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Loss of the Set2 histone methyltransferase increases cellular lifespan in yeast cells

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ABSTRACT

The post-translational modification of histones has been implicated in the regulation of cellular lifespan. Previously, we reported that cellular aging is associated with increased ubiquitylation of histone H2B and methylation of histone H3 at lysines 4 and 79 in yeast telomeric heterochromatin. Here, we show the antagonistic role of Set2 methyltransferase, which is specific for histone H3 at lysine 36, in regulating telomeric silencing and cellular lifespan. We observed that an intermediate state of chromatin, namely, unstable ON telomeres, exists when a gene is switched on near telomeres. This unstable state of chromatin is temporally maintained in a transcription-dependent manner and is preferentially restored to its original heterochromatic state, namely, OFF telomeres. We found that Set2 suppresses the restoration of unstable ON telomeres to the stable OFF state and promotes cellular aging. Our results suggest that the accumulation of unstable ON telomeres maintained by Set2 is one of the features of aged cells.

1. Introduction

Post-translational histone modifications in the nucleosome play a crucial role in the tight control of chromatin structure and gene expression to enable diverse biological processes such as cell differentiation and organism development, while deregulation of these modifications can contribute to diseases such as cancer [1]. In yeast *Saccaromyces cerevisiae*, histone modifications show distinct patterns in chromosomal domains. For example, genome-wide studies of histone modifications have revealed that histone H3 acetylation is correlated with transcriptional activity and that trimethylation of both histone H3 at lysine 4 (H3K4) and histone H3 at lysine 36 (H3K36) is associated with actively transcribed regions of genes. In contrast, heterochromatic regions, such as telomeres, ribosomal DNA and mating-type loci, are deficient in histone methylation and acetylation (for review see [2]). In telomere-proximal regions, low levels of histone H2B ubiquitylation and H3 methylation were shown to correlate with Sir2 association and gene silencing [3]. Recently, we have reported a connection between histone modifications and cellular aging in budding yeast: ubiquitylation of histone H2B at lysine 123 (H2BK123) and methylation of H3K4, H3K36 and H3 at lysine 79 (H3K79) are increased in replicatively aged cells. Disruption of low H2B ubiquitylation levels by deletion of the H2B ubiquitylase complex Rad6/Bre1 or the H2B deubiquitylase Ubp10 reduces normal lifespan by altering H3K4 and H3K79 methylation and Sir2 association. We suggested that the *trans*-tail modification between H2B ubiquitylation and H3 methylation regulates Sir2-mediated telomeric silencing and concurrently, cellular lifespan [4].

Telomeres protect the ends of chromosomes from DNA repair and degradation mechanisms [5,6]. In general, genes located near telomeres are transcriptionally silenced, a phenomenon known as the 'telomere position effect' (TPE) in budding yeast [7]. The stepwise assembly of silent chromatin in budding yeast has been well characterized: the Sir2/Sir4 complex is recruited to telomeric TG_{1-3} repeats through its interactions with the yKu70/yKu80 heterodimer and Rap1. Sir2, an NAD^+-dependent histone deacetylase, removes the acetyl group from lysine 16 of histone H4 (H4K16) and provides a binding site for Sir3. The subsequent multimerization of Sir3 and Sir4 permits additional rounds of this cycle, and Sir proteins spread from telomeres to subtelomeric regions, promoting the hypoacetylation of histones H3 and H4, which leads to transcriptional repression [5,8,9].

The TPE is an example of position-effect variegation, which was discovered in the fruit fly *Drosophila melanogaster*, in which the expression of a gene reversibly switches within heterochromatin [10]. Recently, Grunstein and colleagues reported a mechanism for the epigenetic variegation of gene expression at yeast telomeric

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regions. Using approaches to select cells in the ON and OFF expression states, they found that histone lysine methylation at H3K4, H3K79, and H3K36 is depleted at OFF telomeres but enriched at ON telomeres [11]. This finding suggests that the mechanism that leads to transcriptional silencing near telomeres in budding yeast may involve an extra level of complex epigenetic regulation that may include all known variations of H3 lysine methylation, as well as modification of H2BK123 ubiquitylation or H4K16 acetylation up- or downstream of histone methylation at H3K4 and H3K79.

In the present study, we explored the underlying mechanism by which epigenetic variegation of gene expression is associated with cellular aging. We found that the antagonistic role of Set2 methyltransferase on telomeric silencing negatively contributes to normal lifespan by affecting the restoration of unstable ON chromatin to the stable OFF state. We propose a mechanism that elucidates a possible link between epigenetic variegation of Sir2-mediated telomeric gene expression and cellular aging.

2. Materials and methods

2.1. Yeast strains and plasmids

The yeast strains used in this study are listed in Supplementary Table S1. Strain SY386 containing *sir2Δ*::*KanMX4*::*LEU2* was generated by marker swapping [12]. The plasmid M4755 (*KanMX*::*LEU2*) was used to convert a *sir2Δ*::*KanMX4* strain into *sir2Δ*::*LEU2*. M4755 was cut with *Not*I, ethanol precipitated, and transformed into strain FY186. Transformants were then selected by leucine prototrophy, and a set of Leu^+ transformants were screened for growth on yeast extract peptone dextrose (YPD) plates containing G418. All deletion strains were confirmed by PCR using gene-specific and deletion cassette-specific primers.

2.2. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously [13,14]. Cells for ChIP experiments were grown to mid-log phase (A_{600} = 0.6) in synthetic complete (SC) medium, unless otherwise indicated. The oligonucleotide sequences used in ChIP PCR are listed in Supplementary Table S2. Sir2 antibody (Santa Cruz Biotechnology) was bound to protein A-Sepharose CL-4B (GE Healthcare) and used to precipitate chromatin. To control for differences in the amplification efficiency and label incorporation of different primers, the PCR signals were quantitated and normalized to the internal control and the input DNA.

2.3. Replicative lifespan analysis

The replicative lifespans of the yeast strains were determined as described previously [4,15]. All lifespan experiments were performed on standard YPD plates (2% glucose). A total of 50 virgin daughter cells were isolated from mother cells, and approximately 38-50 mother cells were evaluated in each 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 S3.

2.4. RT-PCR

Total RNA was extracted from cells using the hot phenol method. The primer sequences used in the RT-PCR analysis are shown in Supplementary Table S2. RNA was reverse transcribed using a DiaStar™ RT Kit (SolGent), and the PCR reaction was performed in the presence of [α-^{32}P] dATP. The PCR cycle conditions were 95 °C for 3 min followed by 26 cycles at 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. A final extension was performed for 10 min at 72 °C. The RT-PCR products were then analyzed by electrophoresis using 8% polyacrylamide gels.

3. Results

3.1. Unstable ON telomeres exist during gene switching

Our previous report illustrates that the *trans*-tail regulation of histone modifications at telomere-proximal regions is associated with Sir2-mediated silencing and cellular lifespan [4]. As mentioned earlier, the decreased telomeric silencing in cells lacking histone ubiquitylase complex Rad6/Bre1 is coincided with decreased silencing in *set1Δ* or *dot1Δ* cells, and this reduced telomeric silencing is thought to contribute to shortened lifespan [4]. However, genetic analyses using cells lacking Set2 suggest that Set2 has the opposite effect on telomeric silencing of that of Set1 and Dot1 as well as Rad6/Bre1 [4,16]. In addition, this observation from *set2Δ* cells raises several questions: if Sir2-mediated telomeric silencing is associated with the regulation of cellular lifespan, then could the enhanced silencing caused by *set2Δ* increase cellular lifespan? If so, is there a correlation between telomeric silencing and Sir2 recruitment at telomere-proximal regions in *set2Δ* cells? To address these questions, we first determined the relationship between gene expression and Sir2 recruitment at telomere-proximal regions using a *URA3* reporter. The *URA3* reporter gene embedded in a truncated chromosome end is widely used as a model of TPE that can be selected both positively and negatively; in addition to 5-fluoroorotic acid (5-FOA), which is converted into a lethal metabolite by the active (i.e., not silenced) *URA3* enzyme, media lacking uracil can also be used to evaluate the levels of silencing. At regions near the telomere, *URA3* expression, as determined by RT-PCR analysis, was significantly induced upon the removal of uracil and was maintained for approximately 120 min (Fig. 1B). Consistent with this result, the association of Sir2 with the telomere proximal region was suppressed during the first 60 min upon removal of uracil (Fig. 1C), indicating a correlation between gene silencing and Sir2 association in this region.

However, unexpected results were observed in cells cultured in SC-Ura medium and cells cultured in complete medium (SC) with uracil removed at log phase. *URA3* was highly expressed under the former conditions even without loss of Sir2 association, whereas under the latter conditions, although there was a rigid correlation between elevated levels of *URA3* and reduced Sir2 recruitment upon uracil removal, *URA3* expression returned to basal levels after 240 min followed by the restoration of Sir2 association (Fig. 1B and C). Recent work has provided evidence that natural ON telomeres obtained from cells cultured in SC-Ura medium exhibit certain characteristics in common with OFF telomeres obtained from cells in medium containing 5-FOA, including significant amounts of Sir3 and H4K16 deacetylated nucleosomes [11]. Consistent with this epigenetic variegation of telomeric gene expression, we observed significant levels of Sir2 association in natural ON telomeres. However, we also found that when gene expression was switched on in cells cultured in SC medium, in which telomeres exhibit bistable ON/OFF expression [11], the induction of gene expression was regulated by Sir2 in a transcription-dependent manner but maintained only temporarily. This intermediate telomeric state, hereafter termed unstable ON telomeres, appears to be inclined to regress toward an OFF state,

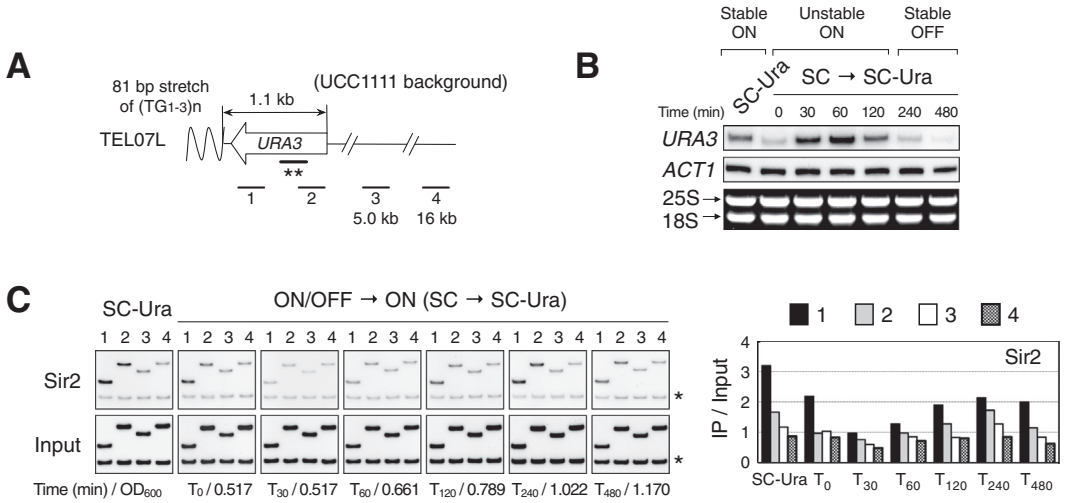

Fig. 1. The intermediate state of unstable ON telomeres exists near telomeres. (A) Schematic representation of the *URA3* reporter at the left end of chromosome VII (TEL07L). The position of the DNA fragment used as a probe in RT-PCR analysis is indicated by a double asterisk. The numbers below *URA3* indicate the relative positions of the PCR products used in the ChIP analysis; these numbers were also used in Fig. 2C. RT-PCR and ChIP analysis were performed in WT cells that contain the *URA3* reporter (UCC1111 background). (B) Cells grown in complete media were transferred to media lacking uracil, incubated for the indicated times, and then *URA3* expression was determined by RT-PCR. The levels of *ACT1* and 25S/18S rRNA transcripts were measured as controls. To obtain cells containing natural ON telomeres, the cells were cultured in SC-Ura medium. (C) ChIP analysis was performed using a Sir2 antibody in WT cells. The cells were incubated as described in (B). Quantitation of the ChIP experiments in (C) is shown in the right pane. 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.

suggesting that the epigenetic variegation of telomere gene expression adopts additional complex pathways.

3.2. Set2 suppresses the restoration of unstable ON telomeres to the stable OFF state

Next, we sought to examine the role of Set2 in maintaining unstable ON telomeres. Previously, an antagonistic role for Set2 in telomere silencing was suggested because mutations in H3K36 or *set2Δ* cause ectopic silencing adjacent to *HMRa*, a silent mating-type locus in yeast, and decrease the expression of native genes proximal to the telomeres of chromosome XIV [17]; *set2Δ* also suppresses the silencing defect caused by *set1Δ* [16]. Again, we used the same *URA3* subtelomeric region reporter in cells deficient for each histone H3 lysine methyltransferase. We previously observed that *set2Δ* suppressed *URA3* expression not only in the absence of uracil but also in the presence of 5-FOA [4]. Consistent with this finding, the loss of Set2 significantly suppressed the induction of *URA3* expression by the removal of uracil until 60 min. In contrast, little change in the expression of *URA3* was observed in *set1Δ* and *dot1Δ* cells until the same point, when telomeric chromatin adopted the unstable ON state (Fig. 2A). Moreover, the expression changes of *URA3* coincided with the observed changes in Sir2 recruitment; in cells lacking Set1 or Dot1, the recruitment of Sir2 remained unchanged, but a significant increase in Sir2 association was observed upon the removal of uracil (T_{30}) in *set2Δ* cells (Fig. 2B and C), suggesting that the gene expression in unstable ON telomeric chromatin is regulated by Set2.

Surprisingly, we also found that the repression of *URA3* expression in *set2Δ* cells was not permanent; at later times of induction (T_{120} and T_{240}), the derepression of *URA3* expression was observed together with decreased Sir2 association (Fig. 2A and B). This result suggests that the loss of Set2 only delayed Sir2 recruitment to telomere-proximal regions and thereby affected the restoration of unstable ON telomeres to the OFF state. Taken together, our results show that gene expression within unstable ON telomeres is regulated by Set2 and suggest that Set2 contributes to the maintenance of the unstable ON state at telomere-proximal regions by inhibiting restoration to the stable OFF state.

3.3. Set2 negatively regulates cellular lifespan

The results obtained above prompted us to investigate whether lysine methyltransferases, including Set2, regulate cellular lifespan through a pathway similar to those involving histone ubiquitylases or deubiquitylases. As previously shown by silencing assay, the *URA3* reporter at TEL07L was less sensitive to 5-FOA in *set2Δ* cells than in *set1Δ*, *dot1Δ* or *sir2Δ* cells [4]. We speculated that if the loss of telomeric silencing in *set1Δ* or *dot1Δ* cells affects aging, then these mutations should also decrease the average cellular lifespan similarly to the observations made in *rad6Δ* or *ubp10Δ* cells. On the other hand, we also expect that if the antagonistic role of Set2 in telomeric silencing affects aging, enhanced lifespan would be observed.

Consistent with these hypotheses, we observed that the replicative lifespan of *set2Δ* cells was significantly extended compared with that of wild type cells. In contrast, the lifespans of *set1Δ* and *dot1Δ* cells were shortened (Fig. 3A). Moreover, the reduced lifespans of the *sir2Δ set1Δ* and *sir2Δ dot1Δ* double deletion mutants were indistinguishable from that of *sir2Δ* alone. Therefore, we reasoned that Set1 and Dot1 must influence lifespan through a Sir2-mediated pathway. However, we found that *set2Δ* partially increased the shortened lifespan of *sir2Δ* cells (Fig. 3B). Additionally, *htb1-K123R set2Δ* double mutants showed a slight extension of the shortened lifespan caused by *htb1-K123R* alone, whereas the lifespans of *htb1-K123R set1Δ* and *htb1-K123R dot1Δ* remained unchanged compared with those of *htb1-K123R* (Fig. 3C). These results show that the histone H3K36 methyltransferase Set2 regulates lifespan partially independently of Sir2 and the histone H2B ubiquitylation pathway and suggest that the aging pathway may involve epigenetic variegation of gene expression regulated by Set2 methyltransferase.

3.4. A proposed model linking the epigenetic variegation of gene expression regulated by Set2 to cellular aging

In the present study, we provided evidence that Set2 methyltransferase inhibits the pathway through which the unstable ON state of yeast telomeric heterochromatin is inclined to return to

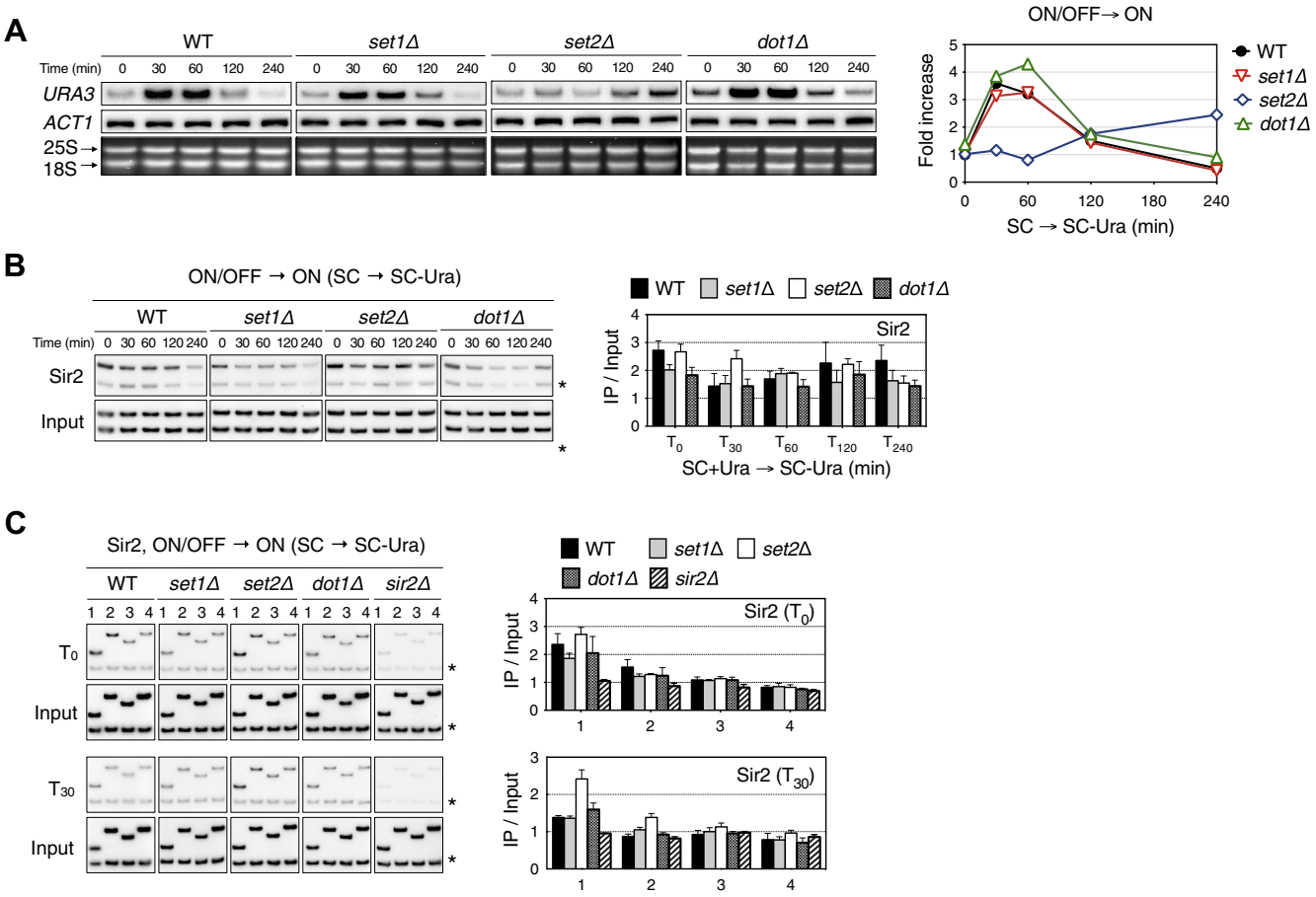

Fig. 2. Set2 maintains unstable ON state chromatin near telomeres. RT-PCR and ChIP analysis were performed in WT, *set1Δ*, *set2Δ* and *dot1Δ* cells, all of which contain the *URA3* reporter (UCC1111 background). (A) RT-PCR analysis of *URA3* mRNA levels in WT, *set1Δ*, *set2Δ* and *dot1Δ*. The differences in the *URA3* transcript levels between WT and mutants are presented as fold increases relative to the WT strain; *URA3* mRNA levels were divided by the level of *ACT1* and further normalized to the 'time 0' signal in WT. The quantitation result from (A) is shown at the right pane. (B) ChIP analysis of Sir2 binding at *URA3*-TEL07L in WT, *set1Δ*, *set2Δ*, and *dot1Δ*. Primer set 1, as shown in Fig. 1A, was used in this ChIP analysis. The error bars indicate the S.D. of three PCRs from two independent preparations. (C) The indicated strains grown in complete media were transferred to media lacking uracil and further incubated for 30 min to evaluate the changes in Sir2 association with telomeric-proximal region. ChIP analysis was performed as described in Fig. 1C. Results from (C) were quantitated and graphed in the right pane. The error bars indicate the S.D. in three PCRs from two independent preparations.

the OFF state. We found that the histone H3 methyltransferases Set1 and Dot1 are required for normal cellular lifespan through a pathway dependent upon Sir2, whereas Set2 methyltransferase negatively regulates lifespan through a mechanism that is partially independent of Sir2 and H2B ubiquitylation and that preserves unstable telomeres in the ON state.

Based on the results obtained in this study, we propose a novel pathway through which the epigenetic variegation of gene expression regulated by histone methyltransferase Set2 contributes to cellular aging (Fig. 4). In highly proliferative young yeast cells, telomeric regions adopt a rigid heterochromatic structure, namely, stable telomeres in the OFF state, that is maintained by low levels of histone modifications such as ubiquitylation at H2BK123 and methylation at H3K4 and H3K79. These histone hypo-modifications support the stable association of Sir2 within telomeric heterochromatin, followed by the deacetylation of histone H4 by Sir2 deacetylase. However, as cells age, genes located near telomeres are likely to escape from the stable OFF state, characterized by hyper-modification of H2B ubiquitylation and H3 methylation; hyperubiquitylated telomeres permit high levels of histone H3 methylation at K4 and K79, which then suppresses the association of Sir2 in this region through the pathway known as '*trans*-tail regulation of histone modification' [18]. Histone methylation at H3K36 by Set2 also increases in aged cells. Unstable telomeres appear to be preferentially restored to the stable OFF state in young cells, but the age-promoting Set2 methyltransferase seems to inhibit this restoration as cells age. Thus, the gradual accumulation of unstable ON telomeres mediated by Set2 function appears to be one of the features of aged cells.

4. Discussion

We and others have previously observed an antagonistic role for Set2 in heterochromatin gene silencing [4,16,17]. However, the underlying mechanism by which Set2 methyltransferase antagonizes gene silencing has remained obscure. We found that loss of Set2 delays the dissociation of Sir2 from telomeric regions when a gene is switched on in a transcription-dependent manner. In addition, gene induction at regions proximal to telomeres appears to be unstable, and the chromatin is preferentially restored to its original heterochromatic state (i.e., the OFF state) through a process that is counteracted by Set2. Furthermore, it seems likely that the accumulation of unstable ON telomeres as cells age depends on Set2, as we previously observed significantly elevated levels of histone H3K36 methylation in aged cells [4]. Thus, the antagonistic role of Set2 in telomeric silencing is attributable to its regulation of epigenetic variegation of gene expression, which eventually contributes to cellular aging.

Budding yeast expresses two ubiquitin-specific proteases, Ubp8 and Ubp10. Ubp8 is a component of the SAGA complex and

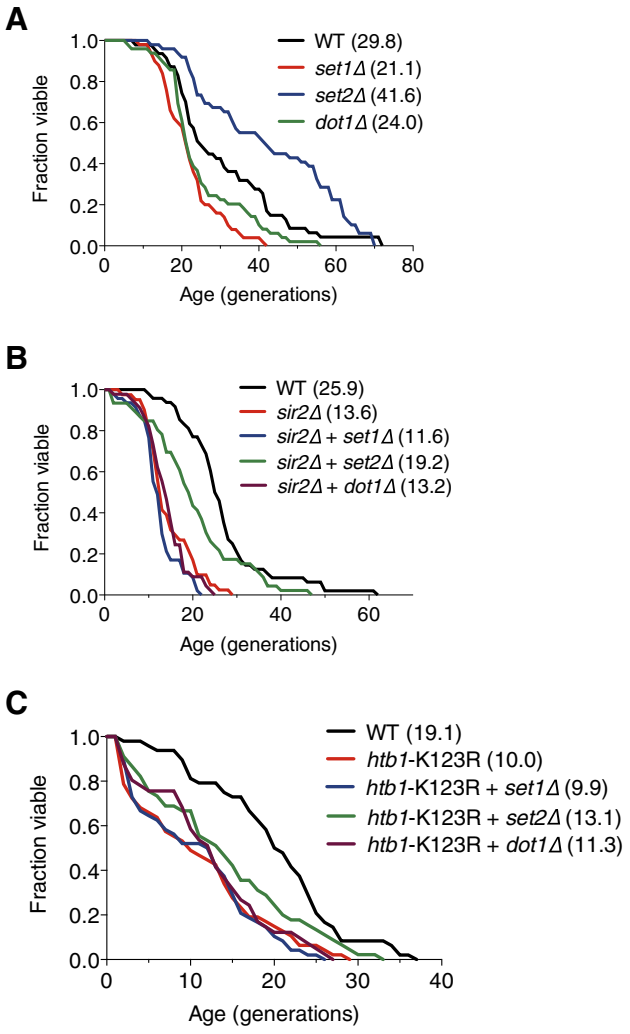

Fig. 3. Cellular lifespan is significantly enhanced by loss of Set2. (A–C) Replicative lifespan analysis of WT, *set1Δ*, *set2Δ*, and *dot1Δ* strains in BY4741 (A), *sir2Δ* (B), and *htb1-K123R* (C) backgrounds. The mean lifespan is shown in parentheses. Lifespan *P* values are listed in Supplementary Table S3.

deubiquitylates ubiquitylated H2B histone *in vitro* and *in vivo* [19,20]. Ubp10 (also known as Dot4) is a deubiquitylase that functions independently of Ubp8 and appears to act on distinct pools of cellular ubiquitylated H2B; cells lacking both Ubp8 and Ubp10 show greater increases in ubiquitylation compared with either of the single deletions alone [21,22]. Several lines of evidence indicate that Ubp10, but not Ubp8, is associated with heterochromatic telomeres. In contrast to Ubp8, which functions in transcription activation, Ubp10 interacts with Sir4 and is important for Sir complex-mediated telomeric and rDNA silencing [23]. In addition, Ubp10 exhibits a preferential association with the telomere-proximal region of the right arm of chromosome VI, whereas Ubp8 does not show a preference for binding to this region [3]. Furthermore, unlike *ubp10Δ* cells, cells lacking Ubp8 do not show increases in steady-state levels of methylation at H3K4 or H3K79 with inconsequential effects on telomeric silencing [21]. In accordance with these reports, we previously showed that that *ubp10Δ*, but not *ubp8Δ*, caused a preferential increase in histone methylation at H3K4 and H3K79 and a decrease in Sir2 association at telomere-proximal regions, which were followed by a silencing defect in this region and a shortened lifespan [4]. Therefore, it is possible that the age-resisting role of histone methylation at H3K4 or H3K79 is maintained by low levels of H2B ubiquitylation, which in turn is counteracted by the role of Set2 methyltransferase in regulating the epigenetic variegation of gene expression in yeast telomeric heterochromatin.

Accumulating evidences have shown that the establishment of Sir protein-dependent silencing in yeast requires progression through cell cycle [24–27]. Particularly, Martins-Taylor et al. showed that transit through M-Phase is necessary and sufficient to establish silencing at telomeres following induction of the Sir3 protein [28]. Furthermore, they show that deletion of gene coding for the histone variant H2A.Z (Htz1 in yeast) abolishes the cell-cycle requirement for the establishment of silencing [29]. Thus, future studies that address a connection between the cell-cycle-dependent establishment of transcriptional silencing and epigenetic variegation of gene expression at yeast telomeric heterochromatin will be interesting and promising.

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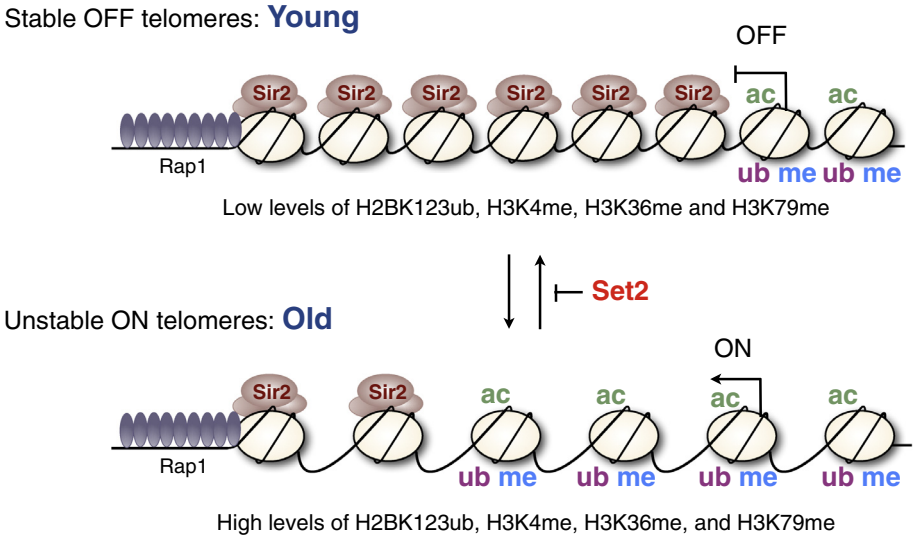

Fig. 4. A proposed model showing that epigenetic variegation of gene expression regulated by histone methyltransferase Set2 contributes to cellular aging. See text for details.

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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.2014.02.061.

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