GGS-22-00100

Genes Genet. Syst.

Changed life course upon defective replication of ribosomal RNA genes

Mei Hattori[1,2], Chihiro Horigome[1†], Théo Aspert[3], Gilles Charvin[3] and Takehiko Kobayashi[1,2,4*]

[1]*Laboratory of Genome Regeneration, Institute for Quantitative Biosciences (IQB), The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan*
[2]*Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan*
[3]*Laboratoire de Génétique Moléculaire, Génomique et Microbiologie, 67000 Strasbourg, France*
[4]*Collaborative Research Institute for Innovative Microbiology, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan*

*(Received 23 August 2022, accepted 9 November 2022; J-STAGE Advance published date: ** ***** 20**)*

Genome instability is a major cause of aging. In the budding yeast *Saccharomyces cerevisiae*, instability of the ribosomal RNA gene repeat (rDNA) is known to shorten replicative lifespan. In yeast, rDNA instability in an aging cell is associated with accumulation of extrachromosomal rDNA circles (ERCs) which titrate factors critical for lifespan maintenance. ERC accumulation is not detected in mammalian cells, where aging is linked to DNA damage. To distinguish effects of DNA damage from those of ERC accumulation on senescence, we re-analyzed a yeast strain with a replication initiation defect in the rDNA, which limits ERC multiplication. In aging cells of this strain (rARS-Δ3) rDNA became unstable, as in wild-type cells, whereas many fewer ERCs accumulated. Single-cell aging analysis revealed that rARS-Δ3 cells follow a linear survival curve and can have a wild-type replicative lifespan, although a fraction of the cells stopped dividing earlier than wild type. The doubling time of rARS-Δ3 cells appears to increase in the final cell divisions. Our results suggest that senescence in rARS-Δ3 is linked to the accumulation of DNA damage as in mammalian cells, rather than to elevated ERC level. Therefore, this strain should be a good model system to study ERC-independent aging.

Key words: ribosomal RNA gene (rDNA), lifespan, budding yeast, ERC, senescence

INTRODUCTION

Genome instability is one of the most influential factors to restrict lifespan (Schumacher et al., 2021). This is supported by evidence that defects in DNA stability-related genes and DNA damaging factors frequently curtail lifespan (Lombard et al., 2005). DNA damage can occur at random sites in the genome, making it complicated to gain insights into mechanisms that explain how this damage affects lifespan. Therefore, the aging process by DNA damage is still poorly understood.

The budding yeast, *Saccharomyces cerevisiae*, is a well analyzed model organism to study the aging mechanism (Kobayashi, 2011). This yeast divides unequally by budding; that is, the nascent daughter is much smaller than the mother. A cell divides twenty times on average before ceasing division and dying. In the last several cell divisions, the doubling time is extended and mother cells display increased nuclear size. These are typical phenotypes for an aging cell and their onset is referred to as the senescence entry point (SEP) (Fehrmann et al., 2013;

Edited by Hiroyuki Araki
* Corresponding author. tako2015@iqb.u-tokyo.ac.jp
† Present address: Laboratory of Molecular Biochemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 980-8572, Japan
DOI: https://doi.org/10.1266/ggs.22-00100

Morlot et al., 2019).

A plausible molecular mechanism whereby the aging phenotype is induced and the lifespan restricted involves the instability of the ribosomal RNA gene repeat (rDNA) on chromosome XII (Saka et al., 2013; Ganley and Kobayashi, 2014). The first evidence connecting rDNA stability and lifespan in yeast was the observation that extra-chromosomal rDNA circles (ERCs) accumulated in aging cells (Sinclair and Guarente, 1997). ERCs are a by-product of recombination in the rDNA (Fig. 1, right) and accumulated when a mutation in the *SGS1* gene, encoding an *Escherichia coli* RecQ helicase homolog, shortened the lifespan (Sinclair and Guarente, 1997). *SGS1* is a homolog of the human *WRN* gene, whose mutation causes Werner syndrome, a premature aging disease (Gray et al., 1997). ERCs accumulate ahead of senescence as detected in aging cells by single-cell imaging analysis of their whole lifespan (Crane et al., 2019; Morlot et al., 2019; Neurohr et al., 2019). The observed exponential formation of ERCs (~1,500 molecules) is thought to titrate factors essential for cell growth such as those involved in chromatin maintenance. ERC production is tightly linked to rDNA recombination (Johzuka and Horiuchi, 2002; Takeuchi et al., 2003), which also occurs to correct rDNA copy numbers by gene amplification. Both ERC formation and gene amplification are hallmarks of rDNA instability (Fig. 1; for a review, see Kobayashi (2011)).

Recombination in the rDNA largely depends on replication fork blocking activity at the replication fork barrier site (RFB) (Kobayashi and Horiuchi, 1996; Kobayashi et al., 1998). The RFB has been identified as a site where collision between the DNA replication and rDNA transcription machineries can be prevented by the function of Fob1, which specifically associates with the RFB (Brewer et al., 1992; Kobayashi et al., 1992; Kobayashi, 2003). The RFB forms a recombination hot spot linked to the onset of rDNA amplification and homogenization (Kobayashi et al., 1998; Ganley and Kobayashi, 2007). When a replication fork is arrested at the RFB, and collapses, a DNA double-strand break (DSB) occurs (Weitao et al., 2003; Burkhalter and Sogo, 2004; Kobayashi et al., 2004; Sasaki and Kobayashi, 2017). The broken ends of the DSB trigger repair, which usually involves equal sister chromatid exchange guided by cohesin and other factors that maintain genome integrity (Kobayashi and Ganley, 2005) (Fig. 1, left). However, when cohesin is displaced by

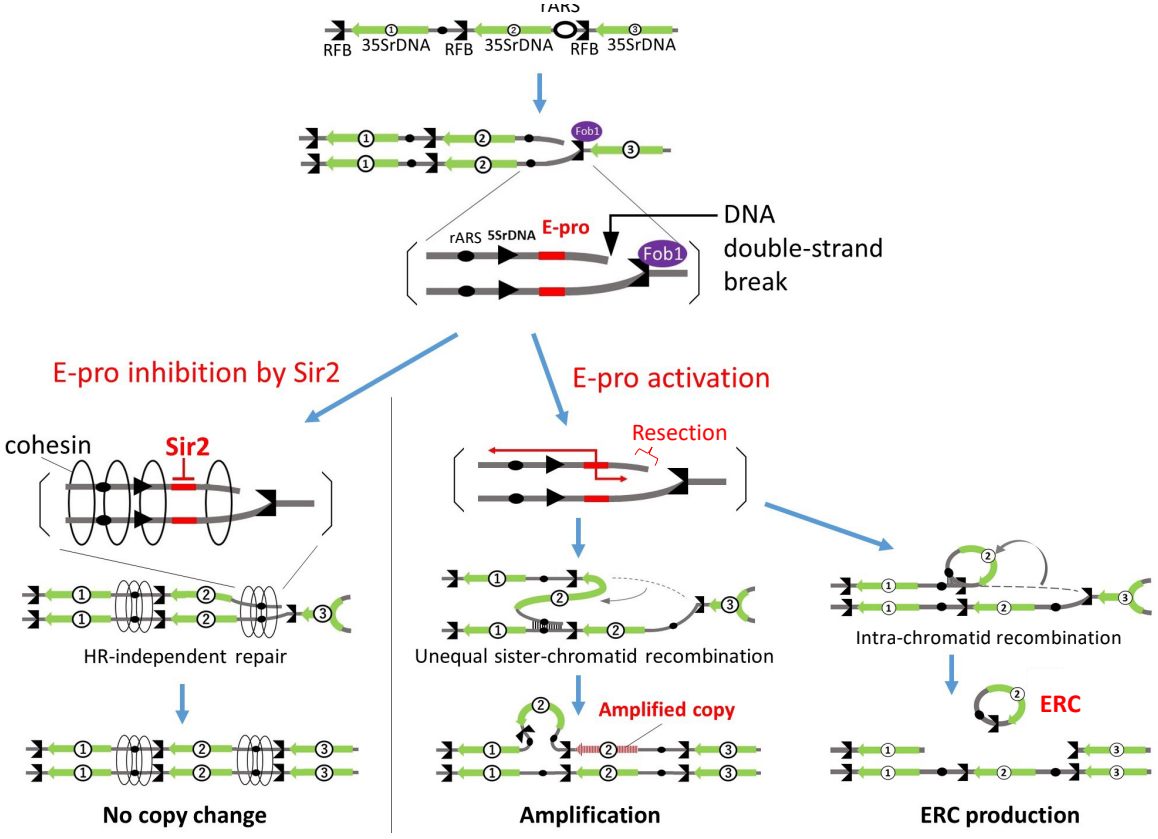

Fig. 1. Yeast rDNA stability. The rDNA is a recombinogenic region in the chromosome. The replication fork barrier (RFB) induces a DNA double-strand break, which triggers recombination. See text for details; only three rDNA copies out of ~150 are shown. rARS: autonomously replicating sequence in the rDNA; ERC: extrachromosomal rDNA circle; E-pro: bidirectional non-coding promoter; HR: homologous recombination.

non-coding transcription from the bidirectional promoter E-pro, the broken end can be resected and repaired by recombination with other homologous copies in the paired sister chromatids (Sasaki and Kobayashi, 2017). In this case, recombination templated from the opposite sister chromatid (Fig. 1, center) results in unequal sister chromatid recombination that leads to gene amplification, while intra-chromatid recombinational repair results in the release of ERCs (Fig. 1, right), which remain in the mother cell during cell division (Sinclair and Guarente, 1997). Due to the replication initiation activity of the autonomously replicating sequence (rARS) in the rDNA, ERCs exponentially accumulate in aging cells.

In the absence of the RFB binding factor Fob1, replication arrest does not occur and ERC levels are significantly reduced (Kobayashi and Horiuchi, 1996; Defossez et al., 1999; Johzuka and Horiuchi, 2002; Takeuchi et al., 2003). In contrast, when the histone deacetylase Sir2, which represses E-pro transcription, is inactive, unequal sister chromatid recombination occurs and ERCs are produced in large quantities (Kobayashi and Ganley, 2005). Compared to wild type, the replicative lifespan of *fob1* deletion mutants becomes longer, while that of *sir2* mutants is shorter (Defossez et al., 1999; Saka et al., 2013). These observations suggest that ERC accumulation can govern replicative lifespan in yeast.

As mentioned above, in addition to ERC formation, Fob1-dependent RFB activity in yeast may stimulate copy number alteration by recombination. This contributes to gene amplification involved in the recovery of copy number after accidental loss of rDNA copies (Kobayashi et al., 1998). Genomic instability due to loss and subsequent recovery of rDNA units possibly affects senescence and lifespan, as is hypothesized to happen in other organisms such as mammals where a pileup of ERCs has not been observed in aging cells (Killen et al., 2009). In yeast, the effects of Fob1-dependent DNA damage to lifespan and ERC-driven senescence can mask each other. Therefore, to assess the precise impact of DNA damage on replicative lifespan in budding yeast, ERC accumulation needs to be suppressed. This cannot be achieved by an approach involving Fob1, as both the formation of ERCs and the DNA damage caused by DSBs stem from RFB activity. To study the effect of rDNA instability on lifespan, we revisited a strain in which the rARS activity has been reduced (Ganley et al., 2009). In this strain (here referred to as rARS-Δ3), one of the three elements in the ARS consensus sequence, ACS-III (Bell and Stillman, 1992), has been deleted, resulting in reduced ERC replication. In this study, we exploited this strain and analyzed rDNA stability in aging cells and followed lifespans of dividing cells in real time by single-cell imaging analysis. Our results show that the absence of ERC accumulation has no impact on replicative lifespan, which instead seems to be affected by Fob1-dependent DNA damage.

RESULTS

ERC levels are reduced in aging rARS-Δ3 cells Ganley et al. (2009) demonstrated that lowering the replication initiation activity of the rARS by ~50% reduces the accumulation of ERCs. These results had been obtained with a strain, rARS-Δ3, which has ~150 rDNA copies containing an ARS with a non-critical conserved element, ACS-III (Bell and Stillman, 1992), replaced by *URA3*. As a control, strain ARS1-R had been constructed with *ARS1* as the origin of replication in the rDNA, which was expected and found to be more active than the original rARS (Ganley et al., 2009). In this study, we used rARS-Δ3 for real-time analysis of dividing single cells to establish whether enhanced ERC levels are required for the onset of senescence in budding yeast or whether other events, such as the accumulation of DNA damage, could determine replicative lifespan.

It has been reported that differences in the length of the rDNA repeat affect ERC formation rate (Mansisidor et al., 2018). Therefore, we spread the rARS-Δ3 strain onto a rich medium and carefully selected a clone whose rDNA was similar in length to wild type for further analysis. As has been described previously for cultures in log phase (Ganley et al., 2009), ERC levels are reduced in cells with an attenuated rARS (rARS-Δ3), while, compared to wild type, enhanced ARS activity (ARS1-R) leads to a two-fold increase in ERC formation (Fig. 2). ERC levels rise four-fold when E-pro-directed transcription is not repressed due to the absence of Sir2 and rDNA instability is enhanced. In contrast, when Fob1 is absent, recombination in the rDNA is reduced and, concomitantly, ERC levels are lower, irrespective of rARS activity (Fig. 2). These results were encouraging for further in-depth analysis of replicative lifespan in rARS-Δ3 cells.

ERC accumulation precedes senescence in aging cells (Crane et al., 2019; Morlot et al., 2019; Neurohr et al., 2019), which form a minor fraction of log phase cultures because most of the cells (~75%) have divided less than twice. Therefore, we compared the accumulation of ERCs between wild type and rARS-Δ3 in young and old cells, which were separated after biotinylation of the inoculum and collection of the old cells on streptavidin-conjugated magnetic beads at the end of an overnight incubation of the culture (Smeal et al., 1996) (Fig. 3A). Young cells comprised the rest of the culture. After 7–8 cell divisions, the ERC level in ARS-Δ3 cells was ~5-fold lower than in wild-type cells, a ratio similar to that found for young cells (Figs. 3B, 3C). These results indicate that ERC accumulation remains low in rARS-Δ3 cells due to attenuation of the rARS activity regardless of their age.

rDNA becomes more unstable in old cells In the rARS-Δ3 strain the rDNA is more unstable than in the wild type as indicated by the loss of a marker gene, which

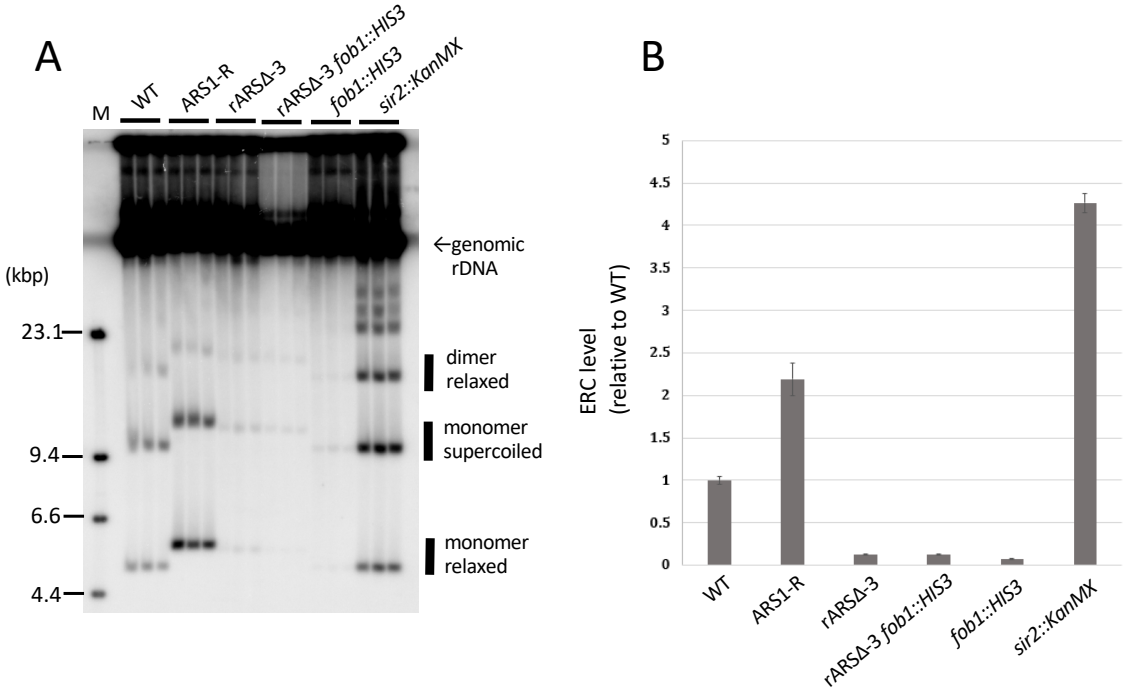

Fig. 2. ERC levels are reduced in rARS-Δ3 cells. (A) ERCs visualized with a rDNA probe (see Materials and Methods). The amount is related to rDNA stability (Fig. 1, right panel). ERC forms, relaxed (open circular) or supercoiled (closed circular), are indicated for cells from three independent colonies of each strain. (B) Intensities of ERC bands were measured and normalized to genomic rDNA {(dimer relaxed+monomer supercoiled+monomer supercoiled)/genomic rDNA}. Amounts are shown relative to wild type (WT). Error bars indicate SEM (n = 3). M is a size marker of HindIII-digested phage lambda DNA.

was six-fold more frequent (Ganley et al., 2009). Reduced replication initiation in the rDNA of the rARS-Δ3 strain prolongs the travel of replication forks initiated at other sites. This is expected to increase replication stress caused by fork arrest, which induces recombination as observed at fragile sites in human genes (Letessier et al., 2011). Actually, compared to wild type, the level of recombination intermediates (such as Holliday structures) increased as determined by two-dimensional (2D) gel electrophoresis, not only in the rARS-Δ3 strain, but also in a *clb5* deletion mutant with inefficient replication initiation (Ganley et al., 2009; Goto et al., 2021). To assess the effect of aging on rDNA stability, we analyzed the shape of chromosome XII by pulsed-field gel electrophoresis (PFGE) in young cells and in old cells after 7–8 cell divisions. Frequent copy number changes, a sign of unstable rDNA, broaden the chromosome XII band (Kobayashi et al., 2004), as was the case for young rARS-Δ3 cells (Figs. 4A, 4B, left panels). In the old cells the chromosome XII bands were almost invisible, although still detectable for two wild-type cultures (Figs. 4A, 4B, right panels, asterisks). ERCs remain in the mother cell after cell division (Sinclair and Guarente, 1997), and were visible as sharp bands (arrowheads in Fig. 4B, right panels) and were far less abundant in old rARS-Δ3 cells. During PFGE of complete chromosomes, recombination intermediates with unusual structures become stuck in the well (Ganley et al., 2009). The noticeable increase in intensities of well signals after hybridization to a rDNA probe compared to DNA staining (cf. Figs. 4A, 4B) shows that after 7–8 cell divisions, recombination intermediates involving rDNA accumulate. Compared to old wild-type cells, the rDNA signal in the case of rARS-Δ3 decreases in the wells, possibly reflecting a reduced amount of ERCs. As in the rARS-Δ3 strain, the shape of chromosome XII was broader than the wild-type strain in the young and old cells, indicating that the rDNA on the chromosome is more unstable. These results indicate that the rDNA becomes unstable with aging, which seems to occur more frequently in rARS-Δ3 cells.

rARS-Δ3 gradually dies after average lifespan Previous assessment of the effect of infrequent replication initiation in the rDNA on lifespan showed that cell viability decreased at a comparable rate as for wild type but that the onset of this decrease occurred after a smaller number of cell divisions, leading to a shorter replicative lifespan (Ganley et al., 2009). In this study, we used a microfluidics system to follow in real time the life course of single cells (Fig. 5A). Exponentially growing cells were trapped on the device and ~20 cells for the wild-type or rARS-Δ3 strain were analyzed by microscopy until they stopped dividing (Fig. 5B, Supplementary Movie S1). We measured the number of cell divisions (replicative lifes-

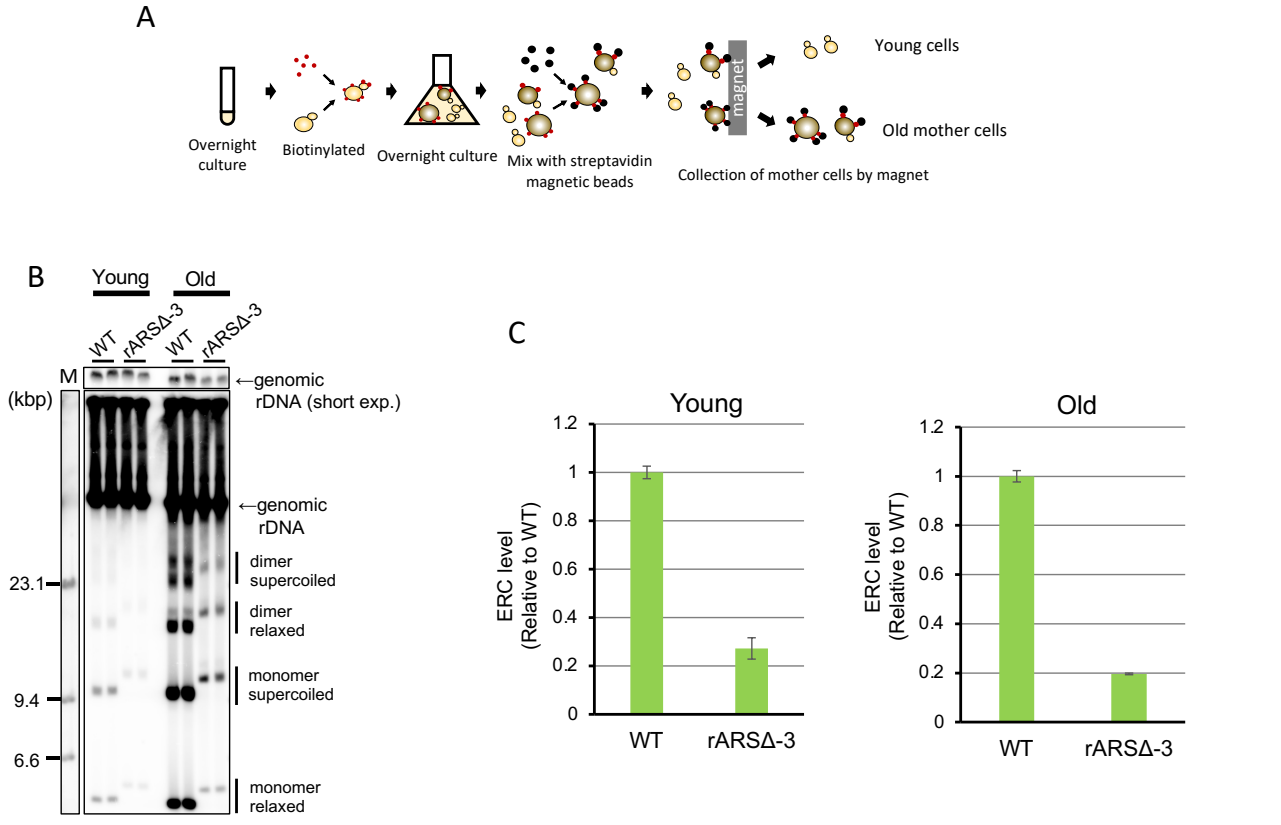

Fig. 3. Reduced ERC levels in both young and old rARS-Δ3 cells. (A) Schematic diagram of old- and young-cell sorting using biotin and streptavidin magnetic beads. See Materials and Methods for details. (B) ERC levels in young and old cells. ERCs in cells from two independent colonies were tested for each strain. (C) Signal intensities of ERCs were measured and normalized as in Fig. 2B. Error bars indicate SEM (n = 2). M is a size marker of HindIII-digested phage lambda DNA.

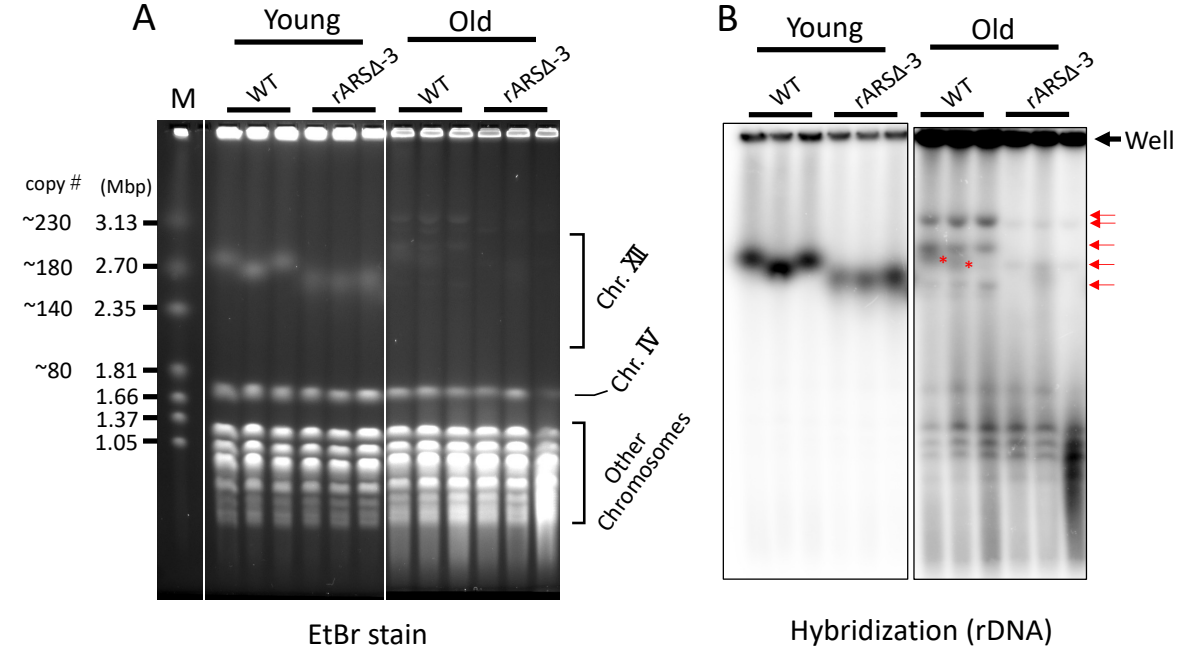

Fig. 4. Reduced rDNA stability in old cells. Genomic DNAs were separated by PFGE. (A) DNA visualized by staining with ethidium bromide (EtBr stain). (B) Southern analysis using a rDNA probe. In old cells, chromosomes with rDNA form fuzzy bands (asterisks) that comigrate with multimeric ERCs (sharp bands, indicated by red arrows). M is a size marker of *H. wingei* DNA (Bio-Rad).

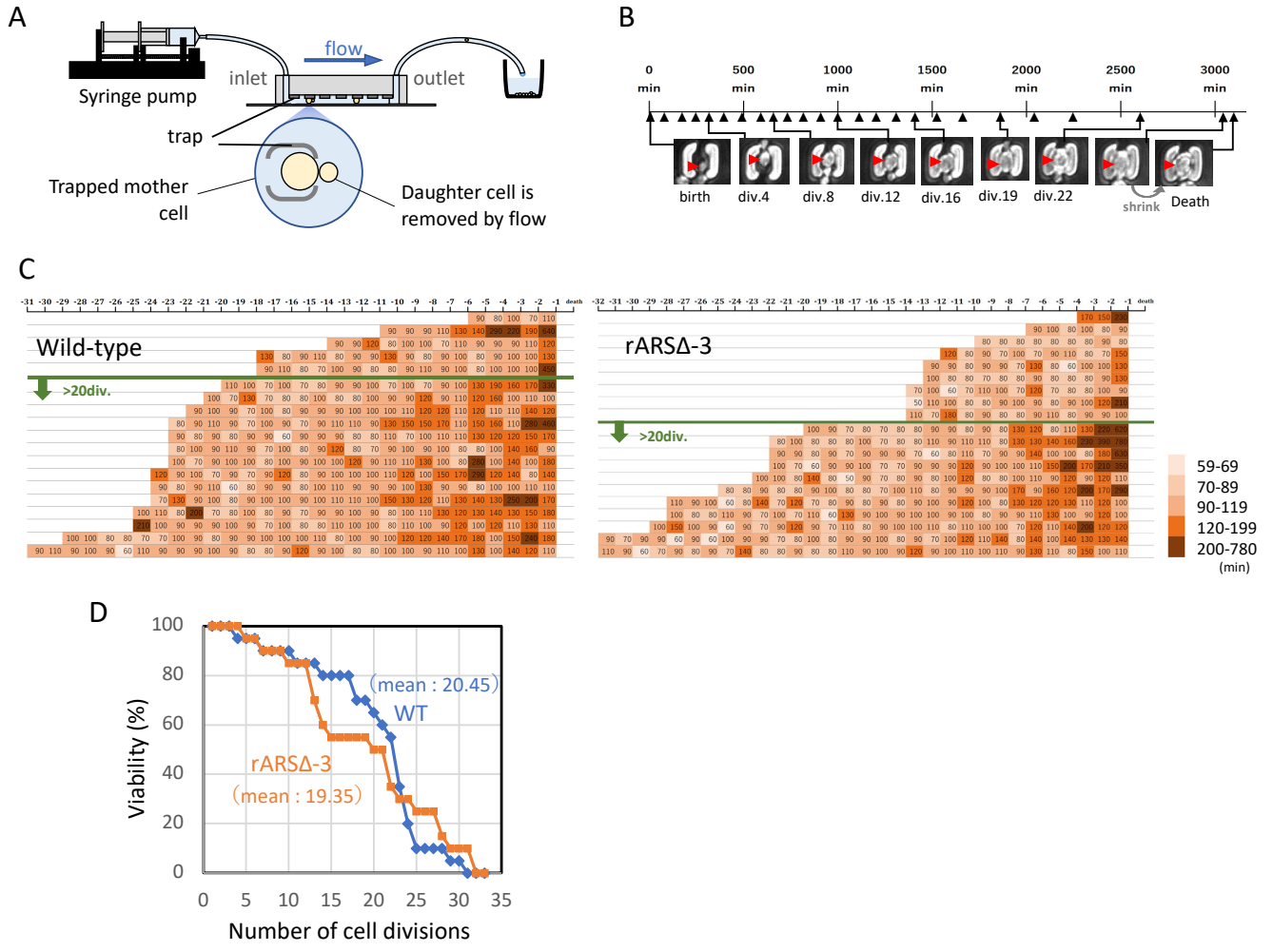

Fig. 5. Single-cell lifespan analysis. (A) Schematic diagram of microfluidics system to observe the complete life course of individual yeast cells. Trap openings allow passage of small, nascent cells. Once a cell is trapped, it spends its whole life in the device. (B) Snapshots of a trapped cell. After a cell becomes trapped (birth = div.0) and starts dividing, nascent cells flow out of the trap (div.4 to 22). In final rounds of division, the nascent cell does not detach from the mother (div.22); the mother "shrinks" and stops dividing (death = div.22). (C) Each cell division (boxes) and its duration (doubling time) in min (numbers) are indicated; the darker the box color, the longer the cell division. The right side of the diagram marks the end of life. (D) Mortality curves for rARS-Δ3 and wild-type (WT) strains based on the number of cell divisions counted for trapped cells. Numbers in brackets indicate the mean replicative lifespan. Twenty mother cells were counted (n = 20).

pan) and doubling time of each division and aligned the single-cell data with respect to their last division (Fig. 5C). This showed that the majority of wild-type cells managed 20–25 cell divisions, while a fraction of rARS-Δ3 cells show early death after 12–13 rounds; the ones that divided more than 20 times died gradually rather than within the span of a few cell divisions. We think this early and gradual death in the rARS-Δ3 strain is due primarily to increased rDNA instability, which was observed already in the young population of the strain (Fig. 4A and 4B).

The time taken for a cell division to complete increased near the end of a cell's lifespan. A similar phenomenon was reported by other groups, and defined as the senescence entry point (SEP) (Fehrmann et al., 2013; Morlot et al., 2019). For rARS-Δ3 cells, the SEP became more noticeable: the doubling times of the final cell divisions were in general longer than in wild-type cells (Fig. 5C). The single-cell data were combined for the construction of mortality curves, which indicated that the rARS-Δ3 strain had a slightly shorter lifespan (mean, 19.35) than the wild-type strain (20.45); this tendency is consistent with previous results (Ganley et al., 2009) (Fig. 5D). In addition, almost half of rARS-Δ3 clones (9/20) did not exceed 20 cell divisions (cf. 5/19 for wild type), which shows poorer fitness. Due to inefficient replication initiation, resulting in longer travel of the replication machinery and increasing replication stress in the rDNA, the rARS-Δ3 cells may have accumulated more DNA damage, which led to a shortened replicative lifes-

pan and extended doubling times for the final cell divisions. The smaller number of ERCs in rARS-Δ3 may have postponed the sudden drop of viability after ~20 cell divisions observed in the wild-type cells. In addition, in the terminal cell morphology, the rARS-Δ3 strain had more large buds (37%) than the wild-type strain (26%). This suggests that the rARS-Δ3 strain has more S/G2-arrested cells due to replication stress. Overall, our results indicate that rARS-Δ3 provides a good model system to study the effect of the accumulation of DNA damage on lifespan.

DISCUSSION

In many organisms, genome instability is a major cause of cellular senescence and is found to restrict the lifespan (Schumacher et al., 2021). The budding yeast *S. cerevisiae* is a good model to study the aging mechanism because of its short lifespan and the availability of effective genetic and molecular biological techniques. For the study of DNA damage-driven cellular senescence that is commonly observed in mammalian cells, wild-type budding yeast is less suitable because of elevated levels of extra-chromosomal rDNA circles associated with entry into senescence. Whether these ERCs are a by-product of DNA damage or can contribute to the onset of senescence is currently unclear. Here, we describe the aging phenotypes of a strain (rARS-Δ3) in which the accumulation of ERCs is suppressed due to attenuation of the ARS in the rDNA. Real-time analysis of single cells demonstrated that rARS-Δ3 cells follow a more linear survival curve than the typical inverted-S-shape observed for the wild type. Furthermore, an increase in doubling times around the end of life was observed, which could be related to replication stress that is expected to occur in this strain. We therefore think that this strain ages due to a damage signal from the rDNA, which makes it a suitable model to study DNA damage-induced cellular senescence.

The rDNA locus is known to be one of the most unstable regions in the genome (Kobayashi, 2014). In this study, we obtained evidence that the stability at this site reduces as the cell ages. As shown in Fig. 4B, in both wild-type and rARS-Δ3 strains, chromosome XII containing the rDNA locus was dramatically smeared after 7–8 cell divisions. This age-dependent rDNA instability is thought to be a cause of cellular senescence.

We propose three possible explanations for age-dependent rDNA instability. One explanation centers on a reduction in the expression level of Sir2, as has been found in old cells (Fine et al., 2019). A decrease in Sir2 levels leads to a rise of E-pro expression, which increases rDNA instability (Kobayashi and Ganley, 2005). We recently found that UAF (upstream activating factor for 35S rDNA) represses *SIR2* expression (Iida and Kobayashi, 2019). The daughter cell, which is smaller than the mother cell, may inherit less UAF and can rejuvenate by increased rDNA stability with enhanced expression of *SIR2*. The second possible reason for age-related DNA instability is an increase of oxidative stress. In old cells, mitochondria become dysfunctional, elevating the levels of reactive oxygen species (ROS; for a review, see Breitenbach et al. (2014)), which oxidize and damage cellular components including DNA (Veatch et al., 2009). The rDNA forms an unstable region by nature, so that it may be more sensitive to oxidative stress from ROS. The third explanation for age-dependent rDNA instability assumes that this is a side effect of ERC accumulation. Additional copies of episomal DNA, such as a plasmids or ERCs, can contribute to lifespan reduction which depends on rDNA stability as the major determinant (Ganley et al., 2009). Episomes, such as ERCs in budding yeast, may titrate factors required for genome maintenance, such as histones, DNA polymerase, topoisomerase and condensin, and thereby reduce DNA stability. The rDNA region is known to need many factors for its maintenance and is sensitive to their shortage (Ide et al., 2007). In line with this, a strain with increased ARS activity in the rDNA (ARS1-R) produced an excess of ERCs (Fig. 2) and had a shorter lifespan, with viability decreasing much faster per cell division than that in wild type or rARS-Δ3 (Ganley et al., 2009).

As mentioned above, rDNA instability can be a result of senescence by a reduction of Sir2, and an increase of ROS and ERC. On the other hand, according to our results here, the instability itself is presumed to accelerate senescence and shorten the lifespan. In the rARS-Δ3 strain, the effect of titrating essential DNA maintenance factors due to ERC accumulation will be much smaller than in the wild type even in old cells (Fig. 4). The increased rDNA instability in cells after 7–8 cell divisions is possibly caused by elevated replication stress due to less frequent replication initiation. Therefore, in the rARS-Δ3 strain, formation of ERCs may be similar to or higher than in the wild-type strain, but they are not maintained due to less frequent replication initiation. The rDNA stability will be further compromised when the normal rDNA copy number can no longer be maintained. Ganley et al. (2009) reported a decrease in the capacity for gene amplification in rARS-Δ3 cultures grown for several generations, which implies that aging cells will struggle to recover from a loss of rDNA copies. For this strain, no deviation from wild-type RFB activity or RFB-dependent DSB formation was found in exponentially growing cells, so that rDNA damage associated with collapsing replication forks can be expected to occur normally. In other words, RFB/Fob1-independent rDNA damage may also affect the stability. Similar Fob1-independent rDNA recombination is observed in the *hpr1* mutant (Merker and Klein, 2002). In this mutant,

because lifespan reduction is not correlated with ERC production, the similar event that occurs in rARS-Δ3 may affect the rDNA stability and lifespan. Suggestively, the survival curve of the mutant has a very similar shape to that of the rARS-Δ3 strain.

Replication stress, which increases rDNA instability, is enhanced due to less frequent replication initiation (Ganley et al., 2009). When ERC-mediated titration of rDNA maintenance factors is reduced, the onset of senescence will predominantly be triggered by DNA damage. Our data, therefore, suggest that rARS-Δ3 cells mainly die from rDNA instability. This is supported by the observation that more rARS-Δ3 cells died with large buds due to replication stress in S/G2 phase. In addition, in terms of the morphology, after the last cell division, the wild-type mother shrinks. In contrast, some of the rARS-Δ3 cells did not shrink, suggesting that the manner of death is different in the two strains.

One of the reasons for elongated doubling time around the end of cellular life is that rDNA instability may inhibit cell cycle progression. Such a phenotype has been observed in mammalian cells, which arrest in the G1 phase upon DNA damage-induced senescence (Johmura et al., 2014). Why the cells arrest in G1 is still unknown, but one possibility is that it takes longer to repair damaged rDNA in old cells. Supporting this idea, another single-cell aging study reported on the kinetics of DNA repair, which is delayed in aged yeast cells (Novarina et al., 2017). As with Sir2, it is known that the levels of repair enzymes decrease in old cells, which reduces the efficiency of DNA repair (Pal et al., 2018).

Another question about rDNA instability and lifespan in budding yeast is how mother and daughter cells are distinguished during cell division. As shown in Fig. 4, the rDNA of dividing cells becomes unstable with age. If rDNA instability determines lifespan, it should not be passed on to the daughter cell, for which the lifespan is reset (Kobayashi, 2014). Compared to old cells, reduced amounts of rDNA recombination intermediates remain stuck in the wells in PFGE of chromosomes of young cells (Fig. 4B). DNA molecules that are unable to migrate into the gel contain unusual structures derived from unstable rDNA (Ganley et al., 2009). Recently, a comparable "nonrandom segregation" of rDNA has been observed for dividing *Drosophila* stem cells (Watase and Yamashita, 2021), suggesting that cells with a higher life expectancy, such as a daughter cell in budding yeast and a nascent stem cell in *Drosophila*, inherit more stable rDNA. Such a mechanism may be critical to maintain the continuity of life.

MATERIALS AND METHODS

Yeast strains, growth conditions and DNA preparation Yeast strains used in this study were derived from NOY408-1b (a W303 derivative) (Nogi et al., 1991). Strains were grown at 30 °C in YPDA (YPD with 0.4% adenine) or synthetic complete (SC) medium lacking the appropriate amino acids when selecting for a gene marker (Sherman et al., 1986). Yeast strains used in this study are listed in Table 1. For selection of the *kanMX* marker, 500 μg/ml G418 (Sigma) was added to the medium. PCR primer sequences used in this study are available on request. Yeast genetic transformation was performed as previously described (Ito et al., 1983). To test rDNA stability by PFGE, DNA was prepared from cells that had divided ~45 times after transformation.

For DNA preparation, low-melting-temperature agarose plugs were used as described previously (Sasaki and Kobayashi, 2017). In short, 5×10^7 cells were suspended in 33 μl of 50 mM EDTA (pH 7.5) at 42 °C, to which 66 μl of solution I (0.83% low-melting-point agarose SeaPlaque GTG, 170 mM sorbitol, 17 mM sodium citrate, 10 mM EDTA pH 7.5, 0.85% β-mercaptoethanol, and 0.17 mg/ml Zymolyase 100T) was added. The mixture was poured into a plug mold (Bio-Rad) and placed at 4 °C for 1 h. The hardened plugs were then treated with solution II (450 mM EDTA pH 7.5, 10 mM Tris-HCl pH 7.5, 7.5% β-mercaptoethanol and 10 μg/ml RNase A) for 1 h at 37 °C. Plugs were then incubated overnight at 50 °C in solution III (250 mM EDTA pH 7.5, 10 mM Tris-HCl pH 7.5, 1% sodium dodecyl sulfate (SDS), 1 mg/ml proteinase

Table 1. Strains used in this study

Name	Name in figures	Genotype	Reference
YMH-41	NOY4081b (wild type)	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Nogi et al., 1991
YMH-10	ARS1-R	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 rARS is replaced with URA3-ARS1*	Ganley et al., 2009
YMH-88	rARSΔ-3	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 ACSIII is replaced with URA3*	Ganley et al., 2009
YMH-20	rARSΔ-3, *fob1Δ::HIS3*	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 ACSIII is replaced with URA3 fob1Δ::HIS3*	Ganley et al., 2009
YMH-30	*fob1Δ::HIS3*	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 fob1Δ::HIS3*	This study
YMH-35	*sir2Δ::KanMX*	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 sir2Δ::KanMX*	This study

K), after which they were washed four times with 50 mM EDTA pH 7.5.

ERC assays ERC assays were done as previously described (Goto et al., 2021; Sasaki and Kobayashi, 2021). In short, an agarose plug (5 mm) was placed on a tooth of a comb. The comb was placed in a 15 × 25 cm gel tray, into which 300 ml of 0.4% agarose in 1× TAE (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA pH 8.0) was poured. Phage lambda HindIII-digested DNA (175 ng) was used as a size marker. Electrophoresis was performed in 1× TAE buffer at 1.0 V/cm for ~60 h at 4 °C with buffer circulation. The buffer was changed every ~24 h.

Pulsed-field gel electrophoresis PFGE was performed as previously described (Sasaki and Kobayashi, 2017, 2021). In short, an agarose plug (3 mm) was placed on a tooth of the comb along with a *Hansenula wingei* CHEF DNA size marker (Bio-Rad). The comb was set in a 15 × 25 cm gel tray, into which 170 ml of 1% agarose gel in 0.5× TBE (44.5 mM Tris base, 44.5 mM boric acid and 1 mM EDTA pH 8.0) was poured. Electrophoresis was performed with a CHEF-MAPPER (Bio-Rad) with 300–900-s pulse time (0.3 V/cm) for 68 h at 14 °C.

Southern analysis Before DNA transfer by Southern blotting, the gel was photographed after staining with EtBr (0.5 μg/ml) for 30 min. Southern analysis was performed as previously described (Kobayashi et al., 1998). In short, the gel was treated for 30 min with, successively, 0.25 N HCl, denaturation solution (0.5 N NaOH, 1.5 M NaCl) and transfer buffer (0.25 N NaOH, 1.5 M NaCl). DNA was blotted onto Hybond-XL (GE Healthcare) by capillary transfer for 12 h, after which the membrane was soaked in 0.4 N NaOH for 10 min and then in 2× SSC for 10 min.

Probes for hybridization were prepared from 50 ng gel-purified DNA that had been amplified from yeast rDNA with primers 5′-CATTTCCTATAGTTAACAGGACATGCC-3′ and 5′-AATTCGCACTATCCAGCTGCACTC-3′ by means of a random primer DNA Labeling Kit (Takara), according to the manufacturer's instructions. Probes were purified with ProbeQuant G-50 Micro Columns (GE Healthcare) and denatured for 5 min at 100 °C.

After pre-hybridization in 25 ml of hybridization buffer (1% bovine serum albumin, 0.5 M phosphate buffer pH 7.2, 7% SDS, 1 mM EDTA pH 8.0) for 1 h at 65 °C, membranes were hybridized in 25 ml of hybridization buffer with the heat-denatured probe at 65 °C for 12 h. The membrane was washed four times in 40 mM phosphate pH 7.2, 1% SDS, 1 mM EDTA pH 8.0 for 15 min at 65 °C and exposed to a phosphor screen for ~5 days. The radioactive signal was detected on a Typhoon FLA 7000 (GE Healthcare).

Collection of biotinylated cells Old mother cells were collected as previously described (Smeal et al., 1996). In short, a single colony was inoculated into 5 ml YPD and grown overnight at 30 °C. Cells (2×10^7) were collected by centrifugation, washed twice in phosphate-buffered saline (PBS) and resuspended in 100 μl PBS. After addition of 1.25 mg sulfo-NHS-LC-biotin (Thermo Fisher Scientific) in 37.5 μl PBS and incubation at room temperature with gentle shaking for 15 min, cells were washed three times with 300 μl PBS, resuspended in 250 ml SD and incubated at 30 °C for 12 h. The OD_{600} was measured and the number of cell divisions (~7) was calculated. Cells were harvested, washed with PBS and resuspended in 3.5 ml PBS; 37.5 μl of streptavidin-coated magnetic beads (PerSeptive Biosystems) was added to the cell suspensions, followed by incubation at room temperature for 15 min with mild agitation. The beads were washed five times, and collected on a magnetic sorter.

Observation of yeast life course by microfluidic device A 10-μl sample of overnight cultured yeast cells was transferred into 5 ml SC medium and incubated for 4–5 h at 30 °C. The exponentially growing cells were diluted 100-fold and injected into the chip of a microfluidic device that was made as previously described (Aspert et al., 2022) by means of a 1-ml syringe. SC medium was continuously streamed into the device at 20 μl/min.

Bright-field images were recorded every 10 min for 96 h on a Nikon ECLIPSE Ti microscope (Plan-fluor ELWD 20×/0.45) with a Roper CoolSNAP HQ-2 using Nikon NIS-Elements software. Doubling times and number of cell divisions were manually determined for each captured cell.

We thank Drs. T. Iida, M. Sasaki, Y. Hori, E. Ohya and other members of the Kobayashi lab for discussion and technical advice. This work was supported by JST CREST (JPMJCR19S3 to T. K.), AMED-CREST (JP21gm1110010), Japan Society for the Promotion of Science (JP18K06056 to C. H., 17H01443 and 21H04761 to T. K.) and the Sumitomo Foundation to C. H.

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