicated uni-directionally, creating a full arc of simple Y-shaped intermediates (Figure 1D, passive replication movement).

We investigated whether natural RPSs (e.g., the inactive RFB, 5S rDNA genes, and silent rDNA replication origins) experience increased pausing in aged cells (Figure 1E) (Lindstrom et al., 2011; Vijg and Suh, 2013). Surprisingly, we observed strongly *reduced* pausing at these sites in old cells except for the active RFB, which was only slightly reduced (Figures 1E and 1F [quantitation], and S1C). The amount of putative rDNA recombination intermediates and converging forks (Figure 1D, RI/CF) was also largely reduced in old cells (Figures 1E and 1F [quantitation]). For example, strains expressing mutated forms of nuclear DNA polymerase α (e.g., *pol12*) highly induce the formation of recombination intermediates in rDNA (Zou and Rothstein, 1997).

The Reduced Replication Pausing/Stalling at Natural RPSs in rDNA Is Independent of Rrm3p and Sir2p, Occurs on Both Chromosomal rDNA and ERCs during Aging, and Is Partly Recapitulated under Caloric-Restricted Growth Conditions

The DNA helicase Rrm3p is proposed to facilitate replication at over 1,400 chromosomal sites (e.g., tRNA genes, silent replication origins, and sites within the rDNA [Figure 1]) by transiently displacing the tightly bound non-histone protein complexes (Azvolinsky et al., 2006; Ivessa et al., 2000, 2002, 2003). Because replication forks paused/stalled with similarly reduced rates at the inactive RFB, the 5S rDNA gene, and the silent replication origin in rDNA of old WT and old mutant *rrm3* (age, ~10 cell divisions) cells, the age-dependent decrease in replication pausing at the RPSs in rDNA does not likely depend on Rrm3p (Figure 2A, *rrm3*; ERCs: Figure S1B).

We used the *fob1* mutant to prevent the age-dependent accumulation of ERCs to address whether replication pausing at the natural RPSs in rDNA occurs preferentially on chromosomal DNA in old cells (ERCs: Figure S1B) (Defossez et al., 1999; Lindstrom et al., 2011; Sinclair and Guarente, 1997). Like in old WT cells, replication did not pause/stall at these sites in old *fob1* mutant cells, suggesting that the loss in pausing/stalling at the natural RPSs in rDNA occurs on both chromosomes and ERCs (Figure 2A, *fob1*).

Mutants lacking the protein deacetylase Sir2p accumulate faster ERCs compared to WT (Figure S1B) and age faster (Kaeberlein et al., 1999). Although we expected increased replication pausing at the RPSs in rDNA of old *sir2* mutant cells, we found no significant difference between WT and *sir2* mutant cells in replication pausing at the rDNA-RPS in young and old (~10 cell divisions) cells, suggesting that Sir2p does not affect the loss in pausing during aging (Figure 2A, *sir2*).

Growth condition of caloric restriction (CR) (0.05% glucose), which extends life span (Lin et al., 2000), resulted in a significant loss in pausing at the 5S rDNA gene; pausing at the other RPSs in rDNA was not affected (Figure 2B).

Reduced Replication Pausing at Natural Non-rDNA Replication Pause Sites in Aging and Caloric-Restricted Cells

We observed that replication pauses less at several natural non-rDNA RPSs including the silent replication origins *ARS313/314* and *ARS608*, and the tRNA genes $tRNA^{A}$, $tRNA^{Y}$, and $tRNA^{F}$ in old cells lacking Rrm3p (Figure 3A) (Azvolinsky et al., 2009; Ivessa et al., 2003). Replication pausing at tRNA genes ($tRNA^{A}$, MEP *rrm3*) was also reduced in CR cells (0.05% glucose; Figure 3B). These results suggest that reduced replication pausing occur genome-wide at natural RPSs in aging cells and is partially recapitulated in CR cells.

(E) Predicted and observed rDNA replication intermediates in aging cells. Right side: 2D gels of *Cla*I-digested DNA of young and old WT cells. Probe: rDNA (*Stu*I-*Bgl*II).

(F) The amount of each replication intermediate is expressed as ratios (i.e., specific intermediate versus total replication structures [i.e., sum of RF (including pauses and RFB), RI/CF, and BU; see cartoon]; "old" is compared to "young"). In this way, we normalized for different amounts of total replication structures. We added up all the intensities of the replication fork pauses (i.e., the in-active RFB [*], the 5S rDNA gene [**], and the silent replication origin [*ARS*, ***]). Standard errors are displayed.

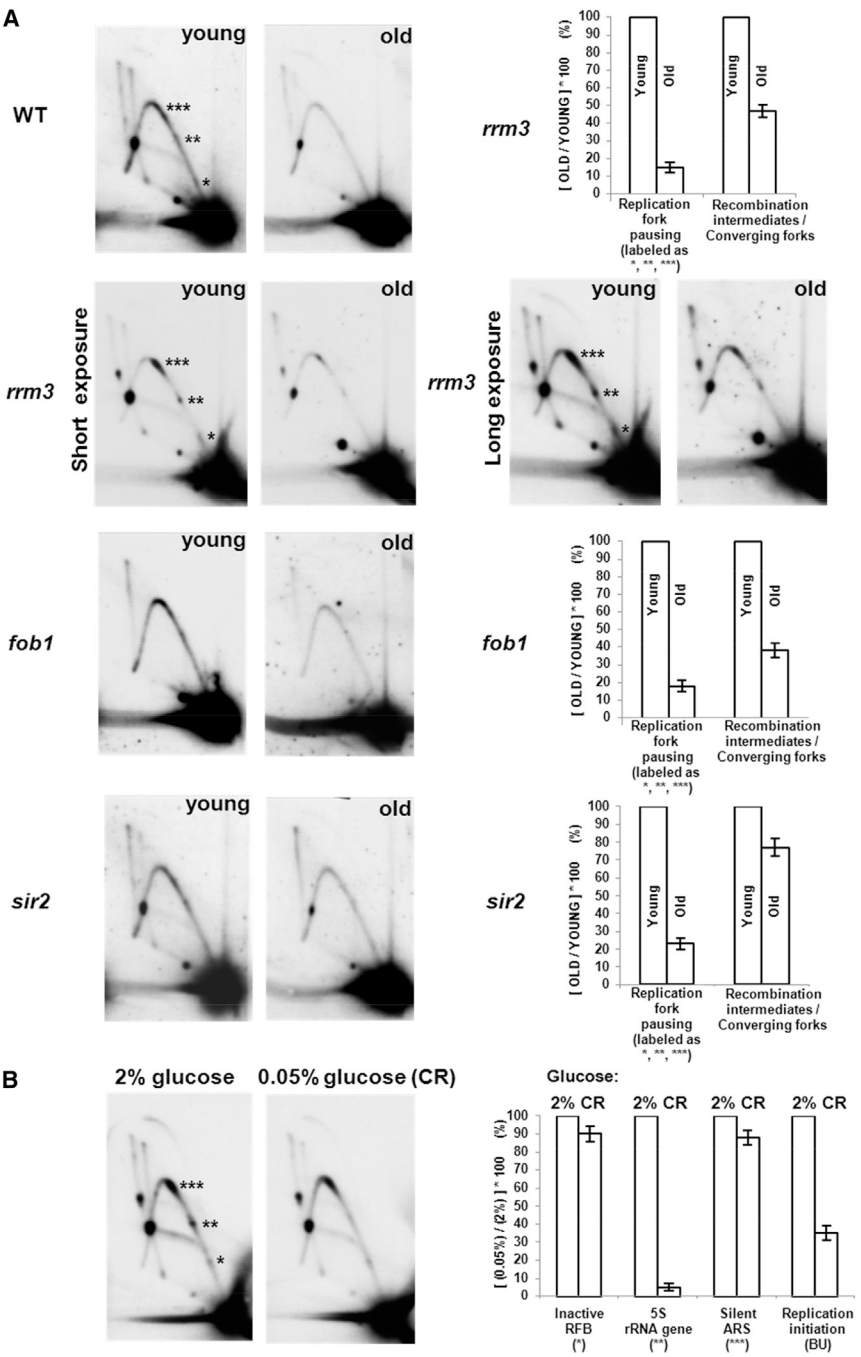


Figure 2. The Reduced Replication Pausing/Stalling at Natural RPSs in rDNA during Aging Is Independent of Rrm3p and Sir2p, Occurs on Both Chromosomal rDNA and ERCs, and Is Partly Recapitulated under Caloric-Restricted Growth Conditions

(A) and (B) display analyses of *Cla*I-digested nuclear rDNA replication intermediates by DNA 2D gels. See Figure 1 for probe, locations of the natural RPSs in rDNA, and quantitation of the replication intermediates. Standard errors are displayed.

(A) Young (not expressing the MEP) and aged (expressing the MEP) cells of the indicated strains (WT, *rrm3*, *fob1*, *sir2*) were used. Age and ERCs enrichment (old versus young; Figure S1B): WT (~8 cell divisions; 8-fold enrichment); *rrm3* (~10 cell divisions; 22-fold enrichment); *fob1* (~12 cell divisions; 2.1-fold enrichment); *sir2* (~10 cell divisions; 27-fold enrichment).

(B) Cells (*rrm3*) were grown in 2% or 0.05% glucose (CR). Initiation of rDNA replication is reduced in CR cells (Kwan et al., 2013).

movement at the mutated tRNA gene (Deshpande and Newlon, 1996; Ivessa et al., 2003). We addressed whether these complexes (Orc, TFIIIC) are absent from the investigated sites in aging cells, thus causing the observed reduction in replication pausing (Figures 1, 2, and 3).

We observed that Orc1p-13Myc is mainly localized in the nucleus in young cells. It is expressed in old cells but has preferential cytosolic/nuclear rim localization in 20%–30% of old cells (~15 cell divisions; Figures 4A and S2). In young cells, an average of about 93% of Orc1p overlapped with the nucleus (DAPI), whereas in old cells only 15% of Orc1p overlapped with the nuclear DAPI.

To localize TFIIIC, we used strains expressing Tfc1p-GFP or Tfc4p-GFP (Breker et al., 2014). On average, about 90% of both Tfc1p and Tfc4p localized to the nucleus in young cells (Figure S3A). Aged cells (approximately eight cell divisions) displayed a heterogeneous expression and localization pattern (Figures 4B, S3B, and S3C). Some cells had Tfc1p and Tfc4p highly expressed in the cytosol, whereas other aged cells exhibit low diffused expression. In some aged cells undergoing mitosis, Tfc1p or Tfc4p was clearly localized to the nucleus in the daughter cell but had diffused localization in the aged mother cell (Figures S3B [Tfc1p] and 4B [Tfc4p]). CR cells (0.05% glucose) compared to cells grown in 2% glucose also had increased cytosolic localization of Tfc1p (Figure S4).

Chromatin immuno-precipitation (ChIP) confirmed that, in aged and CR cells, 5S rDNA and tRNA genes are less occupied by Tfc1p (Figures 4C and S5). The silent replication origin in rDNA

Members of the Non-histone Binding Complexes That Occupy the Replication Pause Sites Exhibit Diffused Cytosolic and/or Nuclear Rim Localization in Aging Cells

Both 5S rDNA genes and tRNA genes are bound by the RNA polymerase III preinitiation complex TFIIIC, and both rDNA and non-rDNA silent replication origins including the silent mating type loci are bound by the origin recognition complex (Orc), which all cause replication pausing (Deshpande and Newlon, 1996; Ivessa et al., 2003; Wyrick et al., 2001). Preventing the binding of the tightly bound TFIIIC leads to unimpeded fork

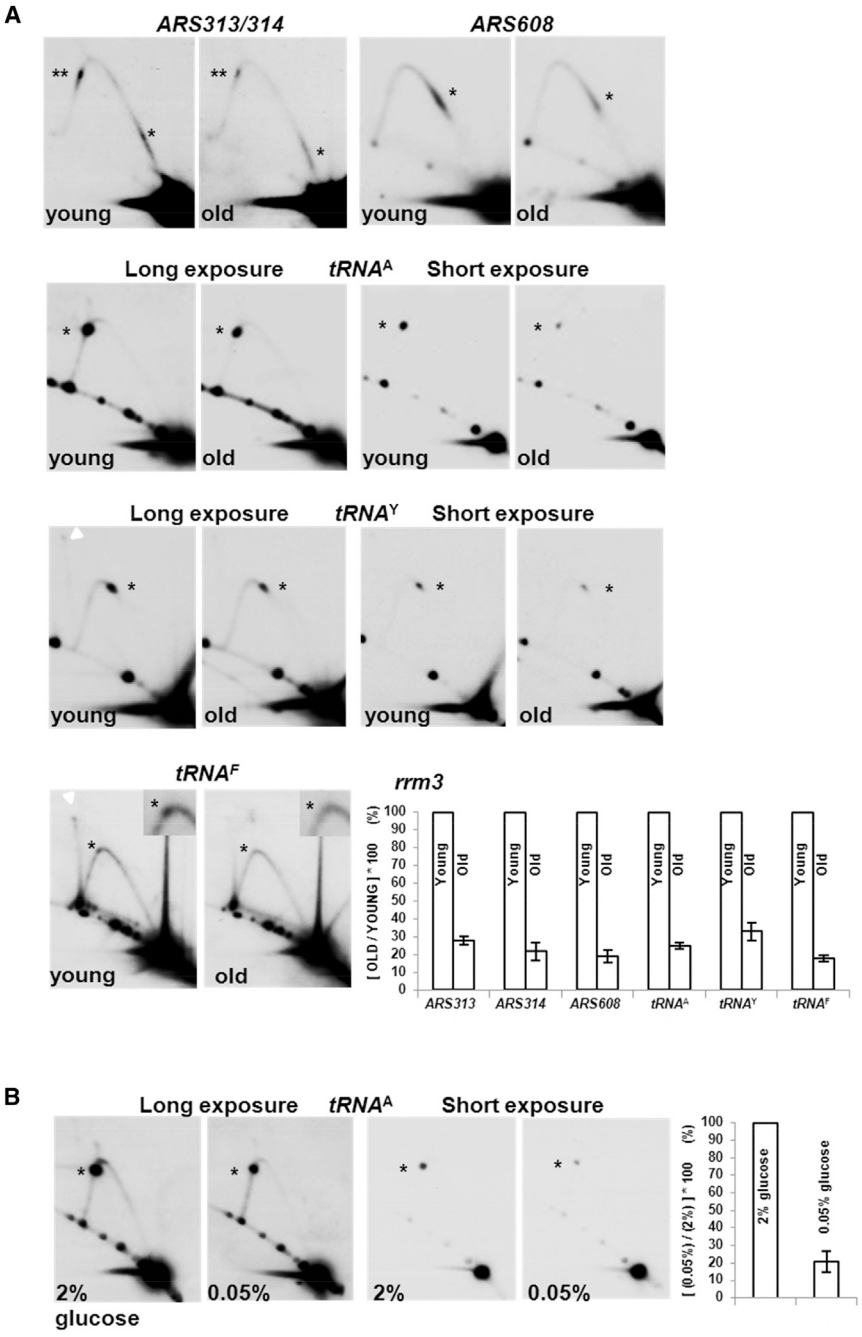


Figure 3. Reduced Replication Pausing at Natural Non-rDNA Replication Pause Sites in Aging and Caloric-Restricted Cells

(A) Young and old mutant *rrm3* cells (~10 cell divisions; expressing the MEP) were analyzed by DNA 2D gels. Investigated sites: the silent replication origins *ARS313/314* (*, **), and *ARS608* (*), and the tRNA genes (*) $tRNA^A$, $tRNA^Y$, and $tRNA^F$. Two different auto-radiographic exposures are displayed for the $tRNA^A$ and $tRNA^Y$ genes. The area of the replication pause for the $tRNA^F$ gene is magnified in the upper right corner. The white triangles (young cells, $tRNA^Y$ and $tRNA^F$) indicate putative recombination intermediates/converging forks, which are reduced in old cells.

(B) CR mutant *rrm3* cells were analyzed by DNA 2D gels at the $tRNA^A$ locus (two different auto-radiographic exposures are shown). In (A) and (B), quantitation of the replication intermediates was carried out as described in Figure 1. Standard errors are displayed.

was only less occupied by Orc2p (part of Orc) in aged cells, but not in CR cells (Figures 4C and S5). The ChIP results are consistent with the above-shown 2D gel data.

DISCUSSION

We demonstrate that in replicative aging yeast cells DNA replication is pausing (or even stalling) with a reduced rate at natural difficult-to-replicate sites. These results were unanticipated, because various forms of DNA damage (e.g., chromosome translocations) are increasing during aging. Because many of these genetic changes require DNA breakage, we expected an increase in pausing/stalling of replication, particularly at the RPSs to induce breakage (Branzei and Foiani, 2010; Lindstrom et al., 2011; Vijg and Suh, 2013).

Tyler and colleagues (Hu et al., 2014) determined that both inter- and intra-chromosomal translocations occur at high rates on the rDNA locus but also on the mitochondrial DNA locus during yeast replicative aging. Some of these translocations occur in the vicinity of replication origins (silent and active), and LTR and tRNA genes. If replication migrates unimpeded at these sites in old cells, then it will be interesting to investigate how breakage and translocation events are induced there. Because the general loss of histones induces transcription of almost all yeast genes, superhelical tension may increase and lead to chromosome breakage (Hu et al., 2014).

We also observed a reduction in replication pausing at the 5S rDNA gene and at a tRNA gene in CR cells (Figures 2B and 3B). Reduced mRNA translation is one of several phenotypes of CR cells (Wasko and Kaeberlein, 2014). Reductions in replication pausing at 5S rDNA genes and tRNA genes may also reflect that. Less replication pausing at chromosomal RPSs and possibly less DNA breakage at those sites may contribute to the life span extension observed in CR cells, which, however, has not been fully addressed yet.

The examined RPSs are occupied by non-histone protein complexes that cause replication pausing (Deshpande and

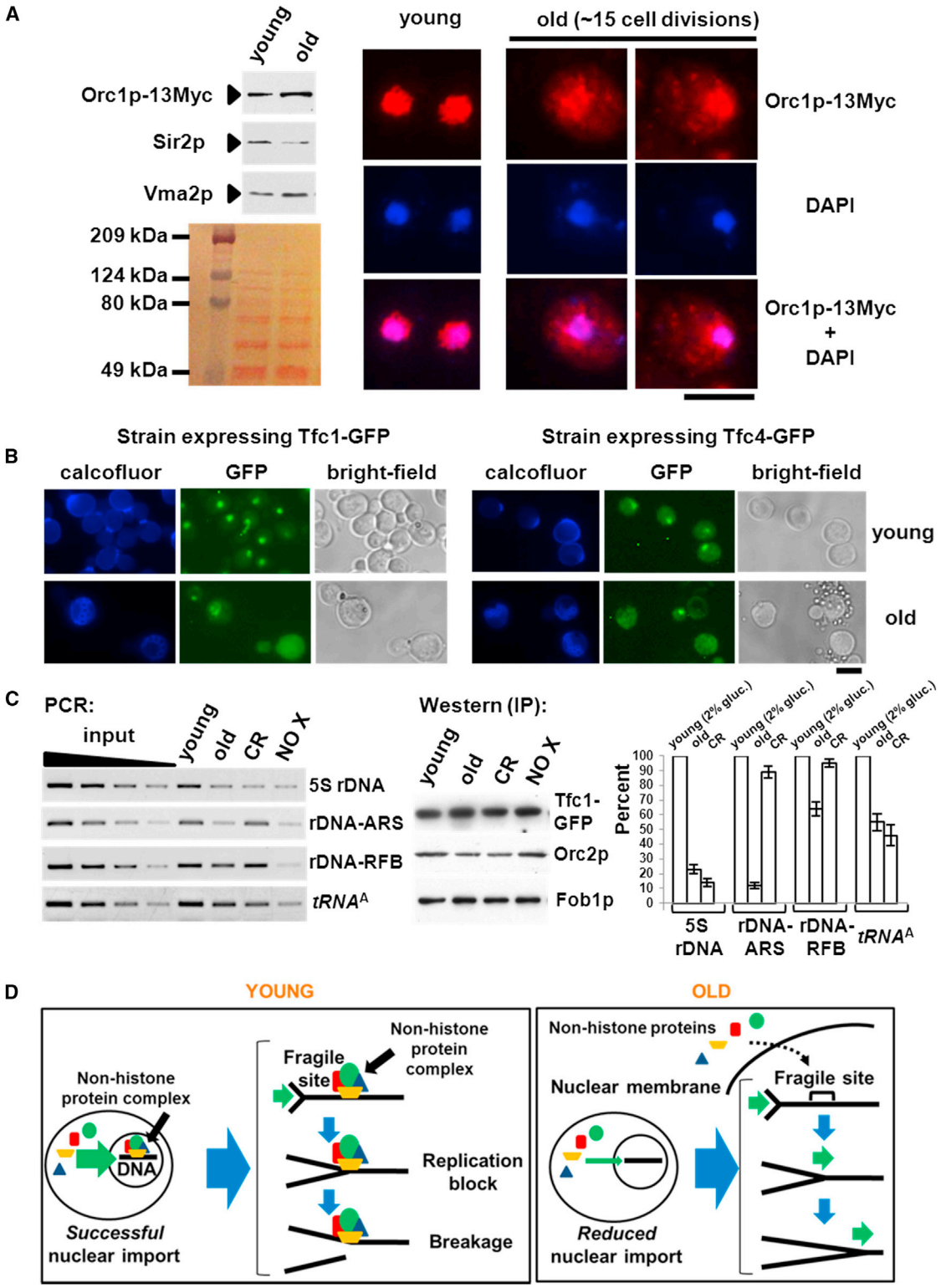


Figure 4. Members of the Non-histone Protein Complexes ORC and TFIIIC That Bind to Natural Replication Pause Sites Display Largely Diffused Cytosolic or Nuclear Rim Localization in Aged Cells

(A) Orc1p-13Myc is detected by western blotting in young and old (~15 cell divisions) diploid WT cells. Controls: expression of Sir2p is largely reduced in old cells, whereas the vacuolar protein Vma2p is still expressed in old cells (Dang et al., 2009; Lindstrom et al., 2011). The Ponceau S-stained western blot demonstrates

(legend continued on next page)

Newlon, 1996; Ivessa et al., 2003). We demonstrate that members of these complexes (Orc1, Tfc1, Tfc4) have a cytosolic/nuclear rim distribution in aged cells, which is consistent with the observation that the nuclear protein import is impaired in aged cells (Lord et al., 2015). Similarly, some proteins (e.g., the nuclear tRNA exporter Los1p) are also excluded from the nucleus in CR cells (McCormick et al., 2015). We also demonstrate by ChIP that Orc and TFIIIC exhibit a reduced presence on the investigated RPSs in aged and CR cells (Figure 4C). This reduced presence of the non-histone protein complexes at the chromosomal RPSs (rDNA, tRNA genes) may reduce replication pausing and potential DNA breakage, which may promote longevity (Figure 4D). However, to maintain essential cellular processes and to propagate, the nuclear protein import can only be reduced to a certain level.

Because the nuclear protein import becomes less efficient during aging, members of various non-histone protein complexes might be present in non-stoichiometric amounts in the nucleus, leading to a failure in the proper formation of mature complexes that can bind DNA (Janssens et al., 2015). In contrast, Fob1p binds to the RFB in rDNA as a single protein, thereby causing replication stalling. Therefore, we still observe replication stalling at the active RFB in rDNA of old cells, although the stalling rate is reduced compared to young cells (Figures 1 and 2). Although the nuclear import efficiency might vary for different nuclear proteins in different cells, the outcome would always be the same, i.e., a failure in the proper assembly of nuclear protein complexes such as Orc, because in each case different single subunits may be present in low amounts. Future studies will investigate whether the reduced replication pausing at the chromosomal RPSs influences the occurrence of DNA breakage at non-fragile sites and whether the reduction in pausing (or stalling) indeed has an impact on longevity.

EXPERIMENTAL PROCEDURES

Yeast Strains and Yeast Methods

A list of used yeast strains and their construction is provided in Table S2.

Determination of the Amount of ERCs and Analysis of DNA Replication Intermediates

DNA was prepared by CsCl-gradient centrifugation (Ivessa, 2013). The amount of ERCs and the structure of DNA replication intermediates were determined by 2D gels (Ivessa, 2013; Ivessa et al., 2000, 2003). Primer sequences for the PCR amplifications of DNA probes are listed in Table S1. The amounts of specific replication intermediates were determined by phosphor-imager analysis.

Other Procedures

Conditions for the MEP, immunofluorescent staining, and fluorescence microscopy of yeast cells, FACS, and ChIP are outlined in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.050.

AUTHOR CONTRIBUTIONS

M.C., X.C., S.S, and A.S.I. conducted the experiments. S.S. carried out the FACS experiments. A.S.I. conceived and supervised the project, designed the experiments, analyzed the data, and wrote the paper.

ACKNOWLEDGMENTS

We are grateful to Dr. Daniel Gottschling (FHCRC, Seattle) for providing us with the MEP-expressing strains. We thank Christopher Brady for critically reading the paper. This work was supported by NIH Grants R21AG047423 (to A.S.I.) and NIH SIG Grant 1S0OD018103-01 (Rutgers NJMS Flow Cytometry and Immunology Core Laboratory) (to S.S.).

Received: May 24, 2016
Revised: September 8, 2016
Accepted: October 14, 2016
Published: November 8, 2016

REFERENCES

Azvolinsky, A., Dunaway, S., Torres, J.Z., Bessler, J.B., and Zakian, V.A. (2006). The *S. cerevisiae* Rrm3p DNA helicase moves with the replication fork and affects replication of all yeast chromosomes. Genes Dev. *20*, 3104–3116.

Azvolinsky, A., Giresi, P.G., Lieb, J.D., and Zakian, V.A. (2009). Highly transcribed RNA polymerase II genes are impediments to replication fork progression in *Saccharomyces cerevisiae*. Mol. Cell *34*, 722–734.

Branzei, D., and Foiani, M. (2010). Maintaining genome stability at the replication fork. Nat. Rev. Mol. Cell Biol. *11*, 208–219.

Breker, M., Gymrek, M., Moldavski, O., and Schuldiner, M. (2014). LoQAtE—Localization and Quantitation ATlas of the yeast proteomE. A new tool for multiparametric dissection of single-protein behavior in response to biological perturbations in yeast. Nucleic Acids Res. *42*, D726–D730.

equal loading (below). Immunofluorescent staining of young and old WT cells (right side): red, Orc1p-13Myc; blue, DNA (DAPI). Bar (bottom right), 5 μm. See also Figure S2.

(B) Strains expressing the nuclear transcription factors Tfc1-GFP and Tfc4-GFP in young and old (approximately eight cell divisions) haploid WT cells. Green: GFP; blue: calcofluor (age determination); gray: bright-field. Avidin-coated magnetic beads that bind to the biotin-labeled old cells can be seen as small, grainy dots surrounding the old cells. Bar, 5 μm. See also Figure S3.

(C) Chromatin immuno-precipitation (ChIP) to assess binding of Tfc1p to 5S rDNA and *tRNA*^{A} genes, of Fob1p to the rDNA-RFB and of Orc2p to the rDNA-*ARS*. A strain expressing Tfc1-GFP was used. Input DNA (before IP) was diluted 1:500, 1:1,000, 1:2,000, and 1:4,000 prior to the PCR to demonstrate the linear range of the amplification. The samples were as follows: young (log-phase cells, 2% glucose), old (8–10 cell divisions), CR (0.05% glucose), no crosslinking agent (i.e., formaldehyde, NO X) added to log-phase cells (2% glucose). Western blots of IP samples: Tfc1p-GFP (100 kDa), Orc2p (71 kDa), and Fob1p (65 kDa). Amount of the PCR products of the input and IP samples were determined by an imaging system and ratios of IP/input were calculated. The IP/input value of the non-crosslinked control (NO X) was subtracted from each IP/input value (young, old, CR), and percentages were calculated for each indicated DNA location as follows: [([young, old, or CR] − [NO X])/([young] − [NO X])] * 100. Standard errors are displayed. The raw IP/input data are shown in Figure S5.

(D) In our model, we propose that in young cells non-histone protein complexes are successfully imported into the nucleus, bind to natural RPSs, and slow/stall DNA replication, which may lead to DNA breakage. In contrast, in old cells the protein complexes are imported with a reduced rate (or not at all) into the nucleus and therefore do not bind to the RPSs to impede DNA replication. Thus, the RPSs may experience only reduced replication stress in aging cells.

Brewer, B.J., and Fangman, W.L. (1988). A replication fork barrier at the 3′ end of yeast ribosomal RNA genes. Cell *55*, 637–643.

Dang, W., Steffen, K.K., Perry, R., Dorsey, J.A., Johnson, F.B., Shilatifard, A., Kaeberlein, M., Kennedy, B.K., and Berger, S.L. (2009). Histone H4 lysine 16 acetylation regulates cellular lifespan. Nature *459*, 802–807.

Defossez, P.A., Prusty, R., Kaeberlein, M., Lin, S.J., Ferrigno, P., Silver, P.A., Keil, R.L., and Guarente, L. (1999). Elimination of replication block protein Fob1 extends the life span of yeast mother cells. Mol. Cell *3*, 447–455.

Deshpande, A.M., and Newlon, C.S. (1996). DNA replication fork pause sites dependent on transcription. Science *272*, 1030–1033.

Di Rienzi, S.C., Collingwood, D., Raghuraman, M.K., and Brewer, B.J. (2009). Fragile genomic sites are associated with origins of replication. Genome Biol. Evol. *1*, 350–363.

Feser, J., Truong, D., Das, C., Carson, J.J., Kieft, J., Harkness, T., and Tyler, J.K. (2010). Elevated histone expression promotes life span extension. Mol. Cell *39*, 724–735.

Greenfeder, S.A., and Newlon, C.S. (1992). Replication forks pause at yeast centromeres. Mol. Cell. Biol. *12*, 4056–4066.

Hu, Z., Chen, K., Xia, Z., Chavez, M., Pal, S., Seol, J.H., Chen, C.C., Li, W., and Tyler, J.K. (2014). Nucleosome loss leads to global transcriptional up-regulation and genomic instability during yeast aging. Genes Dev. *28*, 396–408.

Ivessa, A.S. (2013). Analysis of DNA structures from eukaryotic cells by two-dimensional native-native DNA agarose gel electrophoresis. Methods Mol. Biol. *1054*, 63–81.

Ivessa, A.S., Zhou, J.-Q., and Zakian, V.A. (2000). The *Saccharomyces* Pif1p DNA helicase and the highly related Rrm3p have opposite effects on replication fork progression in ribosomal DNA. Cell *100*, 479–489.

Ivessa, A.S., Zhou, J.-Q., Schulz, V.P., Monson, E.K., and Zakian, V.A. (2002). *Saccharomyces* Rrm3p, a 5′ to 3′ DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA. Genes Dev. *16*, 1383–1396.

Ivessa, A.S., Lenzmeier, B.A., Bessler, J.B., Goudsouzian, L.K., Schnakenberg, S.L., and Zakian, V.A. (2003). The *Saccharomyces cerevisiae* helicase Rrm3p facilitates replication past nonhistone protein-DNA complexes. Mol. Cell *12*, 1525–1536.

Janssens, G.E., Meinema, A.C., González, J., Wolters, J.C., Schmidt, A., Guryev, V., Bischoff, R., Wit, E.C., Veenhoff, L.M., and Heinemann, M. (2015). Protein biogenesis machinery is a driver of replicative aging in yeast. eLife *4* (*e08527*), e08527.

Kaeberlein, M., McVey, M., and Guarente, L. (1999). The *SIR2/3/4* complex and *SIR2* alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. Genes Dev. *13*, 2570–2580.

Kwan, E.X., Foss, E.J., Tsuchiyama, S., Alvino, G.M., Kruglyak, L., Kaeberlein, M., Raghuraman, M.K., Brewer, B.J., Kennedy, B.K., and Bedalov, A. (2013). A natural polymorphism in rDNA replication origins links origin activation with calorie restriction and lifespan. PLoS Genet. *9* (*3*), e1003329.

Lemoine, F.J., Degtyareva, N.P., Lobachev, K., and Petes, T.D. (2005). Chromosomal translocations in yeast induced by low levels of DNA polymerase a model for chromosome fragile sites. Cell *120*, 587–598.

Lin, S.J., Defossez, P.A., and Guarente, L. (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. Science *289*, 2126–2128.

Lindstrom, D.L., and Gottschling, D.E. (2009). The mother enrichment program: a genetic system for facile replicative life span analysis in *Saccharomyces cerevisiae*. Genetics *183*, 413–422, 411SI–413SI.

Lindstrom, D.L., Leverich, C.K., Henderson, K.A., and Gottschling, D.E. (2011). Replicative age induces mitotic recombination in the ribosomal RNA gene cluster of *Saccharomyces cerevisiae*. PLoS Genet. *7* (*3*), e1002015.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. Cell *153*, 1194–1217.

Lord, C.L., Timney, B.L., Rout, M.P., and Wente, S.R. (2015). Altering nuclear pore complex function impacts longevity and mitochondrial function in *S. cerevisiae*. J. Cell Biol. *208*, 729–744.

Magdalou, I., Lopez, B.S., Pasero, P., and Lambert, S.A. (2014). The causes of replication stress and their consequences on genome stability and cell fate. Semin. Cell Dev. Biol. *30*, 154–164.

Makovets, S., Herskowitz, I., and Blackburn, E.H. (2004). Anatomy and dynamics of DNA replication fork movement in yeast telomeric regions. Mol. Cell. Biol. *24*, 4019–4031.

Martín-Parras, L., Hernández, P., Martínez-Robles, M.L., and Schvartzman, J.B. (1992). Initiation of DNA replication in ColE1 plasmids containing multiple potential origins of replication. J. Biol. Chem. *267*, 22496–22505.

Maslov, A.Y., Ganapathi, S., Westerhof, M., Quispe-Tintaya, W., White, R.R., Van Houten, B., Reiling, E., Dollé, M.E., van Steeg, H., Hasty, P., et al. (2013). DNA damage in normally and prematurely aged mice. Aging Cell *12*, 467–477.

McCormick, M.A., Delaney, J.R., Tsuchiya, M., Tsuchiyama, S., Shemorry, A., Sim, S., Chou, A.C., Ahmed, U., Carr, D., Murakami, C.J., et al. (2015). A comprehensive analysis of replicative lifespan in 4,698 single-gene deletion strains uncovers conserved mechanisms of aging. Cell Metab. *22*, 895–906.

McMurray, M.A., and Gottschling, D.E. (2003). An age-induced switch to a hyper-recombinational state. Science *301*, 1908–1911.

Pal, S., and Tyler, J.K. (2016). Epigenetics and aging. Sci. Adv. *2*, e1600584.

Raveendranathan, M., Chattopadhyay, S., Bolon, Y.T., Haworth, J., Clarke, D.J., and Bielinsky, A.K. (2006). Genome-wide replication profiles of S-phase checkpoint mutants reveal fragile sites in yeast. EMBO J. *25*, 3627–3639.

Sinclair, D.A., and Guarente, L. (1997). Extrachromosomal rDNA circles—a cause of aging in yeast. Cell *91*, 1033–1042.

Song, W., Dominska, M., Greenwell, P.W., and Petes, T.D. (2014). Genome-wide high-resolution mapping of chromosome fragile sites in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *111*, E2210–E2218.

Szilard, R.K., Jacques, P.E., Laramée, L., Cheng, B., Galicia, S., Bataille, A.R., Yeung, M., Mendez, M., Bergeron, M., Robert, F., and Durocher, D. (2010). Systematic identification of fragile sites via genome-wide location analysis of gamma-H2AX. Nat. Struct. Mol. Biol. *17*, 299–305.

Vijg, J., and Suh, Y. (2013). Genome instability and aging. Annu. Rev. Physiol. *75*, 645–668.

Wasko, B.M., and Kaeberlein, M. (2014). Yeast replicative aging: a paradigm for defining conserved longevity interventions. FEMS Yeast Res. *14*, 148–159.

Wyrick, J.J., Aparicio, J.G., Chen, T., Barnett, J.D., Jennings, E.G., Young, R.A., Bell, S.P., and Aparicio, O.M. (2001). Genome-wide distribution of ORC and MCM proteins in *S. cerevisiae*: high-resolution mapping of replication origins. Science *294*, 2357–2360.

Zou, H., and Rothstein, R. (1997). Holliday junctions accumulate in replication mutants via a RecA homolog-independent mechanism. Cell *90*, 87–96.