Defects in the NuA4 acetyltransferase complex increase stability of the ribosomal RNA gene and extend replicative lifespan

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Genome instability is a cause of cellular senescence. The ribosomal RNA gene repeat (rDNA) is one of the most unstable regions in the genome and its instability is proposed to be a major inducer of cellular senescence and restricted lifespan. We previously conducted a genome-wide screen using a budding yeast deletion library to identify mutants that exhibit a change in the stability of the rDNA region, compared to the wild-type. To investigate the correlation between rDNA stability and lifespan, we examined deletion mutants with very stable rDNA and found that deletion of *EAF3*, encoding a component of the NuA4 histone acetyltransferase complex, reproducibly resulted in increased stabilization of the rDNA. In the absence of Eaf3, and of other subunits of the NuA4 complex, we observed lower levels of extrachromosomal rDNA circles that are produced by recombination in the rDNA and are thus an indicator of rDNA instability. The replicative lifespan in the *eaf3* mutant was extended by ~30%, compared to the wild-type strain. Our findings provide evidence that rDNA stability is correlated with extended replicative lifespan. The *eaf3* mutation possibly affects the non-coding transcription in rDNA that regulates rDNA recombination through cohesin dissociation.

Key words: Eaf3, genome stability, histone acetyltransferase, replicative lifespan, ribosomal RNA gene (rDNA)

INTRODUCTION

Genome stability is essential to maintain cellular functions. DNA damage is the main inducer of genome instability, as improper repair of DNA damage leads to an accumulation of mutations and chromosome rearrangements. Therefore, organisms have developed effective systems to ensure proper repair of DNA damage in order to maintain genome integrity during the course of evolution (Branzei and Foiani, 2008). However, as the genome maintenance systems cannot repair all DNA damage, mutations gradually accumulate in the genome of each cell. To maintain the fitness of a population, cells that have undergone a particular number of cell divisions are programmed to die. This so-called cellular senescence removes cells with potentially unstable genomes from

the population. For example, by inducing senescence, the risk of cancer is dramatically reduced in mammals (for a review, see Campisi and d'Adda di Fagagna, 2007).

In human tissue cells that lack telomerase activity, telomeres shorten as cells divide. When telomere lengths become too short, cellular senescence is induced (Harley et al., 1990). Thus, programmed cellular senescence can be based on telomere length as a “counter” of the number of cell divisions. In contrast, cells with functional telomerase, in which telomere lengths are maintained, have to induce senescence in a different manner (Kobayashi, 2011a).

The ribosomal RNA gene repeat (rDNA) is the most abundant gene in eukaryotic cells. In budding yeast, the rDNA region is located at a single locus on chromosome XII and contains ~150 rDNA copies (Kobayashi et al., 1998). Due to the repetitive structure, heavy transcription, and deletional intra-repeat recombination, the rDNA copy number frequently changes, making the rDNA region one of the most unstable regions in the genome

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(Kobayashi, 2011b). In budding yeast, the mechanisms that maintain rDNA stability are well studied. One of them is a gene amplification system that recovers lost rDNA copies and relies on several factors.

In the S phase of the cell cycle, one of the DNA replication forks originating from the origin of replication is arrested at the replication fork barrier (RFB) site by Fob1 protein (Fig. 1). At a number of the arrested forks, a DNA double-strand break (DSB) is formed. Although it remains unknown how these DSBs are repaired, we have recently shown that DSB end resection, the initiation event of homologous recombination, is mostly suppressed in cells that carry the normal rDNA copy number (Sasaki and Kobayashi, 2017). However, in certain circumstances, such as when cells carry a low rDNA copy number or lack the replisome component Ctf4, suppression of DSB end resection is relieved to trigger homologous recombination-mediated repair (Sasaki and Kobayashi, 2017). When recombination occurs with the sequence located at an equal position on the sister chromatid, DSBs are repaired without any changes in the rDNA copy number (Fig. 1A). However, when it occurs with the

sequence at an unequal position on the sister chromatid or with another copy on the same chromosome, DSB repair is accompanied by an increase (Fig. 1B, b-1) or a decrease in the rDNA copy number (Fig. 1B, b-2). The intrachromosomal recombination results in the production of extrachromosomal rDNA circles (ERCs), leading to rDNA instability. A non-coding bidirectional promoter, E-pro, which is located in one of the intergenic regions, regulates the outcomes of DSB repair. Transcription from E-pro leads to the dissociation of cohesin that connects sister chromatids and, as a result, an increase in the frequency of unequal sister chromatid recombination events. This type of recombination occurs when the copy number is substantially reduced and helps to recover lost rDNA copies (Fig. 1B, b-1). When the copy number is at the normal level, E-pro transcription is repressed by the histone deacetylase Sir2 and cohesin can be retained to ensure equal sister chromatid recombination (Kobayashi and Ganley, 2005). Recently, we found how the copy number is recognized and adjusted by the cell (Iida and Kobayashi, 2019a). Upstream activating factors (UAFs) bind to the promoter of 35S rDNA and activate transcrip-

Fig. 1. Recombination in rDNA. Three copies of rDNA are shown. (A) Stable recombination pathway. Sir2 represses E-pro and cohesin associates in the intergenic regions. As a result, unequal sister chromatid recombination is restricted, which reduces the frequency of copy number changes. (B) Amplification and ERC production pathway. E-pro is activated and cohesin dissociates. As a result, during repair by homologous recombination, broken ends from a DSB at the RFB can move to homologous sequences elsewhere ('unequally') on a sister chromatid (during amplification, b-1) or on the broken strand itself, leading to the production of ERCs (b-2). For details, see the text. ARS: autonomously replicating sequence; RFB: replication fork barrier; ERC: extrachromosomal rDNA circle; E-pro: non-coding promoter. This figure is modified from Saka et al. (2016).

tion (Keys et al., 1996; Keener et al., 1997; Tongaonkar et al., 2005; Smith et al., 2018). Interestingly, when the rDNA copy number declines, the UAFs are released from the rDNA and move to the promoter of *SIR2* to repress transcription. As a result, the amount of Sir2 is reduced, E-pro is activated and the copy number can be recovered (Iida and Kobayashi, 2019b). When the copy number approaches the wild-type level, UAFs are titrated by the increased number of rDNA copies and *SIR2* repression is lifted, restoring repression of E-pro transcription and thereby preventing further rDNA amplification via recombination. So, in short, Fob1 and non-coding transcription increase rDNA instability that can lead to copy number recovery, while Sir2 suppresses rDNA instability when the copy number is at the normal level.

Fob1 and Sir2 are also known as aging genes (Defossez et al., 1999; Kaeberlein et al., 1999; Takeuchi et al., 2003). In the *fob1* mutant, rDNA is quite stable and the replicative lifespan is extended by ~60%, while it is shortened to half of the wild-type level in the *sir2* mutant with unstable rDNA. These findings strongly suggest that, due to its unstable nature, rDNA is a major region that can produce an “aging signal” to induce cellular senescence. According to this “rDNA theory for aging” (for a review, see Kobayashi, 2008), studying the mechanisms to maintain rDNA stability is important to understand aging.

We previously performed a genome-wide screen to identify mutants that show enhanced rDNA instability or stability, compared to the wild-type strain (Saka et al., 2016). In this screen, we used a budding yeast gene deletion library in which ~4,800 non-essential genes are individually deleted, isolated genomic DNA from each mutant and separated chromosomes by pulsed-field gel electrophoresis (PFGE, Supplementary Fig. S1). Based on the stability of chromosome XII carrying the rDNA array, the mutants were classified into four ranks. We identified ~700 mutants with reduced rDNA stability, and 22 mutants in Rank 1 whose rDNA became more stable than the wild-type (Saka et al., 2016; Kobayashi and Sasaki, 2017; <http://lafula-com.info/kobayashiken/geldata/index.php>).

Here, we examined the effect on rDNA stability of gene deletions that were initially found to increase rDNA stability in a different genetic background, and found that *EAF3* disruption enhances rDNA stability, compared to the wild-type (Supplementary Fig. S1). Eaf3 is a component of the NuA4 protein complex that acetylates histones and thereby regulates gene expression. In mutants lacking Eaf3 and other components of the NuA4 complex, lower levels of ERCs are produced. We also found that the lifespan of the *eaf3* mutant is ~30% longer than that of the wild-type strain. These results support the hypothesis that rDNA stability affects yeast lifespan.

MATERIALS AND METHODS

Yeast strains and culture methods Yeast strains used in this study are derivatives of the laboratory strain W303 and are listed in Supplementary Table S1. Gene replacement was performed by a one-step gene replacement procedure using standard methods. Correct gene replacement was confirmed by PCR-based genotyping. For Figs. 2A and 5, the wild-type and mutant haploid clones were isolated by tetrad dissection of parental diploid cells before further analysis. For PFGE and ERC assays, cells were grown overnight in YP medium (1% yeast extract and 2% peptone) containing 2% glucose (YPD) at 30 °C.

Preparation of genomic DNA For PFGE and ERC assays, cells were collected (5×10^7 cells per plug) and washed twice with 50 mM EDTA (pH 7.5). Genomic DNA was prepared in low melting temperature agarose plugs as described previously (Murakami et al., 2009; Sasaki and Kobayashi, 2017 for Figs. 2 and 5; Takeuchi et al., 2003 for Figs. 3 and 4).

PFGE analysis Genomic DNA as well as *Hansenula wingei* chromosomal DNA markers (Bio-Rad) were electrophoresed in 1.0% agarose gels in $0.5 \times$ TBE (44.5 mM Tris base, 44.5 mM boric acid and 1 mM EDTA, pH 8.0) as

Fig. 2. PFGE analysis of *eaf3* mutants. (A) Genomic DNA was isolated from *EAF3* (WT) and *eaf3* haploid progenies isolated from an *eaf3/EAF3* heterozygous diploid strain and separated by PFGE. The upper panel shows ethidium bromide staining of the gels. The lower panel shows hybridization to probes specific for *YLL058W* (chr XII) and *YDL239C* (chr IV). The size marker consists of *Hansenula wingei* chromosomes (Biorad). (B) Quantification of (A). The signal intensity of chromosome XII was normalized to that of chromosome IV. Error bars (S.E.M.) and *p* value are indicated.

Fig. 3. PFGE analysis of the indicated mutants. The gel was stained with ethidium bromide. The size markers are *H. wingei* chromosomes.

described previously (Sasaki and Kobayashi, 2017). DNA was stained with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide and photographed. DNA was transferred to Hybond-XL (GE Healthcare) using standard methods.

To assess rDNA stability, we quantified the band intensities of chromosomes XII and IV in the ethidium bromide-stained gels, using ImageJ software (NIH). We drew a very narrow rectangle around the band for chromosome IV and measured the signal intensity within the rectangle. Moving the same rectangle below the chromosome XII band, we next measured the background signal, which was subtracted from the chromosome IV signals. In a similar manner, we measured signal intensities of chromosome XII with the same rectangle. We then divided the signal intensities of chromosome XII by those of chromosome IV.

Agarose gel electrophoresis for ERC detection Half of an agarose plug was loaded on 0.4% (Fig. 5B, 5D, 5F) or 0.6% (Fig. 4) agarose gels (in a 15 $\times$ 25 cm gel tray) and separated by electrophoresis in 1 $\times$ TAE (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA) at 1.0 V/cm for ~20 h (Fig. 4) or ~48 h (Fig. 5B, 5D, 5F) with buffer circulation at 4 $^{\circ}\text{C}$, during which the buffer was exchanged every 24 h. DNA was transferred to Hybond XL (GE Healthcare) using standard methods.

Southern blotting PCR products used for radioactive labeling were prepared as described previously (Sasaki and Kobayashi, 2017), using the following primers: *YLL058W* probe to detect chromosome XII in PFGE analysis (5'-GGC-GAGGTTTCAGAAAAGTGTCTG and 5'-AAACGGCAAGAAT-GCGTTGTTTG); *YDL239C* probe to detect chromosome IV

Fig. 4. ERC assays with the indicated mutants. (A) ERCs and genomic rDNA were separated by gel electrophoresis and, after hybridization, visualized by different exposure times. As the genomic rDNA signal is much stronger than the ERC signal, the exposure time for genomic rDNA is shorter. Three independent colonies were analyzed for each genotype. (B) Quantitation of (A). The signal intensity of ERCs was normalized to that of genomic rDNA. Error bars are S.E.M. *P* values of differences from the wild-type: *eaf3* (0.073), *fob1* (0.047), *sir2* (0.122), *eaf3 fob1* (0.046), *eaf3 sir2* (0.973).

in PFGE analysis (5'-TTTGAATAAGCCTGAATCCCTA-AAGG and 5'-TTGATCTCTTCATTTTGGGACTGG); rDNA probe 1 in ERC assay (5'-CATTTCCCTATAGTTAA-CAGGACATGCC and 5'-AATTCGCACTATCCAGCTG-CACTC); and for Fig. 4 (5'-TCTGAAGAGTTAAGCACTCC and 5'-CTTCCCGAGCGTGAAAGGAT). Radioactively labeled probes were prepared using the Random Primer DNA Labeling Kit Ver.2 (TaKaRa) according to the manufacturer's instructions. Hybridization was performed as described previously (Murakami et al., 2009; Sasaki and Kobayashi, 2017).

For ERC assays, the membranes were exposed to phosphor screens for an appropriate exposure time and the screens were scanned before any signal was saturating. From these scanned images, genomic rDNA bands were quantified, using FUJIFILM Multi Gauge version 2.0 software (Fujifilm). To quantify ERCs, membranes were exposed to phosphor screens for several days and the screens were scanned. Signal intensities of four different ERC bands were quantified, including supercoiled monomers, relaxed monomers, supercoiled dimers and relaxed dimers (Fig. 5B, 5D, 5F). We calculated the relative amount of ERCs for each mutant by dividing the sum

Fig. 5. ERC assays in mutants lacking components of the NuA4 and RpdS3 complexes. (A, C, E) Five independent colonies were analyzed by PFGE. Upper panels show ethidium bromide staining and lower panels show hybridization to *YLL058W* (chromosome XII) and *YDL239C* (chromosome IV) probes. Right panels show quantification of the signal intensity of chromosome XII, normalized to that of chromosome IV. Error bars (S.E.M.) and P values are indicated. (B, D, F) ERC assays as in Fig. 4 but with a different method of DNA preparation (see Materials and Methods). The right panels show quantification of ERCs, normalized to genomic rDNA. Error bars (S.E.M.) and P values are indicated.

of the four ERC signal intensities by the signal intensity for genomic rDNA. For Fig. 4, signal intensities of two ERC bands (monomer and dimer) were quantified.

Replicative lifespan analysis Replicative lifespan was measured as previously described (Kennedy et al., 1994). In short, a daughter cell (virgin mother) was isolated on a YPD plate and each daughter cell that budded from this mother cell was removed using a micro manipulator system (Singer) until the mother cell died. The number of removed daughter cells was counted and taken as a measure of replicative lifespan.

Quantification and statistical analysis Statistical analysis was performed using GraphPad Prism software (version 6.0d or 6.0h). For Figs. 2B, 4B, 5 and 6, significance of the difference between WT and mutants was determined by two-tailed, unpaired Student's *t*-test.

RESULTS

rDNA stability is enhanced in the absence of Eaf3 In the 22 Rank 1 mutants where rDNA became more stable than the wild-type (Saka et al., 2016; Kobayashi and Sasaki, 2017; <http://lafula-com.info/kobayashiken/geldata/index.php>), we decided to focus on Eaf3 because the protein is a subunit of a histone acetyltransferase complex, a putative antagonist of Sir2. We tested the reproducibility of the phenotype. To minimize an effect of transformation on rDNA stability (Kwan et al., 2016), we first established a diploid strain heterozygous for *eaf3*, isolated haploid clones by tetrad dissection and tested rDNA stability in five independent clones of *EAF3* and *eaf3* strains by PFGE using genomic DNAs isolated in agarose plugs (Fig. 2A). A comparatively sharp band of chromosome XII indicates stable rDNA and provides a relatively strong signal in a fixed area. Therefore, to assess the stability of rDNA, we measured the band intensity of chromosome XII for a fixed rectangular area (see Materials and Methods) and normalized the value to that of the second-largest chromosome, chromosome IV (Fig. 2B). As shown in Fig. 2, the chromosome XII bands in the *eaf3* mutant appeared to be sharper than those with the wild-type allele, suggesting that the *eaf3* mutation stabilizes the rDNA region.

Eaf3 functions in the same pathway as Fob1 in rDNA instability To investigate the role of *EAF3* in maintaining rDNA stability, we constructed a double mutant with *FOB1*. Fob1 binds the RFB site and inhibits replication fork progression; at some of the arrested forks DSBs are introduced (Kobayashi, 2003; Weitao et al., 2003; Burkhalter and Sogo, 2004; Kobayashi et al., 2004). As rDNA is quite stable in the *fob1* mutant, the formation of a DSB is thought to be a major trigger for

rDNA instability (Kobayashi et al., 1998; Sasaki and Kobayashi, 2017). PFGE analysis of *eaf3*, *fob1* and the *eaf3 fob1* double mutant revealed a comparable level of rDNA stability as evidenced by similarly sharp bands of chromosome XII (Fig. 3).

It is known that the level of ERCs, which are produced from rDNA by intrachromosomal recombination, correlates with rDNA instability (Sinclair and Guarente, 1997; Saka et al., 2016). Analysis of ERCs showed that in the *eaf3* mutant, the signal intensities of ERC bands were much weaker than those in the wild-type strain (Fig. 4). The amount of ERCs in *eaf3* is less than half that of the wild-type (Fig. 4B, $P = 0.073$), although the difference was not statistically significant. This observation supports the idea that rDNA is more stable in the *eaf3* mutant than in the wild-type strain. In the *eaf3 fob1* double mutant, the level of ERCs is as low as that in *fob1*, which suggests that Eaf3 functions in the same pathway as Fob1.

Deletion of EAF3 partially rescues rDNA instability caused by the absence of Sir2 Sir2, a histone deacetylase, functions downstream of *FOB1* by repressing E-pro, the non-coding promoter whose transcription prevents cohesin association and reduces rDNA stability (See Fig. 1 in Kobayashi and Ganley, 2005). As Eaf3 is a component of the NuA4 protein complex, which acetylates histones and activates transcription, it is possible that the *eaf3* mutation affects transcription from E-pro and thereby increases rDNA stability. To test how Eaf3 activity relates to that of Sir2, we generated an *eaf3 sir2* double mutant. PFGE analysis confirmed that rDNA is unstable in the absence of Sir2, as the band of chromosome XII is quite smeared in the case of the *sir2* mutant (See Fig. 3 in Kobayashi et al., 2004). The chromosome XII band in the case of the *eaf3 sir2* double mutant was similarly smeared, indicating frequent changes in rDNA copy number. When we determined the amount of ERCs formed in these mutants (Fig. 4), we observed that in the *sir2* mutant, more ERCs were produced than in either the *eaf3 sir2* double mutant or, as previously reported, the wild-type strain (Kaeberlein et al., 1999; Kobayashi et al., 2004 for Fig. 4A). Quantitation of band intensities indicates that the level of ERCs in the double mutant is about half of that in the single *sir2* mutant (Fig. 4B), which is consistent with the very low level of ERCs formed in the *eaf3* mutant. These results indicate that the *eaf3* mutation suppresses rDNA instability caused by the absence of Sir2 in the ERC assay.

rDNA is stabilized in NuA4 mutants Eaf3 is a component of the NuA4 histone acetyltransferase complex (Reid et al., 2004). It is also known that Eaf3 is included in the Rpd3S histone deacetylase complex (Gavin et al., 2002; Chittuluru et al., 2011). To distinguish which of

these functions is important to regulate rDNA stability, we performed PFGE and ERC assays on mutants lacking other unique components of these complexes (Eaf1 and Eaf5 in the case of the NuA4 complex, and Rco1 in the Rpd3S complex) (Fig. 5). All mutants were obtained by tetrad dissection from heterozygous parental strains. In the case of the *eaf5* mutants, the chromosome XII bands were sharper in the PFGE assay, suggesting that rDNA stability was enhanced in the absence of Eaf5, compared to the wild-type, although the amount of ERCs was not substantially reduced in some clones (Fig. 5A, 5B). In the case of the *eaf1* mutants, the amount of ERCs was significantly lower compared to the wild-type, although no clear difference was observed for the sharpness of the chromosome XII band in the PFGE analysis (Fig. 5C, 5D). The *rco1* mutants did not show statistically significant differences in rDNA stability or in ERC levels, compared to WT (Fig. 5E, 5F). Taken together, these results suggest that at least a defect in the NuA4 complex could increase rDNA stability. We note that some progenies from heterozygous parental strains (*EAF1/eaf1* and *RCO1/rco1*) have several chromosome XII bands whose lengths are quite different from their parents (Fig. 5C, 5E). These observations suggest that the mutations affected chromosome stability during meiosis even in the heterozygous diploid cell.

The absence of Eaf3 extends lifespan rDNA stability is known to affect lifespan (see reviews by Ganley and Kobayashi, 2014; Kobayashi, 2014). As described above, the *eaf3* mutation stabilizes the rDNA to a level higher than that in the wild-type and partially suppresses rDNA instability in the *sir2* mutant (Fig. 4). Therefore, the *eaf3* mutant is expected to have a longer lifespan than the wild-type strain. To test this, we measured replicative lifespan by counting the number of daughters produced from a mother cell (Kennedy et al., 1994). As controls, the lifespans of the *sir2* and *fob1* mutants were also measured (Defossez et al., 1999; Kaeberlein et al., 1999; Takeuchi et al., 2003). As expected, the lifespan was shortened in the absence of Sir2 and extended when Fob1 was not present (Fig. 6). In agreement with the above observed increase in rDNA stability, the lifespan of the *eaf3* mutant was ~30% longer than that of the wild-type (Fig. 6). However, the lifespan was shorter than that of the *fob1* mutant. The *eaf3 fob1* double mutant has a replicative lifespan longer than that of the *eaf3* mutant, but shorter than that of the *fob1* mutant, suggesting that impeding DSB formation at the RFB site in the *fob1* mutant contributes more to extending lifespan than deficiency in the *eaf3* mutant. Notably, the short lifespan observed for the *sir2* mutant was not suppressed in the *sir2 eaf3* double mutant despite the rDNA instability being partly suppressed in the double mutant (Fig. 4B). These results indicate that the *eaf3* mutation

Fig. 6. Replicative lifespan analysis. (A) Mortality curves of the wild-type and mutant strains. The number of analyzed cells is indicated (n). (B) The average lifespan of strains used in (A). Error bars are S.E.M. *P* values of differences from the wild-type are all < 0.01.

extends lifespan mainly through rDNA stabilization, but that this is not sufficient to overcome the lack of E-pro suppression and concomitant ERC production in the absence of Sir2.

DISCUSSION

Ribosomal RNA is the most abundant RNA in a cell. To supply this huge amount of RNA, cells have developed systems to maintain clusters of tandemly repeated rDNA genes (Kobayashi, 2011b). These repeats are prone to recombination, so that fluctuations in rDNA copy number are commonly observed (Zhang et al., 1990; Gibbons et al., 2015). ERCs are formed during recombination that leads to deletion of rDNA units, while rDNA amplification occurs when rDNA copy number is low. These recombination events relate to the regulation of Sir2 (Iida and Kobayashi, 2019a) and the manner of recombination between rDNA units, which depends on the regulation of the non-coding promoter E-pro in one of the non-transcribed spacers (Fig. 1). In a previous screen (Saka et al., 2016), we identified a group of genes whose deletion appeared to increase rDNA stability. Here, we identified

Eaf3, a histone H4/H2A acetyltransferase that forms a subunit of the NuA4 complex and is involved in transcription and DNA repair (Utley et al., 2005), as a gene that affects the regulation of rDNA recombination. Deletion of the *EAF3* gene reduced formation of ERCs and thereby stabilized the rDNA (Fig. 2 and Fig. 4). Deletion of *FOB1* also leads to an increase in rDNA stability, and because this stability was not enhanced in a *fob1 eaf3* double mutant, it seems that the *EAF3* gene functions in the same pathway as *FOB1*; that is, after DSBs, which can occur at the RFB site after replication fork blocking by Fob1, have triggered recombination. Interestingly, the *eaf3* mutation partially rescued the formation of ERCs observed in the absence of Sir2 (Fig. 4). Eaf3 recruits the NuA4 histone acetyltransferase complex to the promoter region of target genes and enhances their transcription (Reid et al., 2004). Because the histone deacetylase Sir2 represses E-pro transcription that stabilizes rDNA (Kobayashi and Ganley, 2005), Eaf3 is likely to enhance E-pro transcription, which would induce unequal sister chromatid recombination and ERC formation (Fig. 7). Unfortunately, we have not been able to detect an effect of the *eaf3* mutation on E-pro transcription (data not shown) because the signal was too low even in the wild-type (Saka et al., 2013).

We note that there is no evidence that the NuA4 complex is located in the rDNA and directly acetylates the histones. Indeed, the NuA4 complex is known to acetylate non-chromatin substrates (Lin et al., 2009; Lu et al., 2011). Therefore, we cannot rule out the possibility that NuA4 affects rDNA stability via another pathway(s).

We tested rDNA stability using PFGE and ERC

assays. In some cases, the results were different in these two assays. For example, using a PFGE assay, we showed that rDNA from the *sir2* single and *sir2 eaf3* double mutants was smeared (Fig. 3). However, results from the ERC assay indicate that the *sir2 eaf3* double mutant has fewer ERCs than the *sir2* single mutant (Fig. 4). In addition, differences among the clones were detected by PFGE (Fig. 5C), although this was not evident using the ERC assay (Fig. 5D). We speculate that PFGE is particularly sensitive to the initial rDNA copy number and that copy number alteration with less ERC production may be induced by the copy number maintenance mechanism (Kobayashi et al., 1998; Iida and Kobayashi, 2019a).

In terms of the relationship between rDNA stability and lifespan, we previously showed that they were correlated using a strain in which E-pro was replaced by an inducible Gal promoter to control expression at this locus (Saka et al., 2013). In this strain, the rDNA was stable when cells were grown on glucose, which represses the Gal promoter. Under these conditions, the lifespan of this strain extended and became comparable to that of the *fob1* mutant, also in a *sir2* defective background. Not only in yeast but also in mammals, rDNA instability has been linked to lifespan. For instance, in cells from patients with premature aging diseases, more non-canonical rDNA repeating units are observed due to mutation of the *E. coli* RecQ homolog, which is involved in DNA repair (Caburet et al., 2005). In aging hematopoietic mouse stem cells, replication stress was found to accumulate in the rDNA region (Flash et al., 2014). In line with these observations that the rDNA is hypersensitive to defects in repair genes or to replication stress, underscoring that the rDNA is one of the most unstable regions in nature, we propose that an accumulation of rDNA damage determines replicative lifespan (Kobayashi, 2008).

Further evidence for this “rDNA theory for aging” is provided by the extended replicative lifespan we observed for the *eaf3* mutant classified in Rank 1 of rDNA-stabilized mutants (see Fig. 6 in Saka et al., 2016). Sen et al. (2015) reported that a deletion of the chromodomain of the Eaf3 subunit shortened the lifespan, although our complete deletion of the gene extended it (Fig. 6). The chromodomain is required for association with H3K36me3 and recruitment of Rpd3S, the histone deacetylase complex. Therefore, we speculate that the chromodomain deletion from Eaf3 would not affect the ability of NuA4 to activate E-pro and shorten lifespan. In contrast, complete deletion of *EAF3* would affect both NuA4 and Rpd3S, and the concomitant decrease in histone acetylation may prevent E-pro activation and thereby extend the lifespan.

In the *fob1 eaf3* double mutant the lifespan was a little shorter than that in the *fob1* mutant despite the finding that the rDNA in these mutants is comparably stable

Fig. 7. Model of rDNA recombination by histone acetylation. A histone deacetylase (HDAC), Sir2, represses E-pro transcription and the NuA4 complex has an opposite effect (histone acetylase, HAT), which regulates rDNA recombination.

(Figs. 4, 6). Although *EAF3* is likely to activate E-pro transcription, and thus to act in the same pathway as *FOB1*, it is known to regulate transcription of other genes and contribute to cell fitness (Reid et al., 2004; Breslow et al., 2008). Therefore, its absence in a *fob1* background can be expected to affect lifespan. This might also be the reason why, despite the observed reduction in ERC accumulation, the lifespan of the *eaf3 sir2* double mutant did not extend in comparison to that of the *sir2* mutant. To reveal the molecular mechanism by which rDNA instability affects lifespan, further analysis will be needed.

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