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.

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 (α1, a2 and α1, α2, 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 telomerases and mating-type loci. Here the nucleolar protein Net1 and the phosphatase Cdc14 recruit Sir2,
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 proteasome 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 osmorepressive 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 RNAPIII were detected in the telomeric region (Figure 2B) and HML (Figure 2C). Moreover, RNAPII occupancy 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
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 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

UBP3

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-fluoroorotic acid (5-FOA). SC medium was used as a 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, a), and the bidirectional E-pro promoter (green). The site of insertion of the silencing reporter is also shown (mURA; 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.
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,
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.).

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
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 Horiiuchi, 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
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 telomeress 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 isofrom 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 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.

Relevance 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 ( Kaebelerlein et al. , 1999 ). The replicative life span of a 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 FO81 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

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**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.

- **Panel A:**
  - **wt (25)**
  - **ubp3Δ (19,3)**
  - **sir2Δ (13,8)**
  - **ubp3Δ sir2Δ (14,8)**

- **Panel B:**
  - **wt (25)**
  - **ubp3Δ (19,3)**
  - **fob1Δ (29,1)**
  - **ubp3Δ fob1Δ (22,9)**

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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, 1x 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 buffer 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). Primer sequences are provided in Supplemental Table S2. 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 using a Bio-Rad Q5 system (Bio-Rad, Hercules, CA). Primer sequences are provided in Supplemental Table S2.

ChIP-seq
ChIP-seq 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, 1x 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 buffer 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). Primer sequences are provided in Supplemental Table S2. 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 using a Bio-Rad Q5 system (Bio-Rad, Hercules, CA). Primer sequences are provided in Supplemental Table S2.

rDNA instability assay
The marker loss assay was performed as previously described (Kaeberlein et al., 1999). A strain (JDB3) 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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