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Cellular aging is associated with increased ubiquitylation of histone H2B in yeast telomeric heterochromatin

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ABSTRACT

Epigenetic changes in chromatin state are associated with aging. Notably, two histone modifications have recently been implicated in lifespan regulation, namely acetylation at H4 lysine 16 in yeast and methylation at H3 lysine 4 (H3K4) in nematodes. However, less is known about other histone modifications. Here, we report that cellular aging is associated with increased ubiquitylation of histone H2B in yeast telomeric heterochromatin. An increase in ubiquitylation at histone H2B lysine 123 and methylations at both H3K4 and H3 lysine 79 (H3K79) was observed at the telomere-proximal regions of replicatively aged cells, coincident with decreased Sir2 abundance. Moreover, deficiencies in the H2B ubiquitylase complex Rad6/Bre1 as well as the deubiquitylase Ubp10 reduced the lifespan by altering both H3K4 and H3K79 methylation and Sir2 recruitment. Thus, these results show that low levels of H2B ubiquitylation are a prerequisite for a normal lifespan and the trans-tail regulation of histone modifications regulates age-associated Sir2 recruitment through telomeric silencing.

1. Introduction

The post-translational modification of histones in the nucleosome provides the tight control of chromatin structure that is associated with gene transcription. The additional complexity provided by different modifications helps to fine-tune the overall control [1]. Cases in which one modification is dependent upon another are referred to as 'trans-tail regulation' of histone modification. This cross-talk was first identified in *Saccharomyces cerevisiae* [2], in which the ubiquitylation of histone H2B on K123 (H2BK123ub) influenced the di- and trimethylation of histone H3K4 and H3K79 [2–4]. Although the precise role of this concerted histone modification in the regulation of gene expression is still unclear, accumulating evidence suggests that the modification is associated with gene transcription or repression over wide regions of chromatin. In telomeric heterochromatin, this sequential modification provides a functional link between Rad6-mediated H2BK123ub, Set1-mediated H3K4 methylation (H3K4me), and transcriptional silencing [2], whereas the requirement of this pathway for active chromatin has also been reported [5–7].

Several cellular pathways in diverse organisms regulate cellular lifespan. For example, dietary restriction reduces the activities of various signal transduction pathways in yeast, nematodes, flies, and mammals [8]. The Sir2 protein has long been associated with the regulation of lifespan in yeast, nematodes, and flies [9]. Additionally, in yeast, the nucleolar accumulation of extrachromosomal rDNA circles generated from recombination between rDNA repeats or mutations in the RNA polymerase II complex component *HPR1* have been associated with aging [10,11]. Furthermore, recent work has described pathways that are distinct from existing aging models, one of which is the epigenetic regulation of lifespan through specific changes in chromatin state. Yeast strains that lack the histone chaperone Asf1 or acetylation at histone H3 on lysine 56 are short-lived, partly due to their decreased histone levels [12]. The trimethylation of H3K4 by the ASH-2 trithorax complex regulates lifespan in a germline-dependent manner in *Caenorhabditis elegans* [13]. The opposing activities of Sir2 and the Sas2 acetyltransferase regulate lifespan by modifying histone H4 at lysine 16 (H4K16) at subtelomeric regions in yeast [14]. The finding that Sir2 protein abundance decreases with age and is accompanied by an increase in H4K16 acetylation and compromised transcriptional silencing at subtelomeric regions [14], together with the observation that low levels of H2B ubiquitylation are required for Sir2-mediated silencing [15], suggests that histone ubiquitylation at these loci may be required for the Sir2-mediated regulation of cellular lifespan. Additionally, as indicated above, the trans-tail regulation of the histone H2BK123ub, H3K4me, and H3K79 methylation (H3K79me) has been suggested to occur near the telomere [2,15]. It remains to be clarified whether these concerted histone modifications on distinct histone tails have roles in the aging program.

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In the present study, we investigated the aging-associated role of histone H2B ubiquitylation and H3 lysine methylation in the regulation of Sir2 recruitment near telomeres. Our results show that these trans-tail histone modifications link Sir2-mediated telomeric silencing with cellular lifespan.

2. Materials and methods

2.1. Yeast strains and plasmids

The yeast strains used in this study are listed in Supplementary Table S4.

2.2. Isolation of old yeast mother cells

Old mother cells were isolated as described previously [16]. Approximately 1.6×10^9 cells (YKH045 strain) were harvested from cultures grown to exponential phase in YPD (1% yeast extract, 2% Bacto-Peptone, and 2% dextrose) at 30 °C. The cell surface was labeled with 48 mg of sulfo-NHS-LC-Biotin (Thermo Scientific) in 10 ml of phosphate-buffered saline (PBS) followed by gentle rotation at room temperature for 30 min. The excess biotin was removed by washing cells 7 times with 1 ml of PBS each time. Biotin-labeled cells were then resuspended in 12 L of YPD and incubated at 30 °C for 12–16 h to limit cell growth to 5–7 doublings. Dynabeads Biotin Binder (2 ml, approximately 8×10^8 beads) (Invitrogen) was used for the first round of sorting. After incubation for 3 h at 4 °C, biotin-labeled cells were collected with a DynaMag-15 (Invitrogen) and subsequently washed 9 times with 10 ml of cold YPD. Even older cells were obtained from the second and third rounds of sorting, inoculated into YPD at 10^8 cells per L and grown for 5–7 doublings. The sorting procedure was then repeated. Dynabeads Biotin Binder was used for the second (1 ml) and third (0.5 ml) round of sorting. Approximately 1.27×10^9 old mother cells were finally obtained from ten repeats of this three sequential rounds of sorting. To assure that the isolated cells were old, approximately 2×10^7 cells were stained with Calcofluor white M2R (Sigma–Aldrich, F3543). The average numbers of bud scars were then counted in composite images created by merging approximately 10–15 z-stack slices with Zen 2009 software (Carl Zeiss) using an inverted Zeiss Axio Observer.Z1 confocal microscope.

2.3. Yeast extract preparation and Western blotting

Yeast whole cell extracts were prepared in young and old cells as described previously [17]. Antibodies used in this study are listed in Supplementary Table S6.

2.4. Chromatin immunoprecipitation (ChIP) and chromatin double immunoprecipitation (ChDIP)

ChIP and ChDIP were performed as described previously [17,18]. Strain YKH045, containing both FLAG-*HTB1* and HA-*UBI4*, was used to detect ubiquitylated histone H2B [19]. The ChDIP PCR signals were quantitated and normalized to the internal control and the input DNA. The observed level of ubiquitylated H2B was further normalized to the FLAG-tagged H2B signal.

2.5. Replicative lifespan analysis

The replicative lifespans of the yeast strains were determined as described previously [20]. A total of 50 virgin daughter cells were isolated from mother cells and subjected to lifespan analysis. To assess the significance of the lifespan differences, a Wilcoxon rank-sum test (the "ranksum" function in MATLAB) was performed with a cut-off of $p = 0.05$. The mean lifespan and p values obtained from these analyses are listed in Supplementary Table S7. The differences of mean lifespan in each wild type strain are due to the different backgrounds used in each lifespan analysis.

2.6. Silencing assays

Heterochromatic silencing at the telomeric, silent mating (HMR and HML), and rDNA regions was evaluated by plate growth assays as described previously [21].

3. Results and discussion

3.1. H2B ubiquitylation and H3 lysine methylation patterns change in aged cells

To examine the changes in various histone modifications within the subtelomeric regions, we first evaluated the levels of histones in replicative aged cells. Aged cells were obtained by isolating biotin-labeled mother cells and confirmed by the relative increase in bud scar number. The strain used in this experiment (YKH045) showed a maximum of approximately 14 bud scars in aged cells after three rounds of sorting, which corresponds to the average lifespan of the yeast strain (Figs. 1B and 2C). Global levels of histone H3 and H2A proteins decrease during aging [12]. Additionally, specific subtelomeric regions in replicatively aged cells contain lower amounts of histone H3, which results in reduced transcriptional silencing [12,14]. Consistent with these findings, we observed that the total amounts of histone H4 and Sir2 proteins were decreased, concomitant with an increase in acetylation at H4K16, in aged cells (Supplementary Fig. S1A). Moreover, the histone H3, H4, or H2B occupancies at most heterochromatic regions, such as those at the subtelomeric, rDNA, or mating-type loci, were observed to be much lower in aged cells (Supplementary Fig. S1C).

We next examined the histone modifications at telomere-proximal regions in young and aged cells using ChIP. We observed high levels of Sir2 occupancy at X core and X repeat elements as well as at regions near the telomeres (at TEL05R, TEL06R and TEL07L) in young cells. These levels were significantly decreased in aged cells, concomitant with an increase in acetylation at H4K16, which was consistent with a previous report [14] (Supplementary Fig. S1D). We then examined histone ubiquitylation. A ChDIP assay involving FLAG immunoprecipitation of H2B-FLAG followed by HA immunoprecipitation of HA-ub was used to monitor H2BK123 ubiquitylation. Consistent with previous descriptions of the distribution of H2B ubiquitylation at TEL06R [15,19], the levels of this modification were relatively low near the end of TEL05R and TEL06R. Surprisingly, however, H2B ubiquitylation was significantly increased at all telomere-proximal regions in aged cells (Fig. 1B, top pane).

These initial observations led us to examine the age-associated changes in histone lysine methylations because histone H3 methylations at K4 and K79 are influenced by H2B ubiquitylation. Histone methylation at H3K4 or H3K79 is low within silenced loci but higher within gene coding regions in the euchromatin [22–24]. Low levels of histone methylation were generally observed at the subtelomeric regions in this study (Fig. 1B). However, the levels of modifications that were low near telomeres were higher in aged cells than in young cells, with patterns reminiscent of the increase in acetylation at H4K16 in aged cells. Additionally, the enrichment of H3K4me and H3K79me in aged cells was greater at regions proximal to telomeres, suggesting that, similar to H4K16ac, these methylations may help to define the boundary between heterochromatin

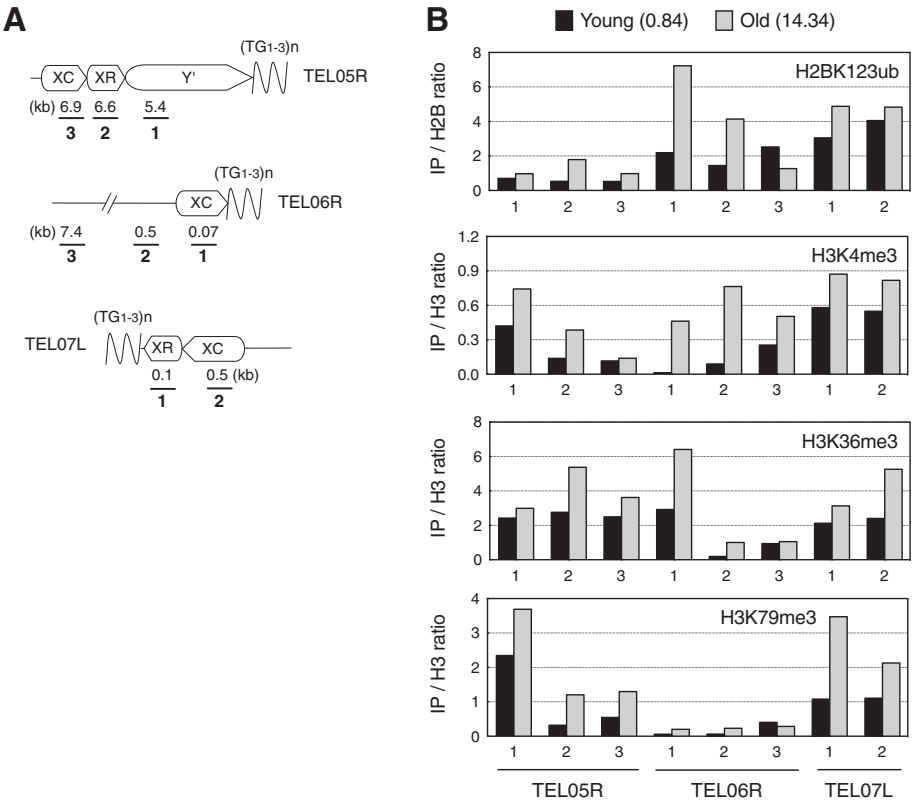

Fig. 1. Age-associated changes in histone H2B ubiquitylation and H3 lysine methylation near telomeres. (A) Schematic diagrams of the TEL05R, TEL06R and TEL07L regions are shown. Subtelomeric repeats are represented as XC (X core), XR (X repeat) and Y′ elements. The numbers below each PCR fragment are used for identification in all subsequent figures. The bars below the subtelomeric regions show the positions of the PCR products used in the ChIP analysis and their distances from their respective telomeric DNA sequences ($TG_{1\text{-}3}$). (B) ChIP analysis of telomere-proximal regions in young and aged cells (strain YKH045). Antibodies against H3K4me3, H3K36me3 and H3K79me3 were used to immunoprecipitate chromatin. A ChDIP assay was carried out with FLAG and HA antibodies to sequentially immunoprecipitate FLAG-H2B and HA-ubiquitin, respectively for the analysis of the levels of ubiquitylated H2B. PCR products amplified from ARS (autonomously replicating sequence), an untranscribed region on chromosome V, were used as an internal background control for all ChIP PCR reactions. PCR signals were quantitated and normalized to the internal control and the input DNA. The results for methylated H3 were further normalized to the total H3 signals.

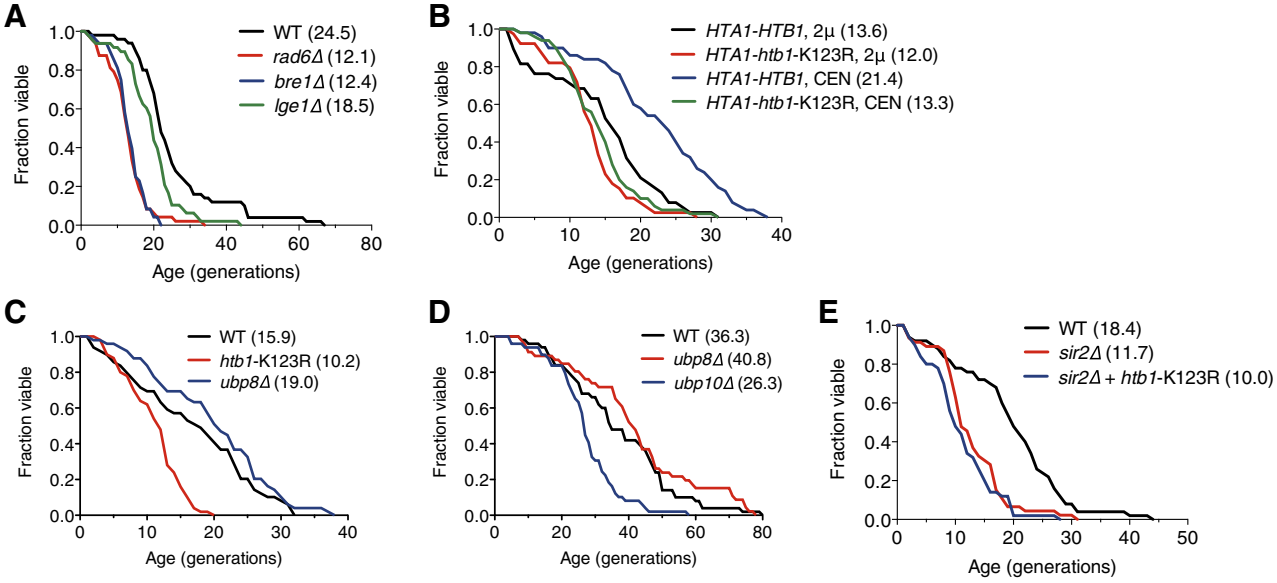

Fig. 2. H2B ubiquitylation regulates lifespan through a Sir2-mediated aging pathway. (A)–(E) Replicative lifespan analysis of WT (wild type), *rad6Δ*, *bre1Δ*, and *lge1Δ* cells (A); strains bearing a WT or point-mutated histone H2B (*htb1-K123R*) in a CEN or 2 μ plasmid (B); WT, *htb1-K123R*, and *ubp8Δ* cells (C); WT, *ubp8Δ*, and *ubp10Δ* cells (D); and WT, *sir2Δ*, and *sir2Δ* in *htb1-K123R* cells (E). The mean lifespan is shown in parentheses. Lifespan *p* values are listed in Supplementary Table S7.

and euchromatin [25]. In contrast, histone H3K36 methylation (H3K36me) was found to be relatively constant near telomeres, at a level slightly higher than that of H3K3me or H3K79me, and was also elevated in aged cells.

In other heterochromatic regions that are regulated by Sir2, e.g., the rDNA and mating-type loci, we observed results that were very similar to those observed in the subtelomeric regions. The occupancy of the Sir2 protein, which is part of the RENT complex that is found at rDNA regions [26] and also plays a silencing role at mating-type loci [27], was significantly decreased with an increase in H4K16ac, whereas modifications such as H2BK123ub, H3K4me, H3K36me, and H3K79me were elevated at both loci in aged cells. The only difference in the pattern of histone modifications was found at the rDNA loci; there was no similarity in the patterns of H3K4me, H3K36me, or H3K79me, suggesting that the aging process in this region is influenced by the distinct functions of each histone H3 lysine methylation (Supplementary Fig. S2). Taken together, these results show that histone H2B ubiquitylation and all H3 lysine methylations are significantly elevated at Sir2-regulated heterochromatic regions, and the co-localization of H3K4 and H3K79 methylation with H4K16 acetylation at the subtelomeric regions of aged cells strongly implies that trans-tail H2B ubiquitylation, H3 methylation and H4 acetylation are functionally related.

3.2. Disruption of H2B ubiquitylation is associated with telomeric silencing

Several lines of evidence have shown that proteins associated with histone H2B ubiquitylation and H3 lysine methylation regulate Sir2-mediated silencing at various heterochromatic regions. Many previous results obtained from *URA3* reporter assays, along with the complementation assay results from this study (Supplementary Figs. S3 and S4), are summarized in Supplementary Tables S1–S3. These results suggest that both ubiquitylating and deubiquitylating enzymes are required to maintain low levels of ubiquitylation and Sir2-mediated silencing at heterochromatic regions. Similar to H2B ubiquitylation, H3K4 and H3K79 methylations are associated with telomeric silencing. In contrast, we further observed that the loss of Set2, a H3K36 methyltransferase, led to an apparent increase in telomeric silencing at TEL07L and TEL05R (Supplementary Fig. S4A and B). Interestingly, we found that *ubp8Δ* showed a significant increase in the silencing of rDNA regions, i.e., the expression of the *URA3* reporter gene was significantly suppressed by the loss of Ubp8 in the absence of uracil, in contrast to the expression pattern observed at subtelomeric or HMR regions (Supplementary Fig. S3B). This result strongly suggests that Ubp8 has a distinct role in gene silencing in rDNA regions. The anti-silencing function of Set2 was observed at both the rDNA and HMR loci, as well as at telomeric regions. Taken together, these results suggest that proteins associated with ubiquitylation, in addition to the Set1 and Dot1 methyltransferases, have silencing functions, whereas the Set2 methyltransferase has an antagonistic function at heterochromatic regions. Additionally, the histone H2B deubiquitylating enzymes Ubp10 and Ubp8 may play different roles in heterochromatic gene silencing depending on the target heterochromatic regions.

3.3. H2B ubiquitylation regulates lifespan through the Sir2 pathway

Having shown that histone ubiquitylation and histone H3 lysine methylation at K4 and K79 are altered in aged cells and that these trans-tail modifications are required for Sir2-regulated telomeric silencing, we next tested the hypothesis that the age-associated change in H2B ubiquitylation regulates lifespan. In budding yeast, the replicative lifespan is determined by the number of times a mother cell divides to give rise to daughter progeny. Upon division, cells segregate older material to the mother, thereby assuring that the daughter cells are renewed [28]. Because Rad6 and Bre1 function together as a histone H2B ubiquitylase, we speculated that the deletion of either gene might reduce lifespan. As expected, the mean lifespans of *rad6Δ* and *bre1Δ* were shorter than that of wild type to a similar degree (Fig. 2A). The loss of Lge1 also decreased the yeast lifespan, but the decrease was not significant compared with those in *rad6Δ* or *bre1Δ*. Similar results were observed in strains that expressed histone H2B with a lysine-to-arginine substitution mutation at residue 123 (*htb1*-K123R) from either a low-copy-number plasmid (CEN/ARS) or a multicopy vector (*HTA1-HTB1* 2 μ) (Fig. 2B). These findings clearly show that H2B ubiquitylation by Rad6/Bre1 regulates replicative aging.

We also speculated that an increase in ubiquitylation caused by the overexpression of histone H2B or the deletion of an H2B deubiquitylase would reduce the mean lifespan relative to wild type if the maintenance of low ubiquitylation under telomeric silencing were required for normal lifespan. Consistent with this prediction, *HTA1-HTB1* 2 μ-transformed and *ubp10Δ* strains were short-lived compared with wild type cells (Fig. 2B and C). In contrast, a strain lacking the ubiquitylase Ubp8 exhibited a subtle increase in lifespan (Fig. 2C and D). Ubp8 is a subunit of the Spt-Ada-Gcn5-acetyltransferase (SAGA) and SALSA-SILK (SAGA altered, Spt8 absent; SAGA-like) complexes, which act mainly during early transcription [29]. Therefore, it seems likely that the loss of Ubp8 alters the expression of age-associated genes that are regulated by SAGA or causes a significant increase in rDNA silencing (as observed in *ubp8Δ*; Supplementary Fig. S3), which presumably influences the lifespan.

It has previously been shown that Sir2 modulates the replicative lifespan by regulating histone H4K16 acetylation at subtelomeric regions [14]. We reasoned that if histone H2BK123 ubiquitylation regulates lifespan through a mechanism that is distinct from the deacetylation of histone H4K16, then *htb1*-K123R should shorten the cellular lifespan in a *sir2Δ* background. However, *htb1*-K123R did not further reduce the lifespan of *sir2Δ* cells, suggesting that *htb1*-K123R reduces the cellular lifespan through a Sir2-related pathway (Fig. 2E). Therefore, these data strongly suggest that the activities of the histone ubiquitylase Rad6/Bre1 and the deubiquitylase Ubp10 are required for lifespan regulation mediated by Sir2.

3.4. Trans-tail modifications regulate Sir2 recruitment

Given that histone H2B ubiquitylation was altered at telomere-proximal regions in aged cells and that such changes are likely to be mediated through the Sir2 pathway, we questioned whether histone H2B ubiquitylation was associated with Sir2 recruitment near subtelomeric regions. To this end, we conducted ChIP analysis of a native telomere-proximal region from the right arm of chromosome VI (TEL06R). This region lacks the repetitive Y′ and X sequences [30]; therefore, regions of Sir2 association or histone modification can be evaluated at various distances from the telomere end. If the lack of H2B ubiquitylation near the telomere were responsible for the shortened lifespan of *rad6Δ* or *bre1Δ* cells, then decreased Sir2 occupancy would be expected at these loci. Consistent with this prediction, each deletion exhibited significantly reduced Sir2 recruitment and increased H4K16 acetylation at the regions near the chromosome end (Fig. 3A and B). Additionally, the disruption of *UBP10*, but not *UBP8*, compromised Sir2 association. These results were further confirmed by similar ChIP experiments in strains bearing a *URA3* reporter gene near TEL07L, which produced similar results (Supplementary Fig. S5). As shown in Supplementary Fig. S3, telomeric gene silencing was strongly reduced in *rad6Δ* and *bre1Δ* cells and moderately reduced in *ubp10Δ* cells. Thus, the reduction in Sir2 association at native or *URA3*-containing subtelomeric regions, which was accompanied by reductions in the mean lifespans of *rad6Δ*, *bre1Δ*, and *ubp10Δ* cells, was paralleled by reduced telomeric silencing. This indicates that histone

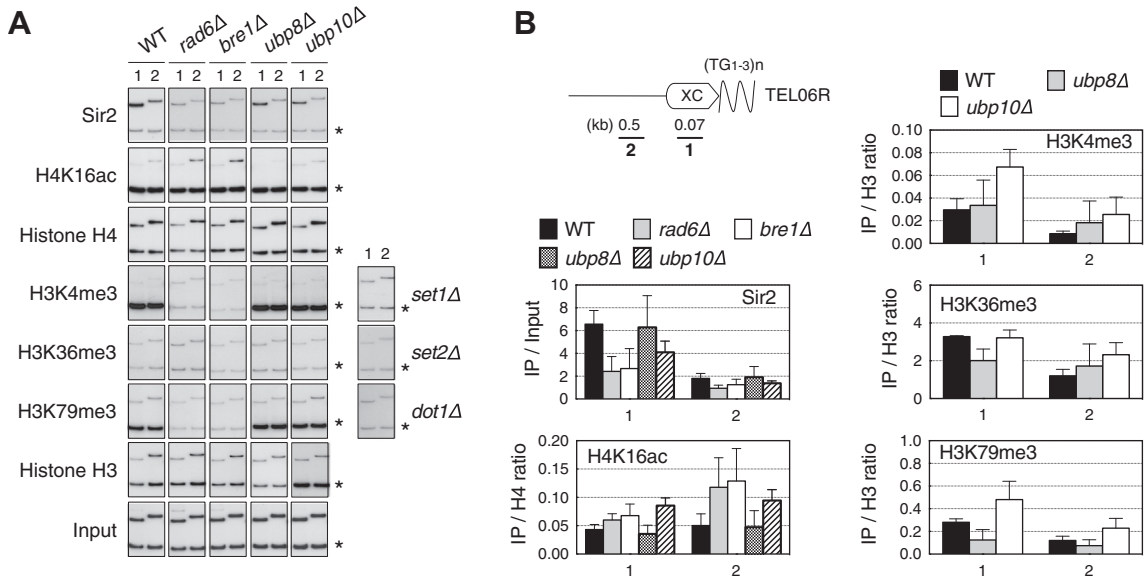

Fig. 3. Histone H2B ubiquitylation regulates Sir2 association through H3 methylation at lysines 4 and 79 near telomeres. (A) Sir2 recruitment and the histone modifications H4K16ac, H3K4me3, H3K36me3, and H3K79me3 near TEL06R in WT, *rad6Δ*, *bre1Δ*, *ubp8Δ*, and *ubp10Δ* cells were evaluated by ChIP. The background levels of histone lysine methylations near the telomeres were measured in cells bearing the deletion of the corresponding methyltransferase (right panel). The asterisk denotes the same untranscribed control described in Fig. 1B. (B) Quantitation of the ChIP experiments in (A). The PCR signals were quantitated and normalized as described in Fig. 1B. Error bars indicate the standard deviation (S.D.) calculated from three PCRs performed using two independent chromatin preparations.

ubiquitylation regulates lifespan through Sir2-mediated telomeric silencing.

Our observations of ubiquitylation-dependent Sir2 association with telomere-proximal regions led us to investigate the changes in histone methylations at H3K4 and H3K79, which are influenced by upstream histone ubiquitylation and are implicated in transcriptional silencing near telomeres. As mentioned above, H3K4 and H3K79 methylation near TEL06R are very infrequent in wild type cells [23,24]. The loss of Rad6 or Bre1 reduced methylation at both H3K4 and H3K79 to background levels but did not affect methylation at H3K36 (Fig. 3A), suggesting a close relationship between histone trans-tail modification and Sir2 occupancy. Moreover, the loss of Ubp10, but not of Ubp8, increased trimethylation at H3K4 and H3K79, whereas trimethylation at H3K36 remained unchanged (Fig. 3A and B). These results indicated that the trans-tail histone modifications of H2B ubiquitylation and H3K4 and H3K79 methylation regulate Sir2 association at telomeric-proximal regions. These results also support the hypothesis that this Sir2 regulation mediates the lifespan reductions observed in *rad6Δ*, *bre1Δ*, *ubp10Δ*, and *htb1*-K12R cells.

In the present study, we provide evidence that histone H2B ubiquitylation regulates the replicative lifespan of *S. cerevisiae*. Our results show that the levels of histone ubiquitylation at H2BK123 and methylations at H3K4 and H3K79 are elevated near the telomeres in aged budding yeast cells, and these changes contribute to Sir2-regulated aging. In this region, low levels of H2B ubiquitylation, which is a prerequisite for a normal lifespan, are maintained by the ubiquitylase Bre1/Rad6 and the deubiquitylase Ubp10. Most importantly, we found that trans-tail histone modifications triggered by H2B ubiquitylation, which include different sequences of modifications at different histone tails (histone H2B ubiquitylation at K123, H3 methylations at K4 and K79 and H4 acetylation at K16), play pivotal roles in lifespan regulation. The trans-tail regulation of histone modifications is conserved in humans [6], suggesting that this regulation is a critical epigenetic feature of aging in most eukaryotes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.017.

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