Loss of Ubp3 increases silencing, decreases unequal recombination in rDNA, and shortens the replicative life span in *Saccharomyces cerevisiae*

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ABSTRACT **Ubp3 is a conserved ubiquitin protease that acts as an antisilencing factor in *MAT* and telomeric regions. Here we show that *ubp3Δ* mutants also display increased silencing in ribosomal DNA (rDNA). Consistent with this, RNA polymerase II occupancy is lower in cells lacking Ubp3 than in wild-type cells in all heterochromatic regions. Moreover, in a *ubp3Δ* mutant, unequal recombination in rDNA is highly suppressed. We present genetic evidence that this effect on rDNA recombination, but not silencing, is entirely dependent on the silencing factor Sir2. Further, *ubp3Δ sir2Δ* mutants age prematurely at the same rate as *sir2Δ* mutants. Thus our data suggest that recombination negatively influences replicative life span more so than silencing. However, in *ubp3Δ* mutants, recombination is not a prerequisite for aging, since cells lacking Ubp3 have a shorter life span than isogenic wild-type cells. We discuss the data in view of different models on how silencing and unequal recombination affect replicative life span and the role of Ubp3 in these processes.**

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INTRODUCTION

In eukaryotes, transcription by RNA polymerase II (RNAPII) is highly influenced by chromatin. In general, eukaryotic chromosomes are organized into transcriptionally active euchromatin and repressed heterochromatin. Placement of nucleosomes—relative binding sites of transcription factors or RNAPII—has a great effect on transcriptional initiation. Thus, simply by impeding access to binding sites of transcription factors, nucleosomes can inhibit transcriptional preinitiation complex formation. In euchromatin, histone acetylation is associated with transcriptional activity, and it has been shown that acetylated histones destabilize their interactions with DNA as well as nucleosomes (Lee *et al.*, 1993; Wang and Hayes, 2008). Even though there are cases in which deacetylation can activate gene transcription (De Nadal *et al.*, 2004), hypoacetylated histones are considered as repressive to transcription. For instance, histone H4 acetylated on lysine 16 (H4K16ac) is found on chromatin throughout the genome except in transcriptionally silent heterochromatin (Suka *et al.*, 2001; Smith *et al.*, 2002).

The domains of chromatin-mediated silencing in *Saccharomyces cerevisiae* are found at the subtelomeric regions, within the ribosomal DNA repeats (rDNAs) and at the cryptic mating-type loci *HMR* and *HML* (Rusche *et al.*, 2003). *HMR* and *HML* comprise genes (**a***1*, **a***2* and α*1*, α*2*, respectively) that encode transcriptional regulators that are controlled by flanking *cis*-acting elements called silencers. Origin recognition complex, Rap1, and Abf1 bind these silencers and initiate formation of silent chromatin by recruiting Sir2, Sir3, and Sir4 (Strahl-Bolsinger *et al.*, 1997). H4K16ac is deacetylated by Sir2, which now, being hypoacetylated, has a higher affinity for the Sir complex. The spreading of Sir complexes propagates through iterative cycles of H4K16 deacetylation by Sir2. Limiting levels of Sir proteins, histone acetylation, and the presence of the histone variant H2A.Z counteract improper spreading of silent chromatin into euchromatin (Suka *et al.*, 2002; Meneghini *et al.*, 2003). A similar mode of action takes place at telomeres, resulting in silent chromatin that gradually decreases with distance from the telomere. In rDNA, the scaffold for Sir2 recruitment is different from telomeres and mating-type loci. Here the nucleolar protein Net1 and the phosphatase Cdc14 recruit Sir2,

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Abbreviations used: ChIP, chromatin immunoprecipitation; RENT, regulator of nucleolar silencing and telephase exit; RLS, replicative life span; TAR, transcription-associated recombination; UPS, ubiquitin–proteasome system.

and together they form the regulator of nucleolar silencing and telophase exit (RENT) complex (Huang and Moazed, 2003). To maintain stringent control of transcription in heterochromatin, all of these silencing factors are required. The importance of maintaining heterochromatin integrity should not be underestimated. For example, in eukaryotes, transcription-associated recombination (TAR) is a fundamental process that is important for DNA integrity. Kobayashi and Ganley (2005) proposed a model of TAR in rDNA in which RNAPII transcription of noncoding RNA (ncRNA) induces recombination, which can lead to accumulation of extrachromosomal DNA and loss of rDNA. In extension, these events may lead to premature aging (Sinclair and Guarente, 1997). Consistent with this, in mutants or cells devoid of any of the RENT subunits, multiple severe phenotypes have been observed, including premature aging, rearrangement and/or loss of genetic material, and disease (Burhans and Weinberger, 2012).

Deubiquitinating enzymes (DUBs) catalyze the reversal of ubiquitination of target proteins. DUBs play a key role in various biological processes, including protein degradation, cell cycle control, stress response, DNA repair, immune response, signal transduction, gene regulation, endocytosis, and vesicle trafficking, and constitute one of the largest classes of proteases and are crucial players in the ubiquitin–proteasome system (UPS). DUBs also process ubiquitin precursors and are responsible for the reversal of ubiquitination to prevent degradation or modify substrate activity (Amerik and Hochstrasser, 2004). In yeast, several ubiquitin-specific processing proteases (UBPs) have been identified. *UBP3*, the yeast homologue of human *USP10*, encodes a 101.9-kDa DUB that, together with its cofactor, Bre5, forms a complex that can regulate 1) anterograde and retrograde transport between endoplasmic reticulum and Golgi (Cohen *et al.*, 2003a,b), 2) DNA repair (Mao and Smerdon, 2010), and 3) protein aggregate clearance (Oling *et al.*, 2014).

Ubp3 has also been shown to participate in and regulate transcription. For instance, Ubp3 has a positive role in activating osmoresponsive genes (Sole *et al.*, 2011) and properly inducing *PHO5* (Kvint *et al.*, 2008). In addition, Ubp3 physically interacts with TFIID (Auty *et al.*, 2004), Tbp1 (Chew *et al.*, 2010), and RNAPII (Kvint *et al.*, 2008). Both Tbp1 and RNAPII can be saved from 26S proteasomal degradation by Ubp3 (Kvint *et al.*, 2008; Chew *et al.*, 2010). Moreover, loss of Ubp3 increases silencing at silent mating-type loci and telomeric DNA regions (Moazed and Johnson, 1996).

In this work we report that Ubp3 acts as an antisilencing factor at all three loci that display silencing in *S. cerevisiae* based on growth assays on selective media using reporter genes. Using chromatin immunoprecipitation (ChIP) assays, we demonstrate that RNAPII occupancy is diminished in heterochromatic DNA in cells lacking Ubp3. The results suggest that the reduction in RNAPII levels residing in rDNA in *ubp3Δ* mutants may be caused by increased Net1 bound to rDNA. Furthermore, in line with previously published data suggesting that the frequency of unequal recombination in rDNA is regulated by RNAPII activity (Kobayashi *et al.*, 2004; Kobayashi and Ganley, 2005), unequal crossover was mitigated in a *ubp3Δ* mutant. Of interest, *ubp3Δ* mutants display shortened replicative life span, raising questions about whether and to what extent increased silencing and lower recombination frequency in rDNA suppress aging.

RESULTS

ubp3Δ mutants are silenced more than wild-type cells in all heterochromatic regions

Moazed and Johnson (1996) demonstrated that in *ubp3Δ* mutants, expression of mRNA-encoding genes at the *MAT* locus (*HML* and *HMR*) and in telomeric regions are negatively regulated. This is consistent with our data showing that *ubp3Δ* mutants carrying an inserted *URA3* allele at *HMR* (Ehrenhofer-Murray *et al.*, 1999) grow poorly on media lacking uracil compared with wild-type cells (Figure 1A). Ubp3 forms a complex with Bre5 (Cohen *et al.*, 2003a), and a number of reports show that cells lacking *BRE5* display an identical phenotype to *ubp3Δ* mutants (Cohen *et al.*, 2003a; Kvint *et al.*, 2008). Therefore we tested whether *BRE5* also had an effect on silencing of a *URA3* gene integrated proximal to telomere VII-L (Gottschling *et al.*, 1990; Kaufman *et al.*, 1997). Indeed, a *bre5Δ* mutant phenocopied cells lacking *UBP3* with regard to silencing of telomeric regions (Figure 1B). Moreover, the *ubp3Δ bre5Δ* double mutant displayed an identical growth pattern to the individual mutants (Figure 1B). The third region that resembles heterochromatin in *S. cerevisiae* is the tandemly repeating rDNA on chromosome XII (Figure 1C). To test whether *UBP3* also has an effect on silencing here, we deleted *UBP3* in a strain carrying a *URA3* gene integrated in rDNA (JS306; Smith and Boeke, 1997). Similar to the effects on telomeric and mating-type loci, cells lacking *UBP3* displayed a near-complete loss of growth on plates lacking uracil (Figure 1D), and this could be reversed if a copy of *UBP3* was introduced on a *CEN* plasmid (Figure 1E), indicating that the increased silencing was specific for *UBP3*. Together these data show that Ubp3 affects gene silencing in a negative way in the heterochromatic regions (of telomeres, rDNA, and *MAT* locus) of *S. cerevisiae*.

RNAPII occupancy in heterochromatic regions is reduced in cells lacking Ubp3

To understand how Ubp3 affects transcription of silenced genes, we measured RNAPII abundance at the promoter and in the coding sequence of a *URA3* gene inserted in rDNA (JS306; Smith and Boeke, 1997) by ChIP assay. Previously, it was shown that heterochromatin influences DNA shearing (Teytelman *et al.*, 2009). Therefore we tested whether our DNA shearing method was sufficient to generate properly sized DNA fragments for the ChIP assay. We used Southern blotting, and the result verified that the technique we used (see *Materials and Methods* and Supplemental Materials and Methods) was adequate (Supplemental Figure S1). ChIP analysis showed a clear reduction in RNAPII occupancy both at the *URA3* promoter and in the open reading frame in the *ubp3Δ* mutant compared with an isogenic wild-type strain (Figure 2A). Similarly, reduced levels of RNAPII were detected in the telomeric region (Figure 2B) and *HML* (Figure 2C). Moreover, RNAPII occurrence outside of the *URA3* locus proximal to the telomere (i.e., in the truncated, promoter-less *ADH4* gene) was also reduced in *ubp3Δ* cells compared with the wild-type strain (Figure 2B), indicating that not only are potentially transcribed DNA or promoter regions affected by Ubp3, but heterochromatin per se is less accessible to RNAPII. Indeed, when we examined the distribution of RNAPII at the right arm of chromosome VI, we found that in *ubp3Δ* mutants, there was less RNAPII present than for wild-type cells (Figure 2D). This was also true in rDNA. In fact, RNAPII levels are quite scarce across the entire rDNA region in *ubp3Δ* mutants compared with wild-type cells (Figure 2E). Accordingly, measures of ncRNA levels expressed from the E-pro promoter by reverse transcription PCR (RT-PCR) using strand-specific primers showed a clear reduction in a *ubp3Δ* mutant (Supplemental Figure S2A). PCR coamplifications with oligonucleotide pairs for *ACT1* and *CUP1* were used for normalizations. In addition, RT-PCR measures of expression of the *mURA3* gene showed similar results (Supplemental Figure S2A). Overall our results suggest that in *ubp3Δ* mutants, increased silencing is caused by a reduction in RNAPII occupancy in heterochromatic

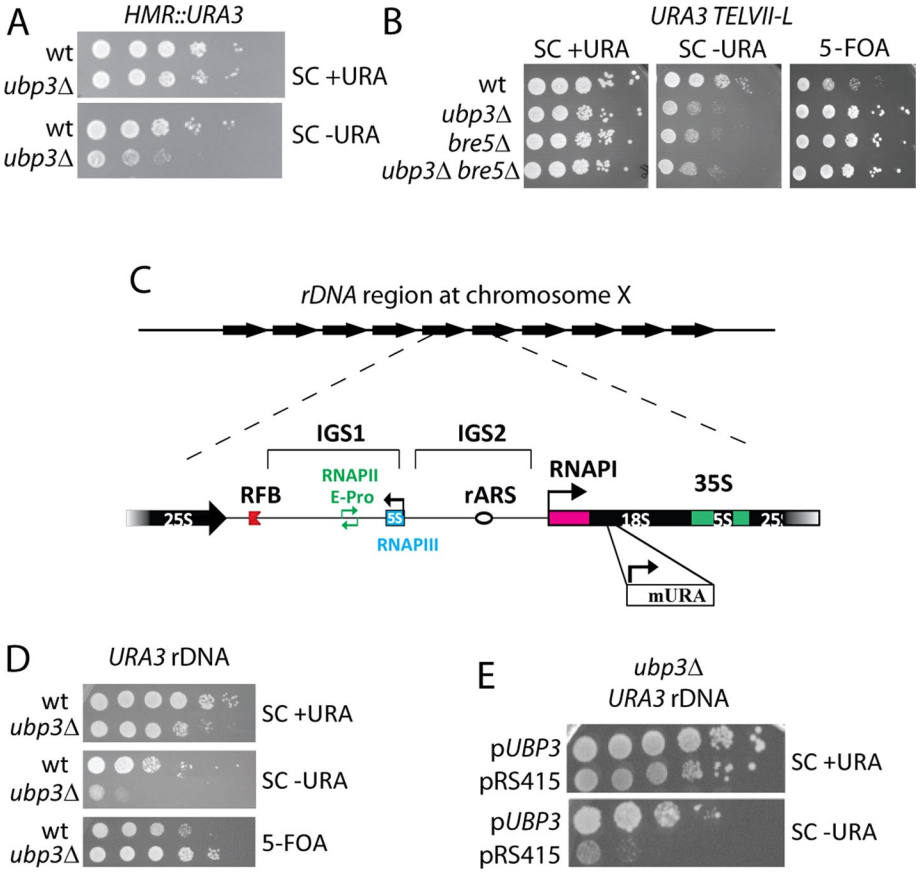

FIGURE 1: Ubp3 antagonizes silencing in telomeric regions, mating-type loci, and rDNA. (A) Ubp3 suppresses silencing at *HMR*. Silencing was measured by monitoring growth of 10-fold serial dilutions of cells plated on SC medium with and without uracil. (B) Ubp3 and Bre5 suppress silencing. Silencing was measured by monitoring growth of 10-fold serial dilutions of cells plated on medium lacking uracil or containing 5-flouroorotic acid (5-FOA). SC medium was used as a plating control. (C) Physical structure of the tandemly repeating rDNA. Bottom, a single unit depicted in detail. Each unit contains an RNAPI-transcribed 35S precursor rRNA and a divergently RNAPIII-transcribed 5S rRNA (blue). Each unit is divided by a nontranscribed unit (NTS), which is divided in two (NTS1 and NTS2, respectively) by the 5S gene. Also shown is the RFB, ◤, the autonomously replicating sequence (rARS, ⊃), and the bidirectional E-pro promoter (green). The site of insertion of the silencing reporter is also shown (m*URA*; JS306). (D) Spot test of JS306 cells (wild type and *ubp3Δ* mutants) on SC plates with or without uracil. (E) Growth on SC medium with or without uracil of *ubp3Δ* mutants carrying an empty vector or a plasmid containing *UBP3*.

regions. Note that overall levels of RNAPII in whole-cell extracts are not different between wild-type cells and *ubp3Δ* mutants (Kvint *et al.*, 2008), that RNAPII was equally well immunoprecipitated from wild-type and *ubp3Δ* mutant ChIP extracts (Supplemental Figure S2B), and that RNAPII occupancy in the actively transcribed gene *ACT1* did not differ between wild-type cells and *ubp3Δ* mutants (Supplemental Figure S2C), suggesting that no obstructive factors influenced the immunoprecipitation differentially (between wild-type and *ubp3Δ* cells) and that Ubp3 predominantly affects RNAPII occupancy in heterochromatin.

Sir2 distribution is altered in heterochromatic regions in cells devoid of *UBP3*

Because *ubp3Δ* mutants consistently display reduced levels of RNAPII attached to rDNA, telomeric regions, and *MAT*, we examined whether Sir protein levels were also affected, given that Sir proteins are refractory to transcription. At *MAT* and telomeres, Sir3/Sir4 recruits Sir2, whereas in rDNA, Sir2 is recruited by the RENT complex (Strahl-Bolsinger *et al.*, 1997; Huang and Moazed, 2003). Sir proteins were monitored by ChIP using tandem affinity purification (TAP)–tagged strains (Sir2-TAP and Sir3-TAP). At the right arm of chromosome VI, Sir2 and Sir3 levels were slightly higher in *ubp3Δ* mutants than with wild type (Figure 3, A and B), whereas at *HML* there was approximately two times as much Sir2 and Sir3 in the *ubp3Δ* mutant (Figure 3, C and D). Next we examined rDNA. In rDNA, not Sir3 but the RENT complex recruits Sir2 (Huang and Moazed, 2003), and thus, as expected, we detected very low levels of Sir3 here in both wild type and *ubp3Δ* (Figure 3D). Sir2 was previously shown to support transcriptional silencing in rDNA (Fritze *et al.*, 1997). However, we detected, if anything, less Sir2 protein at rDNA in the *ubp3Δ* mutant than with wild-type (Figure 3E). Of importance, there was no difference in total levels of Sir2 or Sir3 between wild-type cells and *ubp3Δ* mutants, as measured by Western blotting of whole-cell extracts (Supplemental Figure S3).

To test the epistasis between *UBP3* and *SIR2*, we measured RNAPII occupancy in rDNA in *sir2Δ* mutants by ChIP. Consistent with previous reports, the levels of RNAPII cross-linked to rDNA in *sir2Δ* mutants were higher than with wild-type cells (Figure 3F). Of interest, in the *ubp3Δ sir2Δ* double mutant, RNAPII occupancy was lower than in a *sir2Δ* single mutant (Figure 3F), and, accordingly, *ubp3Δ sir2Δ* double mutants grew poorly on media lacking uracil (Supplemental Figure S4A). As expected and previously shown (Fritze *et al.*, 1997), a *sir2Δ* mutant lost its capacity to silence the reporter gene in rDNA (Supplemental Figure S4B). This suggests that Ubp3 partly functions in a Sir2-independent pathway in allowing RNAPII access to rDNA.

In addition to blocking access to DNA by RNAPII, Sir2 has also been proposed to hamper the transcriptional activity of RNAPII by deacetylating lysine 16 of histone 4 (H4K16) in heterochromatin (Cesarini *et al.*, 2012). Hence we tested whether there were any differences in H4K16 acetylation (H4K16Ac) in these regions (Supplemental Figure S5). The levels of H4K16Ac did not differ significantly between wild type and *ubp3Δ* mutants at the right arm of chromosome VI. When *SIR2* was knocked out, however, H4K16Ac levels increased as expected in both wild-type and *ubp3Δ* mutant cells (Supplemental Figure S5A). Conversely, at *HML*, the degree of H4K16 acetylation was drastically reduced in a *ubp3Δ* mutant compared with a wild-type strain (Supplemental Figure S5B). The ratio of H4K16Ac/H4 in the *ubp3Δ* mutant was ~30% of wild-type levels, whereas in the *sir2Δ* mutant the ratio was significantly higher than in wild-type cells (Supplemental Figure S5B). In rDNA there was no difference in H4K16Ac levels between wild-type and *ubp3Δ* mutants, but levels were high in cells devoid of *SIR2*, as expected (Supplemental Figure S5C). Thus the increase in silencing seen in *ubp3Δ* mutants is likely mediated by the effects on Sir2

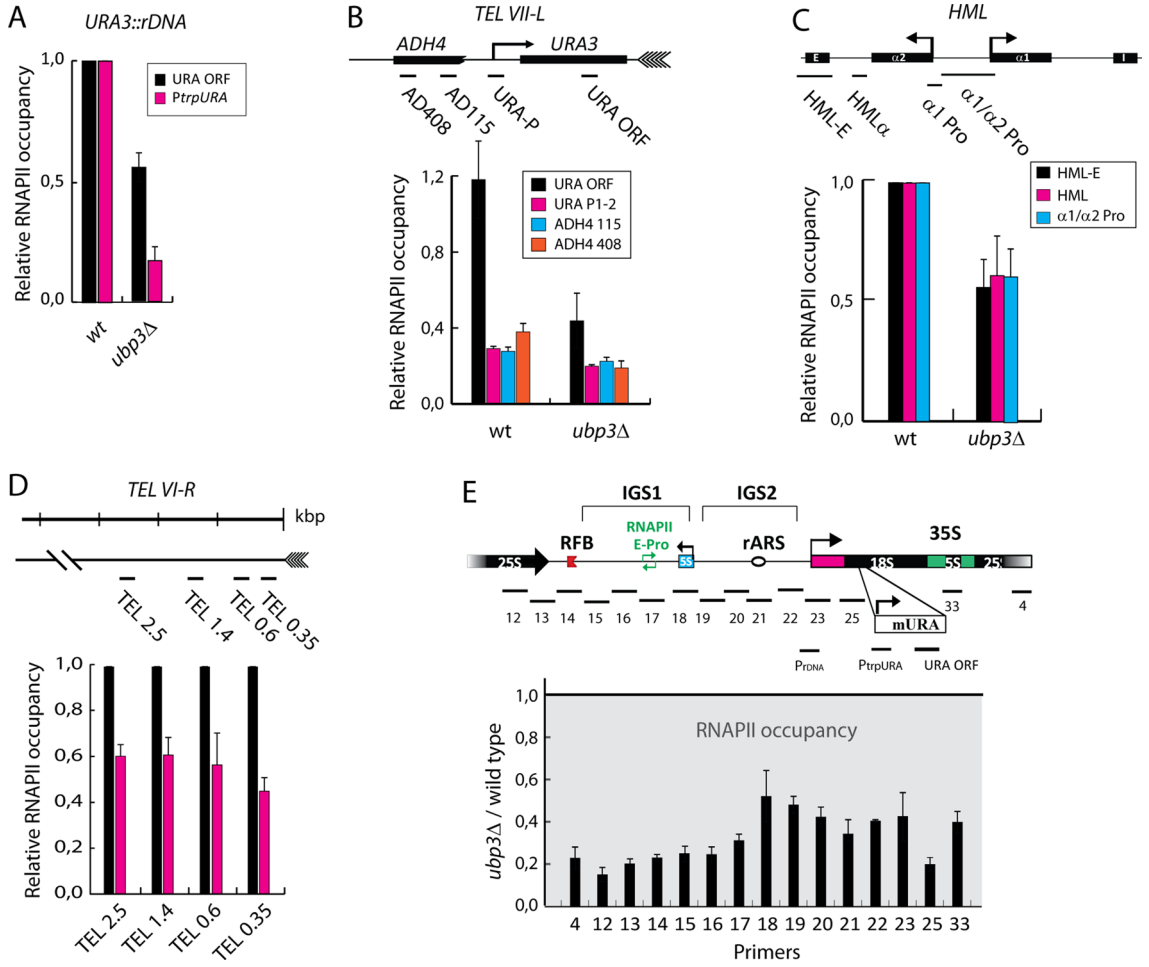

FIGURE 2: RNAPII occupancy in cells lacking Ubp3 is lower than in wild type cells. (A) Relative RNAPII occupancy in the promoter (P_{trp}*URA*) and the open reading frame (ORF) of the *mURA* construct in wild type and *ubp3Δ*. RNAPII levels in wild type are set to 1. (B) Schematic diagram of the chromosomal location of a *URA3* gene integrated at the telomeric region of chromosome VII (Gottschling *et al.*, 1990). Relative RNAPII levels detected in the promoter and the ORF of the *URA3* gene integrated at the telomeric region of chromosome VII as depicted in wild type and *ubp3Δ*. (C) Schematic diagram of the *HML* locus at chromosome III. Detected levels of RNAPII at different positions at *HML*. Wild-type levels are set to 1. (D) Physical structure of the right end of chromosome VI (*TEL VI-R*). Relative RNAPII occupancy at *TEL VI-R* region in the *ubp3Δ* mutant and wild type. Wild-type levels are set to 1. (E) Physical structure of a single unit of tandemly repeating rDNA. RNAPII levels across an rDNA repeat in a *ubp3Δ* mutant (black bars) and a wild-type strain (gray area topped with black line). RNAPII levels in wild type are set to 1. Values are calculated as (RNAPII/input)/($RNAPII_{Cup1}$/$input_{Cup1}$). PCR products analyzed in the ChIP assays are depicted below each physical structure presented (B, C, and E). The numbered PCR products in E are the same as in Huang and Moazed (2003). Data are represented as the mean (± SD) of at least two experiments.

levels and H4K16 acetylation in *HML* but not in telomeric regions or rDNA.

Net1 and Fob1 levels at rDNA are altered in *ubp3Δ* mutants

In rDNA, Fob1 facilitates Net1/Cdc14 (RENT) binding, which in turn recruits Sir2 (Straight *et al.*, 1999). Therefore we next investigated the abundance of the RENT complex in rDNA by ChIP using C-terminally Myc-tagged Net1. In agreement with previous findings (Huang and Moazed, 2003), we observed a pattern with two peaks (NTS1 and NTS2) of intense Net1 occupancy (Figure 4A). In *ubp3Δ* mutants, we discovered that Net1 levels in rDNA were higher than in wild-type cells (Figure 4A). This increase was also observed when Net1 was TAP tagged (unpublished data). Next we measured Fob1 levels bound to rDNA. As previously reported (Huang and Moazed, 2003; Ha *et al.*, 2012), Fob1 amassed around the replication fork barrier (RFB) in NTS1 (Figure 4B). In *ubp3Δ* mutants this accumulation was somewhat enhanced (Figure 4B). The elevated levels of Net1 (and Fob1) in rDNA could thus be a reason for the reduced RNAPII presence/activity in cells devoid of *UBP3*.

Ubp3 suppresses unequal recombination in rDNA in a Sir2-dependent manner

It was reported that transcription by RNAPII from the E-pro promoter in the nontranscribed spacer in rDNA (Figure 1C) has a positive effect on unequal recombination between sister chromatids (Kobayashi *et al.*, 2004; Kobayashi and Ganley, 2005). Consequently, in cells lacking Sir2, hyperrecombination in rDNA occurs (Kaeberlein *et al.*, 1999), and ncRNA expression from the E-pro promoter is elevated (Cesarini *et al.*, 2012). In contrast, *FOB1* is required for recombination in rDNA (Kobayashi and Horiuchi,

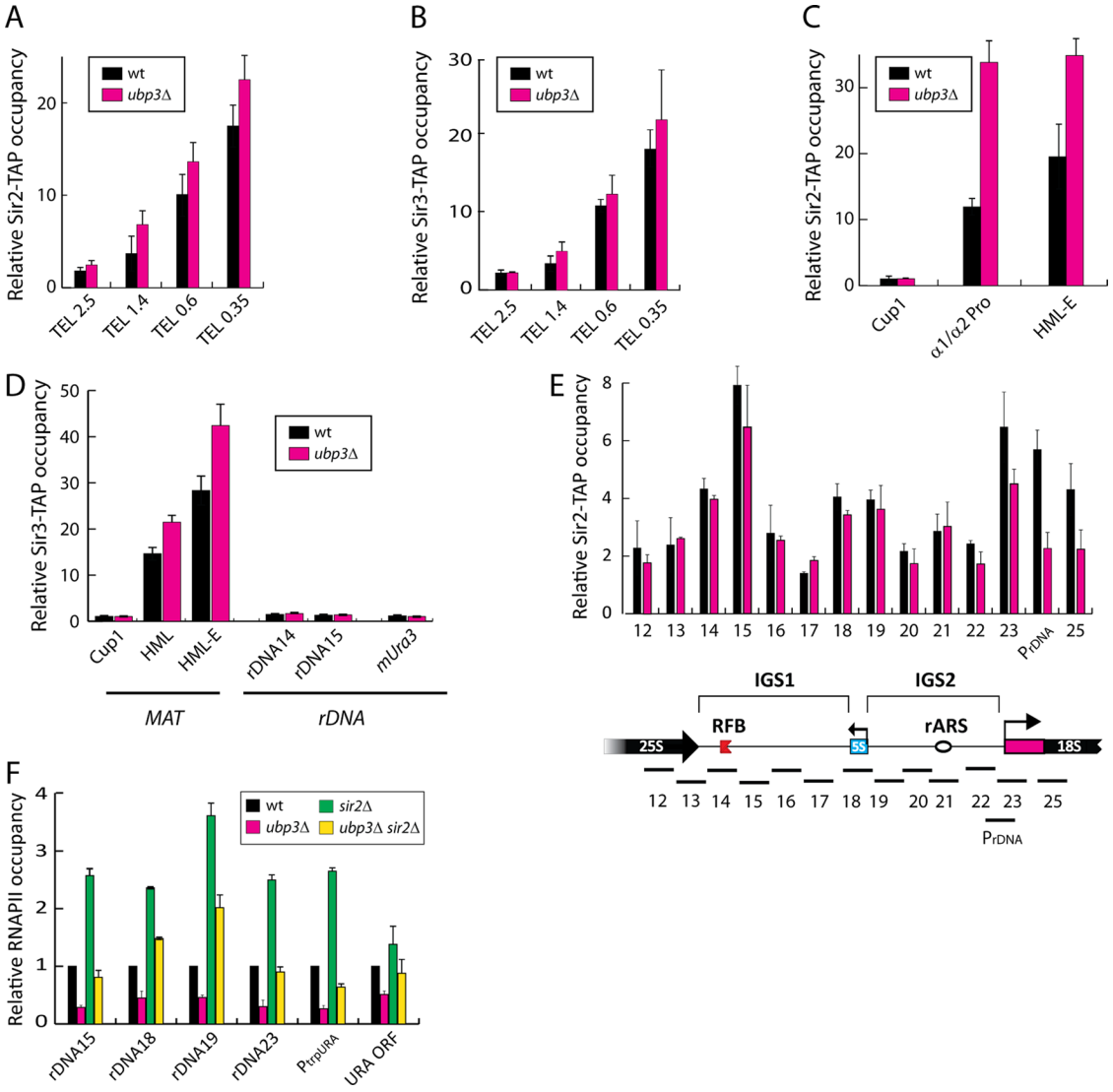

FIGURE 3: Sir2 and Sir3 occupancies in wild-type and *ubp3Δ* mutants. (A) Relative Sir2-TAP occupancy at indicated places of the right end of chromosome VI (Figure 2D) in the *ubp3Δ* mutant and wild-type cells. (B) Relative Sir3-myc occupancy at the indicated places of the right end of chromosome VI in the *ubp3Δ* mutant and wild-type cells. (C) Representative graphs showing relative Sir2-TAP occupancy at the indicated places in the *HML* locus (Figure 2C) in the *ubp3Δ* mutant and wild-type cells, respectively. (D) Relative Sir3-myc occupancy at the indicated places in the *HML* locus and rDNA (Figure 2, C and E). (E) Relative Sir2-TAP levels across an rDNA repeat in a wild-type strain and *ubp3Δ* mutant. Physical structure of a single unit of the tandemly repeating rDNA, with PCR products analyzed in the ChIP assay indicated as horizontal bars below. (F) RNAPII levels across an rDNA repeat in wild type (set to 1) and *ubp3Δ* , *sir2Δ*, and *ubp3Δ sir2Δ* mutants. Values are calculated as (Sir2-TAP or Sir3-myc/input$_{Cup1}$) divided by (Sir2-TAP or Sir3-myc $_{Cup1}$/input$_{Cup1}$) or (RNAPII/input)/(RNAPII$_{Cup1}$/input$_{Cup1}$). Data are represented as the mean (± SD) of at least three experiments.

1996). However, Fob1 appears to counteract its own recombination activity by recruiting Net1 and Sir2 to RFB, which promotes silencing and rDNA stability. Therefore we tested whether a strain lacking *UBP3* had an altered frequency of unequal crossover as assayed by marker loss using a strain (JD83) with an *ADE2* gene inserted in rDNA (Kaeberlein *et al.*, 1999). As predicted, due to the increased silencing and reduced expression of ncRNA (from E-pro), *ubp3Δ* mutants displayed significantly decreased recombination in rDNA (Figure 5). In addition, the rDNA copy number is not affected in cells lacking Ubp3 and thus this cannot be the cause of the lower recombination rate (Supplemental Figure S6). The reduced recombination frequency could be reversed if a copy of *UBP3* was introduced on a *CEN* plasmid (Figure 5), indicating that the reduced recombination frequency was specific for *UBP3*. However, in cells devoid of both Sir2 and Ubp3 the recombination frequency was quite similar to that for a *sir2Δ* single mutant (Figure 5). Thus *SIR2* is epistatic to *UBP3* with regard to unequal recombination in rDNA. In summary, our results suggest that frequency of recombination is not necessarily directly proportional to RNAPII transcription in rDNA (Figure 5 and Supplemental Figure S2A). It is worth noting that often in the analysis of this type of screen it is not entirely clear which colonies are exactly half red/half white. Frequently, the percentage of red to white does not match the expected theoretical outcome (i.e., 50/50, 25/75, etc.). Sometimes

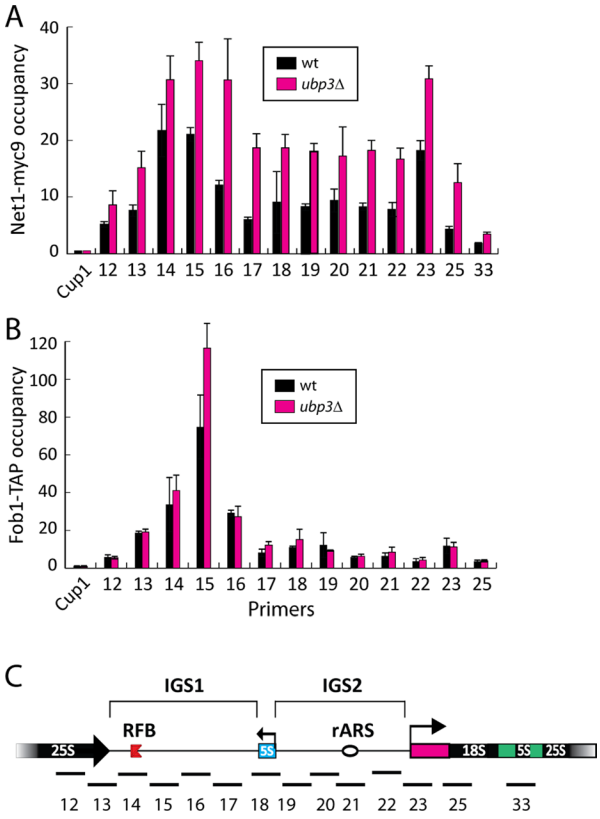

FIGURE 4: Net1 and Fob1 are abundant at rDNA in *ubp3Δ* mutants. (A) Net1-myc9 occupancy across an rDNA repeat in wild-type strain and a *ubp3Δ* mutant. (B) Fob1-TAP occupancy across an rDNA repeat in a wild-type strain and a *ubp3Δ* mutant. (C) Physical structure of a unit of the tandemly repeating rDNA. PCR products analyzed in the ChIP assay are indicated as bars below. Values are calculated as (Net1-myc or Fob1-TAP/input)/(Net1-myc or Fob1-TAP$_{Cup1}$/input$_{Cup1}$). Data are represented as the mean (± SD) of at least three experiments.

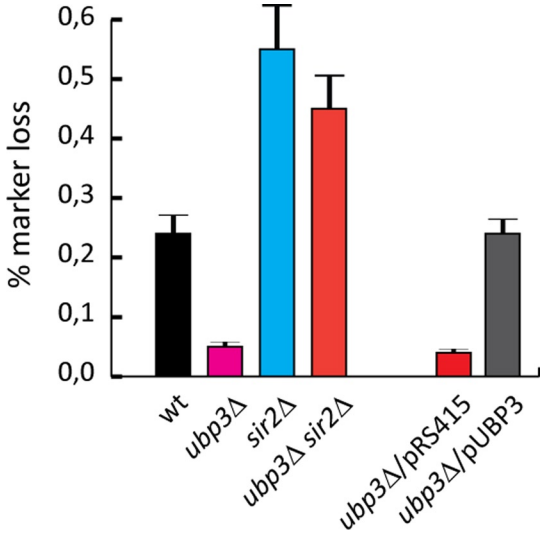

FIGURE 5: *ubp3Δ* suppresses unequal recombination in rDNA in a Sir2-dependent manner. Unequal recombination is represented as percentage marker loss, calculated as the ratio of half-sectored colonies to the total number of colonies, excluding entirely red colonies. See *Materials and Methods* for further details.

colonies have a mosaic pattern, or even 40% white/60% red sectors or vice versa. In the literature, however, the overall trend is obvious, in that there is a significant difference between wild-type and *sir2Δ* mutant strains, but the degree of variance can differ.

ubp3Δ mutants are short lived compared with wild-type cells

Numerous studies on rDNA integrity report a correlation between disrupted silencing, increased recombination, and shortened replicative life span (RLS). For instance, *sir2Δ* mutants have a significantly shorter life span than wild-type cells, whereas *fob1Δ* mutants have an extended life span. *FOB1* is partly epistatic to *SIR2* for life span, since a *sir2Δ fob1Δ* double mutant has a life span similar to that of wild-type cells (Defossez *et al.*, 1999). In addition, in *sir2Δ fob1Δ* double mutants, recombination frequencies are similar to those in wild-type cells, whereas in a *fob1Δ* single mutant, recombination is low (Kobayashi and Horiuchi, 1996; Smith *et al.*, 2009). To test how Ubp3 affects replicative life span, we tested our *ubp3Δ* mutant strains. Surprisingly, the augmented silencing and the decreased recombination frequency taking place in *ubp3Δ* mutants did not cause an extended replicative life span. Instead, the life span of *ubp3Δ* mutants was ~70% of that of wild-type cells (Figure 6A). Moreover, no difference in life span was observed when a *ubp3Δ sir2Δ* double mutant was compared with a *sir2Δ* single mutant (Figure 6A). Thus *SIR2* is epistatic to *UBP3* in determining RLS.

Next we measured RLS in cells devoid of *FOB1*. As expected, cells lacking *FOB1* had an extended life span compared with wild-type cells (Figure 6B). When *UBP3* was deleted in a *fob1Δ* mutant, RLS approached that of wild-type cells. Thus it is likely that reduced transcriptional activity and diminished recombination frequency, as are phenotypes of cells devoid of *UBP3* or *FOB1*, are not a prerequisite for extended life span. Instead, one interpretation is that Ubp3 takes part in another process affecting RLS that overrides the beneficial effects that silencing and recombination in rDNA have on RLS.

In aging cells, gradual decline in protein quality control and accumulation of protein aggregates occur (Heeren *et al.*, 2004; Erjavec *et al.*, 2007). Ubp3 forms a complex with Bre5 (Cohen *et al.*, 2003a), and a number of reports shown that *bre5Δ* and *ubp3Δ* mutants display similar phenotypes, such as deubiquitinating Sec23 (a COPII subunit; Cohen *et al.*, 2003a) and 6- azauracil sensitivity (Kvint *et al.*, 2008). Furthermore, *ubp3Δ* and *bre5Δ* mutants have identical phenotypes regarding clearance of protein aggregates (Oling *et al.*, 2014). Therefore we tested RLS in cells devoid of *BRE5* and found that they also have a shortened life span compared with wild-type cells (Supplemental Figure S7).

DISCUSSION

In this study we elucidated how silencing is altered in cells lacking Ubp3. We confirmed, as described by Moazed and Johnson (1996), that *ubp3Δ* mutants display more silencing at the *MAT* loci and in telomeric regions. In addition, we found that cells lacking *UBP3* are also more silenced in rDNA. There are several hypotheses explaining how and why heterochromatin is silent. One idea is that the Sir complexes (or RENT complexes at rDNA) and hypoacetylated histones sterically hinder access to specific DNA sequences recognized by transcription factors (e.g., RNAPII). However, there are several sequence-specific factors that are permitted access, such as homologous or site-specific recombination enzymes and retrotransposon integration factors (Holmes and Broach, 1996; Zou *et al.*, 1996). In *ubp3Δ* mutants, the levels of H4K16Ac and Sir2 associated with heterochromatin were altered differentially in different heterochromatic

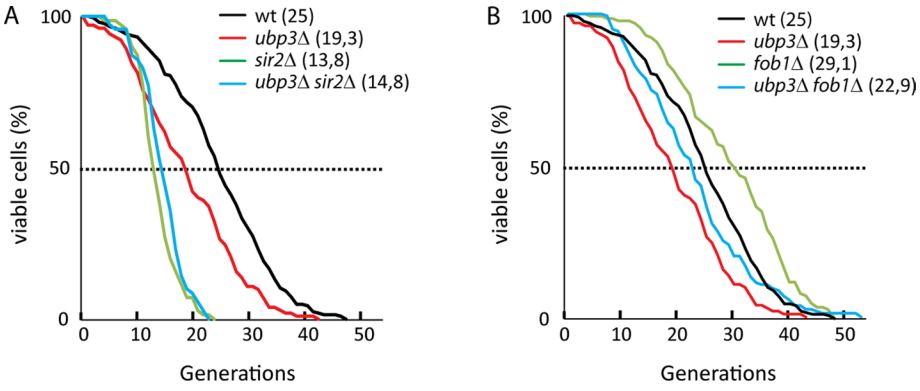

FIGURE 6: Ubp3 is required for longevity. (A) Replicative life span of wild type (black, $n = 204$) and *ubp3Δ* (red, $n = 100$), *sir2Δ* (green, $n = 70$), and *ubp3Δ sir2Δ* (blue, $n = 70$) mutants. (B) Replicative life span of wild type (black, $n = 204$) and *ubp3Δ* (red, $n = 100$), *fob1Δ* (green, $n = 106$), and *ubp3Δ fob1Δ* (blue, $n = 119$) mutants. Average life spans are shown in brackets.

regions. At *HML* the ratio of H4K16Ac/H4 was much lower in cells devoid of *UBP3* than with wild-type cells. Similarly, Sir2 levels at *HML* were almost doubled in the *ubp3Δ* mutant. Thus in *HML* the elevated levels of Sir2 and deacetylated H4K16 may indeed hinder transcription factor access (i.e., RNAPII, and more so in cells lacking Ubp3). In contrast, in rDNA and in telomeric regions, the H4K16Ac/H4 ratio was not significantly affected, whereas Sir2 levels were slightly lower (in the *ubp3Δ* mutant) or largely unchanged, respectively. Thus Sir2 and/or hypoacetylated H4K16 cannot explain the increased silencing in *ubp3Δ* mutants at telomeres and in rDNA.

In a study on heterochromatin, it was shown that general transcription factors TATA-binding protein (TBP) and RNAPII assembled at silent promoters in *HMR* but that no initiation ensued, suggesting that Sir-generated heterochromatin suppresses transcription at a subsequent step (Sekinger and Gross, 2001). Later this model was reinforced when the same lab showed that TFIIH and the Ser5-phosphorylated isoform of RNAPII could be detected at Sir-mediated silent promoters, suggesting that RNAPII was poised to start transcription (Gao and Gross, 2008). However, another lab published data showing that none of TFIIB, TFIIE, or RNAPII occupy silenced promoters; only an activator (Ppr1) was able to associate at the promoters (Chen and Widom, 2005). Thus how silent chromatin inhibits RNAPII-dependent transcription is obscure in many regards. With this in mind, note that many reports and different labs use different strain backgrounds in their studies on gene silencing, which could potentially lead to indistinct results. In this study two different strain backgrounds (BY strains and W303) were used (see Supplemental Table S1), and the results did match.

In this work we present data that match those of Gross and coworkers (Sekinger and Gross, 2001; Gao and Gross, 2008). We find experimental support for their model and that it is applicable to all silent loci in *S. cerevisiae*. Essentially, increased silencing in *ubp3Δ* mutants is likely to be caused by lower levels of RNAPII in these heterochromatic regions, suggesting that RNAPII is indeed active in wild-type cells in these regions. In addition, Sir3 and RENT occupancy is generally higher in *ubp3Δ* mutants in *MAT* and rDNA, respectively, suggesting that silent chromatin is not saturated completely with silencing factors in wild-type cells. Thus we propose that RNAPII is in fact present and active in heterochromatic DNA in wild-type cells and that this is dependent on Ubp3 (Figures 1, A, C, and E, and 2, A–E). Whereas the effect on silencing by Ubp3 in *MAT* and rDNA may be explained by elevated levels of silencing factors (i.e., Sir3 and Net1), in telomeric regions our data are not sufficient for any interpretations. However, it is possible that a countered RNAPII (i.e., such as an RNAPII trying to transcribe in dense heterochromatin in telomeric regions) is likely to be ubiquitinated and thus prematurely terminated, as previously suggested (Kvint *et al.*, 2008). Although these data demonstrate that in wild-type cells RNAPII can assemble and engage in transcription at *MAT*, telomeric regions, and rDNA, we also found that Ubp3 is required for nonequal recombination in rDNA in a Sir2-dependent manner, whereas *sir2Δ* mutants are dependent on *UBP3* for full derepression of a *URA3* allele in rDNA. This contrasts with the observation that recombination is directly proportional to the levels of transcriptional activity in the nontranscribed spacers in rDNA, as was previously proposed (Kobayashi *et al.*, 2004; Kobayashi and Ganley, 2005). However, Sir2 may have a second function in inhibiting recombination that is partly independent of its role in silencing.

Replicative aging has been associated with loss of silencing and increased nonequal recombination in rDNA and vice versa (Burhans and Weinberger, 2007). Of interest, our data demonstrate that *ubp3Δ* mutants age faster than wild-type cells despite increased silencing and reduced recombination in rDNA. One possible explanation for this could be that *ubp3Δ* mutants have delayed clearance of protein aggregates and premature accumulation of Hsp70 aggregates during aging (Oling *et al.*, 2014). Proper clearance of protein aggregates is a requirement to combat premature aging (Heeren *et al.*, 2004; Erjavec *et al.*, 2007; Kruegel *et al.*, 2011). Furthermore, deletion of *FOB1* did not fully suppress the reduced life span of a *ubp3Δ* mutant. This further implies that Ubp3 has another function central for sustaining longevity that bypasses the potential benefits that increased silencing and reduced recombination have on senescence (e.g., corrupt protein quality control). It also suggests either that loss of *FOB1* further decreases recombination in *ubp3Δ* mutants (potentially causing RLS extension) or that Fob1 also affects life span extension via a pathway other than rDNA recombination.

Cells devoid of Sir2 have a shortened life span (Kaeberlein *et al.*, 1999). The replicative life span of a *ubp3Δ sir2Δ* double mutant is similar to that of a *sir2Δ* single mutant. Thus, in cells lacking both *UBP3* and *SIR2*, life span correlates with higher recombination frequency. In contrast, the life span of cells devoid of *FOB1* and *SIR2* is longer than that in a *sir2Δ* mutant but shorter than that in a *fob1Δ* mutant. However, it has been proposed that this is due to Sir2 having other functions important for longevity that cannot be compensated for by a deletion of *FOB1*. For example, Sir2 is required for asymmetric segregation of aberrant proteins during mitosis (Aguilaniu *et al.*, 2003) and has been linked to defense against reactive oxidative species (ROS; Erjavec *et al.*, 2007), both of which have been connected to aging. In addition, as mentioned, *UBP3* and *BRE5* have been implicated in protein quality control, and thus impairment of this process by loss of these genes could have a negative effect on RLS (Oling *et al.*, 2014). Similar to *ubp3Δ* mutants, we found that cells lacking Bre5 age faster than wild-type cells (Supplemental Figure S7), although large-scale analysis of single-gene deletions found that *bre5Δ* cells had increased life span (Kaeberlein *et al.*, 2005), a discrepancy we cannot explain. Taken together, the effect on life span by the loss of both *UBP3* and *SIR2* may be due to

a combination of rDNA recombination defects, deficiency in dealing with ROS, and insufficient protein quality control.

MATERIALS AND METHODS

Yeast strains and procedures

Strains used in this work are derived from S288C and W303 genetic backgrounds (Supplemental Table S1). *S. cerevisiae* strains were grown and manipulated by using standard techniques (Sherman, 1991). Deletion mutants were constructed either by PCR-mediated knockout or sporulation. Transformants and dissected spores were verified by PCR. For growth in rich media, strains were grown in yeast extract/peptone/dextrose (YPD) with 2% glucose. Cells grown in synthetic defined medium were grown with yeast nitrogen base plus ammonium sulfate and 2% glucose.

ChIP

ChIP assays were performed essentially as previously described (Kristjuhan *et al.*, 2002). Briefly, cultures were cross-linked with 1% formaldehyde for 15 min at room temperature. Cross-linking was quenched with 200 mM glycine for 5 min. Cells were pelleted by centrifugation at 4500 rpm for 4 min at 4°C and then washed once with phosphate-buffered saline and then resuspended in FA lysis buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na deoxycholate, 1× protease inhibitors). After disrupting cells with glass beads, the chromatin was sonicated using the Biorupter UCD-200, Diagenode system (power, high; time, 30 s on/30 s off for 15 min) to yield DNA fragments of ~100–700 base pairs. The resulting extract was centrifuged twice at 14,000 rpm to remove debris. The immunoprecipitate (extract, protein G [or A] beads and antibodies) was washed at 4°C after incubation (>4 h) as follows: 1 × 10 min in FA lysis buffer, 3 × 10 min in FA500 (FA lysis with 500 mM NaCl), 3 × 10 min in ChIP wash buffer (10 mM Tris, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Na deoxycholate, 1 mM EDTA), and twice in TES (10 mM Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl). The precipitate was eluted in 100 µl of elution buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS) at 30°C. DNA–protein cross-links were reversed by incubating at 65°C for 6 h, and the DNA was isolated using a PCR Purification Kit (Qiagen, Valencia, CA). 4H8 (anti-Rpb1 antibody) was from Upstate Biotechnology (Lake Placid, NY), and the H3C antibody was a gift from Claes Gustafsson (University of Gothenburg). Immunoglobulin G Sepharose 6 Fast Flow from GE Healthcare (Piscataway, NJ) was used to immunoprecipitate (IP) TAP-tagged proteins. Coprecipitated DNA was analyzed in triplicate by quantitative PCR using SYBR green and the ABI Prism 7000 Sequence detection system (Applied Biosystems, Foster City, CA) or a Bio-Rad Q5 system (Bio-Rad, Hercules, CA). Primer sequences are available upon request. To exclude detection from the *ura3-1* allele in PKY090, a specific primer for *URA3* was used (Supplemental Figure S8). In general, values were normalized to inputs, and as an internal control, the *CUP1* gene was used unless otherwise stated. Relative fold enrichment was determined by calculating the ratio (IP/input)/($CUP1_{IP}$/$CUP1_{input}$) unless otherwise stated. All values expressed in bar graphs are results from at least two independent ChIP experiments and three independent PCR assays.

rDNA instability assay

The marker loss assay was performed as previously described (Kaeberlein *et al.*, 1999). A strain (JD83) with a single *ADE2* marker gene inserted in one rDNA copy was used (Kaeberlein *et al.*, 1999). Strains were grown to mid log phase in YPD medium, diluted, and plated onto synthetic complete (SC) solid medium (250–350 cells/plate). Colonies were grown at 30°C for 3 d and then transferred to 4°C for 4 d, at which point half-sector (red/white) colony formation was detected visually. Experiments were performed at least three times per strain. At least 30,000 colonies were scored per strain. The unequal sister chromatin crossover rate was calculated by dividing the number of half-red/half-white colonies by the total number of colonies (excluding fully red colonies).

Replicative life span assay

A micromanipulator (Singer Instruments, Watchet, United Kingdom) was used to measure replicative life span (Kaeberlein *et al.*, 1999). Briefly, cells were grown overnight in YPD, diluted, plated, and allowed to recover on YPD plates before being assayed for RLS, which was performed independently at least twice for each strain.

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