Deletion of Ogg1 DNA glycosylase results in telomere base damage and length alteration in yeast

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Telomeres consist of short guanine-rich repeats. Guanine can be oxidized to 8-oxo-7,8-dihydroguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG). 8-oxoguanine DNA glycosylase (Ogg1) repairs these oxidative guanine lesions through the base excision repair (BER) pathway. Here we show that in *Saccharomyces cerevisiae* ablation of Ogg1p leads to an increase in oxidized guanine level in telomeric DNA. The *ogg1* deletion (*ogg1Δ*) strain shows telomere lengthening that is dependent on telomerase and/or Rad52p-mediated homologous recombination. 8-oxoG in telomeric repeats attenuates the binding of the telomere binding protein, Rap1p, to telomeric DNA *in vitro*. Moreover, the amount of telomere-bound Rap1p and Rif2p is reduced in *ogg1Δ* strain. These results suggest that oxidized guanines may perturb telomere length equilibrium by attenuating telomere protein complex to function in telomeres, which in turn impedes their regulation of pathways engaged in telomere length maintenance. We propose that Ogg1p is critical in maintaining telomere length homeostasis through telomere guanine damage repair, and that interfering with telomere length homeostasis may be one of the mechanism(s) by which oxidative DNA damage inflicts the genome.

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Introduction

Genome integrity is constantly challenged by both endogenous and environmental oxidative stress. Cells have developed various defence pathways to repair oxidative DNA damage. Defects in these pathways often cause genome instability. 8-oxo-7,8-dihydroguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) are among the most common oxidative base damages observed in DNA (Klungland and Bjelland, 2007; Dizdaroglu *et al*, 2008). Base excision repair (BER) is the primary DNA repair pathway for non-bulky damaged bases. The initial step in BER

is base removal by a DNA glycosylase. The subsequent BER repair steps involve incision of the phosphodiester backbone by the abasic endonuclease APE1, DNA termini processing by a diesterase or lyase, repair synthesis by a DNA polymerase, and nick sealing by a DNA ligase (Memisoglu and Samson, 2000; Klungland and Bjelland, 2007). In eukaryotes, Ogg1 DNA glycosylase is conserved from budding yeast, plants, insects to mammals and initiates the first step of 8-oxoG and FapyG repair through BER by excising oxidized bases in double-stranded DNA (Boiteux *et al*, 2002; Klungland and Bjelland, 2007).

Telomeres are proteinaceous–DNA structures. In *Saccharomyces cerevisiae*, telomeric DNA is composed of double-stranded tandem repeats (TG_{1–3})_n, about 256–375 bp in length, followed by a single-stranded 3′ overhang (Zakian, 1995). Centromeric to terminal telomere repeats are two classes of subtelomeric repeats called X and Y′ elements (Chan and Tye, 1983). X elements are present in all chromosome ends, whereas Y′ elements can be found in about two-thirds of telomeres. Telomeres have an essential role in protecting the linear chromosome ends from being recognized as double-strand breaks (Greider, 1996; Palm and de Lange, 2008). Telomere dysfunction emanating from loss of telomere repeats can trigger a DNA damage response and subsequently lead to cell death, cell proliferation defects, and genome instability (Palm and de Lange, 2008).

Telomere length in *S. cerevisiae* is maintained through a balance between telomere elongation and shortening (Greider, 1996; Blackburn, 2001). Telomere repeat elongation requires a special enzyme—telomerase. Telomerase is composed of two core components, a reverse-transcriptase protein subunit (encoded by *EST2*) and a template RNA (encoded by *TLC1*). Telomerase deficiency leads to replication-dependent telomere shortening and eventually cell death (Singer and Gottschling, 1994; Lendvay *et al*, 1996). However, surviving cells can emerge by maintaining telomeres through recombination that requires the recombination protein, Rad52p (Lundblad and Blackburn, 1993; Lendvay *et al*, 1996). Double-stranded telomeres are bound by an array of a specialized protein complex, Rap1p and its interacting proteins, Rif1p and Rif2p (Conrad *et al*, 1990; Hardy *et al*, 1992; Wotton and Shore, 1997). These proteins negatively control telomere length through a protein-counting mechanism, in which the number of Rap1p–Rif1p–Rif2p complexes bound to a telomere limits the action of telomerase *in cis* (Bianchi and Shore, 2008). Rap1p also negatively regulates telomere length in another yeast, *Kluyveromyces lactis* (McEachern and Blackburn, 1995; Krauskopf and Blackburn, 1996, 1998). In human cells, overexpression of the telomere-binding proteins: TRF1 and TRF2 causes telomere shortening, whereas a decrease in telomere-bound TRF1 promotes telomere lengthening (van Steensel and de Lange, 1997; Smith and de Lange, 2000; Smogorzewska *et al*, 2000; Cook *et al*, 2002).

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In most organisms, telomere repeats contain guanine triplets (Zakian, 1995). Several studies have shown that oxidative stress induces oxidative DNA base damage, preferentially in telomeric triple guanines *in vitro*. In addition, oxidized guanines occur at either the 5' or the middle G in telomeric guanine triplets (Henle *et al.*, 1999; Kawanishi *et al.*, 1999; Oikawa and Kawanishi, 1999), and thus may act as traps of electrons transferring across the base stack (Saito *et al.*, 1998; Yoshioka *et al.*, 1999). It is not yet known whether 8-oxoG or other oxidative guanine lesions accumulate in telomeres *in vivo*. Given the substrate specificity of Ogg1p in excising 8-oxoG and FapyG, we tested whether ablation of Ogg1p might lead to increased oxidative guanine lesions in telomeres, which in turn could affect telomere length in *S. cerevisiae*. Here we present the evidence that loss of Ogg1p results in an increase in oxidized guanines in telomeres. Such base damage attenuates the telomeric association of telomere protein complex and alters telomere length homeostasis through promoting telomerase- and recombination-dependent pathways of telomere elongation.

Results

Ablation of Ogg1p augments oxidative guanine lesions in telomeres of yeast cells

To determine the level of oxidative guanine lesions in telomeres, restricted genomic DNAs were treated with *Escherichia coli* Fapy DNA glycosylase (Fpg). Fpg excises oxidative guanine lesions, resulting in abasic sites that are further processed by the lyase activity of Fpg to single-strand breaks (Klungland and Bjelland, 2007). The extent of DNA fragmentation caused by Fpg reflects the frequency of oxidative DNA lesions, that is, DNAs harbouring oxidative base lesions appear as smaller fragments after Fpg treatment (Figure 1A). Supplementary Figure S1 illustrates the method of estimating Fpg-sensitive lesions in the telomeric DNA according to the published formula (Kruk *et al.*, 1995).

To validate the method, *S. cerevisiae* genomic DNA was treated *in vitro* with increasing concentration of hydrogen peroxide (H_2O_2) plus Cu^{2+} and analysed as described above. As shown in Figure 1B, higher doses of H_2O_2 treatment caused Fpg-dependent increase of shorter telomere DNA fragments. Using the quantification and calculation formulae described in Supplementary Figure S1, we observed that more Fpg-sensitive lesions were detected in the telomeric DNA exposed to higher doses of H_2O_2 . We next examined the genomic DNA isolated from cells briefly treated with 5 mM H_2O_2 . Similar to *in vitro* treatment, *in vivo* H_2O_2 treatment also enhanced the level of Fpg-sensitive lesions in the telomeric DNA (Figure 1C). Thus, the method is feasible in estimating Fpg-sensitive lesions in telomeres. The *in vivo* data also support that telomeric oxidative guanine damage in *S. cerevisiae* can be enhanced by exogenous oxidative stress.

Next, we compared the level of oxidative guanine lesions in wild-type and *ogg1Δ* cells at ~90 generations after sporulation. Although there were fluctuations in the frequencies of Fpg-sensitive lesions among individual clones, *ogg1Δ* cells repeatedly showed more Fpg-sensitive lesions than wild-type cells (Figure 1D). Thus, deletion of *OGG1* associates with a reproducible increase in Fpg-sensitive lesions in the telomeric DNA. The complementation of *ogg1Δ* strain with the wild-type *OGG1* gene adjusted the frequency of base lesions

Figure 1 Augmentation of oxidative guanine lesions in the telomere of *ogg1Δ* strain. (A) Schematics of telomere guanine damage detection. A telomere DNA fragment with guanine lesions is converted into smaller fragments by Fpg treatment. (B) Genomic DNA was treated with indicated concentrations of H_2O_2/Cu^{2+} . Telomeric DNA fragments with (+) or without (–) Fpg treatment were detected by Southern blot analysis using a radiolabelled telomere probe. Fold changes in H_2O_2 -treated samples were derived from calculation against the frequency of Fpg-sensitive lesions in the untreated sample. (C) Wild-type cells in log-phase growth were treated with 5 mM H_2O_2 . Genomic DNA was analysed as in (B). (D) Genomic DNA from wild type (WT), *ogg1Δ* mutant, and *ogg1Δ* mutant complemented with the wild-type *OGG1* gene was analysed as in (B). Representative clones are shown. Values shown as mean $\pm$ s.e. were obtained from three experiments with independent clones.

to about the wild-type level (Figure 1D). These results support that Ogg1p has an important role in excising oxidized guanines in telomere in *S. cerevisiae*.

OGG1 deletion results in telomere lengthening and increased telomere heterogeneity

To study the impact of *ogg1* deficiency on telomere length homeostasis, we examined telomere length in wild-type and *ogg1Δ* strains. The heterozygous *ogg1::HIS3MX/OGG1* diploid strain was sporulated to isolate *ogg1::HIS3MX* and

wild-type spores. These spores were successively streaked and their genomic DNAs were isolated at different generations. Genomic DNA was digested with *XhoI* that cuts in *Y'* elements. Telomere length was examined by Southern blot analysis using a telomere-specific probe. The terminal restriction fragments (TRFs) around 1.2 kb represent about two-thirds of the telomeres that contain one or more *Y'* elements (Louis and Haber, 1990; Yamada *et al*, 1998). In the following analysis, the length of TRFs was subtracted from that of *Y'* element fragment (~875 bp) to yield *Y'*-containing telomere length (LeBel *et al*, 2006).

Through a genome-wide screen for telomere length in *S. cerevisiae* deletion mutants, Askree *et al*, (2004) observed that *ogg1Δ* strain displayed longer *Y'*-containing telomeres. Similarly, we observed that deletion of the *OGG1* gene led to lengthening of *Y'*-containing telomeres (Figure 2A). Telomere lengthening was also observed when the *OGG1* gene was deleted using different gene targeting markers (see below) and in a different strain background (data not shown). Furthermore, telomere lengthening seemed to be uneven among *Y'*-containing telomeres, as some telomeres remained

in the wild-type length and others were longer than wild type. These unevenly elongated telomeres caused increased telomere heterogeneity in *ogg1Δ* strain. Moreover, although wild-type cells showed relatively stable telomere length (*Y'*-containing telomeres ranging from 335 to 344 bp), the telomere length of *ogg1Δ* strain increased with progressive passages, reaching a plateau (370–407 bp) after ~65 generations (Figure 2A). This limited telomere lengthening may be because endogenous oxidative damage is limiting or a compensatory response is induced to set base damage and repair to a new equilibrium. Surprisingly, non-lethal doses of H_2O_2 did not lead to telomere length change in wild-type and *ogg1Δ* strain (data not shown). We thus tested whether an increase in endogenous oxidative stress could enhance telomere-lengthening phenotype in *ogg1Δ* strain. Tsalp, one of peroxiredoxins, has the most potent ability to scavenge H_2O_2 (Park *et al*, 2000). To increase endogenous H_2O_2 in *ogg1Δ* strain, *ogg1Δ tsalΔ* double mutant was generated. The *tsalΔ* mutant showed longer telomeres than wild type, and *ogg1Δ tsalΔ* double mutant displayed longer telomeres than either single mutant (Supplementary Figure S2). Although deletion of

Figure 2 Telomere lengthening in *ogg1Δ* strain. (A) Wild-type and *ogg1Δ* spores from *ogg1Δ/OGG1* diploid strain were re-streaked successively on YEPD plates. Genomic DNA isolated at indicated generations was digested with *XhoI* and analysed by Southern blotting using a radiolabelled polyTG₁₋₃ probe. Representative clones are shown. *Y'*-containing telomere length is subtracted from the *Y'* element fragment (~875 bp) and is shown at the bottom. The graphs represent the mean ± s.e. from at least three experiments. (B, C) Three independent, freshly dissected wild-type and *ogg1Δ* spores were re-streaked successively on YEPD plates. At ~115 generations telomeres were measured by Southern blotting and PCR analysis. (B) Genomic DNA was digested with *XhoI* and analysed by Southern blot analysis using a radiolabelled *Y'* probe (upper panel) and a *TUB2* probe for DNA loading control (lower panel). The ratio of subtelomeric *Y'* elements and *TUB2* DNA is shown at the bottom. (C) PCR products of telomeres at the left arm of chromosome 15 (upper panel) and the right arm of chromosome 6 (lower panel) were separated on 2% agarose gel and stained with ethidium bromide. Brackets: *Y'*-containing telomeres. Open arrows: non-*Y'* telomeres. Asterisks (*): *Y'* elements. Arrowhead: 700-bp telomeric DNA fragment as migration control. M: molecular weight marker.

TSA1 may affect telomeres length through mechanisms other than telomere DNA damage, these data nevertheless imply that increased endogenous oxidative stress can exacerbate telomere lengthening in *ogg1Δ* strain.

Unlike Y'-containing telomeres, subtelomeric Y'-elements did not show obvious amplification in *ogg1Δ* strain (Figure 2A) and therefore unlikely contribute to telomere elongation. To further confirm this, three individual spores of wild-type and *ogg1Δ* strains were re-streaked on plates successively. At ~115 generations, the length of Y' elements was measured by Southern blot analysis using a probe specific for the Y' elements (Maxwell *et al.*, 2004). This probe detects two classes of Y' elements, namely, Y' long (~6.7 kb) and Y' short (~5.2 kb). It also detects the ~1.2-kb telomere restriction fragments containing telomere repeats and part of Y' element (Louis and Haber, 1990; Yamada *et al.*, 1998). Similar to the result shown in Figure 2A, the Y'-containing telomeres in all three *ogg1Δ* clones were longer, 426 ± 55 bp in *ogg1Δ* cells and 336 ± 35 bp in wild-type cells (Figure 2B). In comparison to the control DNA (*TUB2*), the level of subtelomeric Y' elements was not altered in *ogg1Δ* cells, demonstrating that lengthening did not occur in the sub-telomeric region, but was limited to telomeres (Figure 2B). This conclusion was further confirmed by sequencing analysis of telomeres (Supplementary Figure S3 and see below). These results suggest that *OGG1* deletion leads to lengthening of telomeric DNA repeats.

The individual bands above the Y'-containing telomere fragments represent non-Y'-containing single telomeres (Lundblad and Blackburn, 1993), some of which also appeared longer in *ogg1Δ* strain (Figure 2A). We further investigated lengthening of non-Y'-containing telomeres by measuring telomere length of chromosome 6 right arm (VIR) and chromosome 15 left arm (XVL) from three individual clones of wild-type and *ogg1Δ* strains. The length of VIR telomere increased in all three *ogg1Δ* clones and XVL telomere was slightly lengthened in one of three *ogg1Δ* clones (Figure 2C). The PCR products from VIR telomere were cloned into plasmids and sequenced. Sequencing revealed that the length of VIR telomeres was 379 ± 35 bp in *ogg1Δ* mutant and 263 ± 30 bp in wild type (Supplementary Figure S3). Thus, similar to Y'-containing telomeres, non-Y'-containing telomeres also displayed variable elongation in *ogg1Δ* strain.

Telomere lengthening in *ogg1* strain is due to *OGG1* deficiency

To test whether telomere lengthening in *ogg1Δ* strain resulted from ablation of Ogg1p, we performed complementation assay by introducing the wild-type *OGG1* gene into *ogg1Δ* strain. Although *ogg1Δ::KanMX4* haploid strain carrying empty vector showed elongated telomeres, *ogg1Δ::KanMX4* haploid strain carrying the plasmid with the *OGG1* gene displayed wild-type telomere length (Figure 3A). Thus, disruption of *OGG1* contributes to telomere lengthening in *ogg1Δ* strain. As the *OGG1* gene deletion using different targeting markers gave rise to the same telomere-lengthening phenotype (Figures 2 and 3), these results further strengthen the idea that telomere lengthening is the result of *OGG1* deletion, but not of specific marker insertion at the *OGG1* locus.

Besides nuclear DNA repair, Ogg1 is also known to repair oxidative base damage in mitochondrial DNA (Singh *et al.*,

2001). To test whether *ogg1* deficiency mediated-mitochondrial DNA damage affected telomere length, we deleted mitochondrial DNA in wild-type and *ogg1Δ* strains. Loss of mitochondrial DNA (*rho*⁰) did not affect telomere length in wild-type and *ogg1Δ* cells (Supplementary Figure S4), suggesting that telomere lengthening in *ogg1Δ* strain is independent of mitochondrial DNA damage.

One complicating factor in characterizing *ogg1Δ* strain phenotype is the vicinity of the *PIF1* gene. As Pif1p has been shown to be a negative regulator of telomere length (Schulz and Zakian, 1994; Boule and Zakian, 2007; Vega *et al.*, 2007; Smith *et al.*, 2008), Askree *et al.* (2004) have suggested that the telomere-lengthening phenotype observed in *ogg1Δ* strain could be caused by altered *PIF1* expression or function. We thus checked whether the expression of *PIF1* was altered in *ogg1Δ* strain. The mRNA level of *PIF1* did not show any detectable changes between wild-type and *ogg1Δ* strains (Figure 3B). Thus, disruption of *OGG1* does not alter *PIF1* expression. To further verify whether telomere lengthening in *ogg1Δ* strain depended on Pif1p function, we generated *ogg1Δ pif1Δ* double mutant. The *pif1Δ* mutant showed significant longer telomeres than *ogg1Δ* mutant. Moreover, the double mutant displayed longer telomeres than either single mutant (Figure 3C), establishing that *ogg1Δ* and *pif1Δ* mutants affect telomere length through different mechanisms. On the basis of these findings, we conclude that deregulation of telomere length in *ogg1Δ* strain is Pif1 independent.

Telomere lengthening in *ogg1Δ* strain is dependent on telomerase and/or homologous recombination

Telomerase and Rad52p-mediated homologous recombination have key roles in telomere length maintenance (Greider, 1996; Blackburn, 2001). To investigate whether these pathways are responsible for telomere lengthening in *ogg1Δ* strain, we removed *EST2* (the catalytic subunit of telomerase) or *RAD52* (a key component of the homologous recombination pathway) in *ogg1Δ* strain. Heterozygous *ogg1Δ/OGG1 est2Δ/EST2* and *ogg1Δ/OGG1 rad52Δ/RAD52* diploid strains were sporulated to obtain single and double mutants. These strains were streaked on YEPD plates successively and their telomere length was measured at different passages. As described previously (Lendvay *et al.*, 1996; Counter *et al.*, 1997; Lingner *et al.*, 1997), telomeres in *est2Δ* strain shortened after successive re-streaks (Figure 4A). Telomere length in *ogg1Δ est2Δ* clones was significantly shorter than that of *ogg1Δ* cells but similar to *est2Δ* cells (Figure 4A), suggesting that telomerase participates in telomere lengthening in *ogg1Δ* strain.

Deletion of *RAD52* alone had no effect on telomere length in *S. cerevisiae* with intact telomerase (Dunn *et al.*, 1984; Le *et al.*, 1999) and Figure 4B). Rad52p is, however, essential in telomere length maintenance in the survivors of telomerase-defective strains, after telomeres are exhausted and trigger cell death (Lundblad and Blackburn, 1993; Lendvay *et al.*, 1996). Interestingly, telomere length in *ogg1Δ rad52Δ* clones was shorter than that of *ogg1Δ* cells, but similar to that in wild-type and *rad52Δ* cells (Figure 4B). It is possible that Rad52p may mediate telomere lengthening in the presence of telomerase, when *OGG1* is deleted.

It is noteworthy that some *ogg1Δ est2Δ* clones showed a heterogeneous telomere length population, ranging from

Figure 3 Telomere lengthening in *ogg1Δ* strain is Ogg1 dependent and Pif1 independent. (A) Freshly dissected spores with indicated genotype were re-streaked three times on SC-Ura plates. Genomic DNA was digested with *XhoI* and analysed by Southern blotting using a radiolabelled polyTG₁₋₃ probe. (B) PCR products of *PIF1* and *TUB1* cDNA from wild-type and *ogg1Δ* strain were separated on 1.5% agarose gel. Gel was stained with ethidium bromide (top panel). The bands were quantified using Quantity One software and plotted (bottom panel). (C) Spores with the indicated genotype were generated from *ogg1Δ/OGG1 pif1Δ/PIF1* diploid strain and re-streaked successively on YEPD plates. Genomic DNA isolated at indicated generations was digested with *XhoI* and analysed by Southern blot using a Dig-labelled polyUG₁₋₃ probe. Representative clones are shown. Calculated Y'-containing telomere length is shown at the bottom. The graphs represent the mean $\pm$ s.e. from at least three experiments. Brackets: Y'-containing telomeres.

longer telomeres to shorter telomeres, although with a peak value of telomere length similar to that in *est2Δ* strain (Supplementary Figure S5A). Thus, telomere lengths in these *ogg1Δ est2Δ* clones had been either fully or partially reset to that of *est2Δ* strain. Similarly, some *ogg1Δ rad52Δ* clones showed a heterogeneous telomere length population with a peak value of telomere length intermediate between those in wild type and *ogg1Δ* strains (Supplementary Figure S5B). To test whether the heterogeneous telomere length population reflects that both telomerase and Rad52p may be required for a complete rescue of telomere length in these *ogg1Δ est2Δ* and *ogg1Δ rad52Δ* clones, we deleted the *OGG1* gene in *est2Δ rad52Δ* strains. All the clones of *ogg1Δ est2Δ rad52Δ* triple mutant displayed identical telomere length and telomere length heterogeneity as *est2Δ rad52Δ* double mutant (Supplementary Figure S5C). This result suggests that inactivation of Ogg1p may activate both telomerase- and Rad52p-mediated homologous recombination pathways, and that rescue of *ogg1Δ*-mediated telomere lengthening requires

inactivation of both pathways. However, as a complete rescue of *ogg1Δ*-mediated telomere lengthening was observed in some *ogg1Δ est2Δ* and *ogg1Δ rad52Δ* clones (Figure 4), it is possible that only one of the telomerase and Rad52p pathways was activated in these clones. Mechanism(s) underlying the selective activation of different pathways (telomerase, Rad52, or both) in individual *ogg1Δ* clones remain(s) to be determined.

In telomerase defective strain, two types of survivors can emerge by maintaining telomeres via recombination. Type I survivors maintain telomeres by Y' element amplification, whereas type II survivors have increased telomere repeats. Type I survivors depend on Rad51p and Rad52p, whereas type II survivors depend on Rad50p and Rad52p (Le *et al.*, 1999; Teng and Zakian, 1999). To verify whether Rad50p and/or Rad51p may participate in the recombination of telomeres in *ogg1Δ* strain, heterozygous *ogg1Δ/OGG1 rad50Δ/RAD50* and *ogg1Δ/OGG1 rad51Δ/RAD51* diploid strains were sporulated to create single and double mutants.

Figure 4 Telomere lengthening in *ogg1Δ* strain depends on telomerase and Rad52p-mediated homologous recombination. Spores with the indicated genotype were generated from (A) *ogg1Δ/OGG1 est2Δ/EST2* diploid strain and (B) *ogg1Δ/OGG1 rad52Δ/RAD52* diploid strain. Freshly dissected spores were re-streaked successively on YEPD plates. At ~40 and 90 generations, genomic DNA was digested with *XhoI* and analysed by Southern blot using Dig-labelled polyUG₁₋₃ probe. Representative (A) *ogg1Δ est2Δ* clones and (B) *ogg1Δ rad52Δ* clones with fully rescued telomere lengthening are shown. Calculated Y'-containing telomere lengths (mean ± s.e.) from at least three experiments are shown at the bottom. Brackets: Y'-containing telomeres.

These strains were streaked on YEPD plates successively and their telomere length was measured at late passages (~115 generations) by Southern blot analysis. Removal of *RAD50*, but not *RAD51*, completely abolished telomere lengthening in some *ogg1Δ* clones (Supplementary Figure S6). Thus, deletion of the *OGG1* gene may trigger telomere recombination that mimics type II survivors of telomerase-defective mutants. These results further strengthen the idea that recombination is involved in telomere lengthening of *ogg1Δ* strain. As telomere recombination in *ogg1Δ* strain uses Rad50p, an HR protein that can maintain telomeres by telomere repeat amplification in type II survivors, it further supports the observation by Southern blot and sequence analysis that telomere repeats, but not subtelomeres, are amplified in *ogg1Δ* strain (Figure 2 and Supplementary Figure S3).

Telomeric guanine oxidation decreases the affinity of telomere protein complex to telomeres

Rap1p directly binds telomeric DNA and recruits its interacting partners, Rif1p and Rif2p, to telomeres, at which they negatively regulate telomere length (Blackburn, 2001; Bianchi and Shore, 2008). In *ogg1Δ* strain, oxidative guanine lesions may directly affect the binding of Rap1p to telomeres, which may diminish Rap1p's control in telomere lengthening. Thus, telomere lengthening could reflect decreased Rap1p level at telomeres in *ogg1Δ* strain.

We first examined whether 8-oxoG would structurally have any impact on telomeric DNA's interaction with Rap1p. The crystal structure of the DNA-binding domain of Rap1p in complex with telomeric DNA has been reported previously (Konig *et al.*, 1996). The DNA-binding domain of Rap1p is made of two HtH motifs. Each of them specifically associates with a G-rich region in the telomeric DNA (Supplementary Figure S7A and B). In both cases arginine (Arg) sidechains have a critical role in forming hydrogen bonds with O6 and N7 of two or three consecutive guanines (Supplementary Figure S7A and B). In addition, protein backbone carbonyl oxygen and Arg sidechains also form van der Waals contacts with the C8 of guanine and with the phosphosugar backbone. These interactions require guanine bases to form normal Watson-Crick base pairs and to assume the *anti* conformation. When guanine is oxidized to 8-oxo-G, three changes occur with the nucleotide (Supplementary Figure S7C): the N7 becomes a hydrogen bond donor and can no longer accept a proton from the guanidinium group of Arg; the C8 atom acquires an additional oxygen and thus becomes bulky and polar; and finally the phosphate backbone is pushed away from C8 by ~ 0.5 Å to accommodate the additional oxygen (Rechkeblit *et al.*, 2006). All these changes could cause steric and charge clashes with Rap1 protein, and thereby prohibit the protein-telomere DNA interactions (Supplementary Figure S7D).

To test whether oxidative guanine lesions indeed affect the binding of Rap1p to telomere DNA, various telomeric duplex oligonucleotides with two Rap1-binding sites, either carrying no 8-oxoG (unmodified, duplex 1) or 8-oxoG at different positions (modified, duplex 2–4) were used to examine their relative affinity to Rap1p by electrophoretic mobility shift assay (EMSA) (Figure 5A). In the presence of increasing amounts of various unlabelled competitors, the amount of radiolabelled unmodified duplex 1 bound by Rap1 was determined (Figure 5B). In comparison to the unmodified duplex 1, duplex 2 and 3 with a single or two 8-oxoG in one of two Rap1-binding sites showed decreased Rap1 binding by approximately three-fold, whereas duplex 4 with 8-oxoG in two Rap1-binding sites had an approximately 100-fold reduced Rap1 binding (Figure 5B and C). Thus, 8-oxoG in the Rap1p-binding sites adversely affects the binding of Rap1p to telomere DNA *in vitro*. This finding is consistent with the previous *in vitro* observation that the amount of 8-oxoG in telomere repeat sequences affects the affinity of mammalian telomere binding proteins, TRF1 and TRF2, to the telomeric DNA (Opresko *et al.*, 2005).

To test further whether binding of Rap1p to telomeres may be altered in *ogg1Δ* strain, we performed chromatin immunoprecipitation (ChIP). Formaldehyde-cross-linked chromatin DNA was sheared to a standard size (500–1000 bp)

Figure 5 Oxidized guanines in the telomeric DNA inhibit Rap1 binding *in vitro*. **(A)** Sequence of the oligonucleotides used in the EMSA assay. Boxed sequence indicates Rap1-binding sites. G = O denotes 8-oxoG. **(B)** EMSA analysis of the affinity of purified Rap1p on the oligonucleotides with or without 8-oxoG. Unmodified oligonucleotides (duplex 1) were 32 P-labelled, incubated with purified Rap1 protein in the absence or presence of 1-, 3-, 10-, 30-, 100-, 1000-, or 3000-fold molar excess of unlabelled competitor oligonucleotides. Free and Rap1-bound 32 P-labelled duplex 1 are indicated by arrows and arrowheads, respectively. Unlabelled competitor oligonucleotides competed with 32 P-labelled duplex 1 in Rap1 binding and led to a decrease in the amount of Rap1-bound 32 P-labelled duplex 1 at various degrees. **(C)** Autoradiography in **(B)** was quantified using ImageQuant software. The fraction of Rap1-bound 32 P-labelled duplex 1 was plotted against fold change of molar excess of competing duplexes. The value for samples in which no competing oligonucleotide was added was set to 100%. IC₅₀ of each competing duplex was calculated.

to keep most telomeres intact. Telomere DNA bound by Rap1p was immunoprecipitated using antibody against Rap1p and assessed by dot-blot using a telomere-specific probe. *ARO1* was probed as an internal control. To account for differences in telomere length in wild-type and *ogg1Δ* strain, the amount of precipitated fragments was expressed as a percentage of the input telomeric DNA. Under these conditions, the efficiency of ChIP is proportional to the amount of Rap1p bound to telomeres. The *ogg1Δ* strain displayed a moderate decrease in the amount of telomere DNA bound by Rap1p (Figure 6A). This reduction is consistent with a mild increase in telomere length in *ogg1Δ* strain. Thus, *in vitro* and *in vivo* data support the idea that oxidative guanine lesions in telomeres reduce telomere-bound Rap1p level, which may consequently discharge Rap1p from negatively regulating telomere length in *ogg1Δ* strain. In addition to telomere DNA repeats, Rap1p can also bind non-telomeric regions (Lieb *et al.*, 2001). To further strengthen our observation that decrease in Rap1p binding to DNA occurred in the telomeric region, we examined the binding of Rif2 to telomeres in *ogg1Δ* strain by ChIP. Similar to Rap1, telomere-bound Rif2 decreased in *ogg1Δ* strain. Furthermore, the level of Rap1p and Rif2p and their pull-down efficiency were comparable between wild-type and *ogg1Δ* strains (Supplementary Figure S8). These results support that the affinity of telomere protein complex, Rap1p-Rif1p-Rif2p, to telomeres is decreased when Ogg1 is depleted.

OGG1 deletion does not significantly alter telomere position effect (TPE), but prolongs cell viability of telomerase null strain

To study the biological consequence of *OGG1* deletion in telomere function, we investigated telomere position effect (TPE) and telomere-related cell viability. Genes inserted close to telomeres are transcriptionally silenced, which is termed TPE. Telomere length and Rap1 binding can influence sub-telomeric transcription silencing (Kyrion *et al*, 1993). To determine whether increased telomere length and decreased telomere-bound Rap1 in *ogg1Δ* strain had any impact on TPE, we examined the extent of telomeric silencing of *URA3* and *ADE2* in *ogg1Δ* strain. Deletion of *OGG1* in a strain carrying *URA3* near a telomere did not cause significant increase in the survival on 5FOA plates (average 15% in *ogg1Δ* strain and average 9% in the parental strain; Supplementary Figure S9A). Similarly, an *ogg1Δ* mutant carrying *ADE2* near a telomere showed similar frequencies of red or sectorized colonies as its parental strain (average 30% in *ogg1Δ* strain and average 21% in the parental strain; Supplementary Figure S9B). In both assays, the marginal difference between wild-type and *ogg1Δ* strains was on borderline. These results suggest that ablation of Ogg1p does not significantly affect telomeric silencing.

In *S. cerevisiae*, telomerase deficiency can lead to progressive telomere shortening and eventually a dramatic decline in cell viability (Singer and Gottschling, 1994; Lendvay *et al.*, 1996). As *ogg1Δ* strain displayed altered telomere length and recombination, we examined whether *OGG1* deletion could

Figure 6 Telomere-bound Rap1p and Rif2p decreased in *ogg1Δ* strains. ChIP analysis of the association of (A) Rap1p and (B) Rif2p with the telomeric DNA (left, upper panel) or a control locus *ARO1* (left, lower panel) in two independent wild-type and *ogg1Δ* strains. Right panel: ChIP efficiency represents the fraction of telomeric DNA immunoprecipitated with anti-Rap1p or anti-HA antibody after normalization to the value for wild type in each independent experiment. The wild-type value was set to 1. Error bars represented s.e. values from at least three independent experiments. A paired *t*-test was used to determine significance.

have any impact on cell viability of *est2Δ* strain. Similar to previous observations, *est2Δ* strain displayed loss of cell viability after serial passages in culture and subsequent emergence of survivors in continuous culture (Figure 7). Interestingly, some colonies of *ogg1Δ est2Δ* double mutant showed delayed loss of cell viability, in comparison with *est2Δ* single mutant (Figure 7). There was no difference in the growth rate between *ogg1Δ est2Δ* double and *est2Δ* single mutants (data not shown), which excludes the possibility that *OGG1* deletion may alter cellular proliferation of *est2Δ* strain, which may in turn lead to delay in senescence. The delayed senescence in *ogg1Δ est2Δ* double mutant correlated with the existence of longer telomeres within the heterogeneous telomere length population (Supplementary Figure S5A). Thus, prolonged cell viability in *ogg1Δ est2Δ* strain may be attributed to these long telomeres. Deletion of *OGG1* in *est2Δ rad52Δ* strains did not lead to prolonged cell viability (Figure 7) or telomere elongation (Supplementary Figure S5C). It is thus possible that the delayed loss of cell viability in *ogg1Δ est2Δ* strain may be due to the Rad52p-mediated telomere lengthening.

Discussion

Telomeric sequences have a high G content and may thus be particularly prone to 8-oxoG and FapyG lesions. The impact

of these base lesions and their corresponding repair on telomeres are not clear. Here, we report that (1) oxidized guanines increase in the telomeric region of *S. cerevisiae* when a DNA glycosylase, Ogg1p, is inactivated, (2) *OGG1* deletion leads to telomere lengthening that depends on telomerase and/or homologous recombination pathways, (3) telomeric, oxidized guanines attenuate telomere-bound telomere protein complex, which may perturb their regulation of telomere length. To our knowledge, this is the first study demonstrating that oxidative guanine lesions occur in telomeres and have an impact on telomere length regulation *in vivo*, and that the BER pathway functions in telomere DNA damage repair and telomere length regulation.

Telomere lengthening in *ogg1* deletion mutant

Telomere lengthening in *ogg1Δ* strain exhibits several characteristics. First, Southern blot analysis shows that telomere length seems to be heterogeneous, with telomere sizes ranging from wild type to longer than wild type. PCR and sequencing analysis also confirms that telomere lengthening is unequal in single telomeres (chromosome 6 right arm and chromosome 15 left arm). This unequal telomere lengthening may result from differential oxidative base damage across telomeres, that is, some telomeres have no base damage, and others harbour more or less base damage. The recombination-dependent telomere elongation may also contribute to telomere length heterogeneity in *ogg1Δ* strain. Second, both Southern blot and sequence analysis reveal that an increase in telomere length is the consequence of increased telomere repeats and that the length of the subtelomeric region is not affected. Third, telomere lengthening is not accompanied by significant changes in telomere silencing.

Our data support the idea that telomere lengthening is associated with increased oxidative guanine damage in *ogg1Δ* strain. It is noteworthy that previous findings suggest that oxidative stress leads to telomere shortening in mammalian cells (von Zglinicki, 2000). However, given the fact that the previous experiments in mammalian cells were conducted under oxidative stress conditions that could cause DNA strand breaks and that telomere lengthening is also observed in *Ogg1*-deficient mice (Wang *et al*, unpublished data), it is possible that different types of oxidative DNA damage, for example, DNA strand breaks or base lesions may have different impact on telomere length (either lengthening or shortening). Single-strand DNA breaks (SSBs) resulting from Ogg1 lyase activity (i.e. incising the backbone at sites of oxidized bases) may also impact telomere length differently than the oxidized bases themselves. In the absence of Ogg1, there may be less incision of oxidized bases, and thus fewer SSBs and less telomere attrition.

Telomere lengthening is a bona fide phenotype of the *OGG1* gene deficiency

Several lines of evidence support that telomere lengthening is due to ablation of Ogg1p function in repairing telomeric base damage. We demonstrate that telomere lengthening in *ogg1Δ* strain can be complemented by *OGG1* and can occur without mitochondrial DNA. Furthermore, we exclude the possible involvement of the adjacent *PIF1* gene (Askree *et al*, 2004).

Pif1p is a helicase, which negatively regulates telomere length by removing telomerase from chromosome ends through unwinding of the telomeric DNA and telomerase-

Figure 7 Cellular viability in *ogg1*, *est2*, *rad52* single, double and triple deletion mutants. Spores with indicated genotype were generated from heterozygous *OGG1/ogg1 EST2/est2 RAD52/rad52* diploid strain. Freshly dissected spores were inoculated at $\sim 10^4$ cells per ml and grown overnight to $\sim 10^8$ cells per ml. The culture was then diluted to 10^4 cells per ml and continued to grow. The cycles were repeated for 8 days. At day 2, 4, 6 and 8, aliquot of cultures was taken. Cells were five-fold serially diluted and spotted onto YEPD plates. The plates were incubated for 2 days at 30°C before photography. At day 4, loss of cell viability was observed in *est2Δ* and *est2Δ rad52Δ* mutants, and survivors appeared only in *est2Δ* mutants during continuous culture. Removal of *OGG1* delayed the loss of cell viability in *est2Δ* strain, but not in *est2Δ rad52Δ* strain. NA: no survivors.

RNA duplexes (Boule *et al.*, 2005; Zhang *et al.*, 2006; Boule and Zakian, 2007). Our data suggest that deletion of *OGG1* may lead to telomerase-dependent telomere overelongation. Although both Pif1p and Ogg1p can regulate telomere length through telomerase-mediated telomere elongation, they may act on telomerase by distinct mechanisms. We suggest that deletion of *OGG1* may promote the access of telomerase to telomeres through attenuation of Rap1p–Rif1p–Rif2p complex to telomeres.

Mechanisms of telomere lengthening in *ogg1Δ* strain

The *ogg1Δ* strain displays telomere lengthening that is telomerase- and/or recombination dependent, suggesting that telomerase and recombination pathways become more active in this mutant strain. Telomere length homeostasis is maintained through interplay among telomerase extension, telomere replication and end processing, and capping by the telomere protein complex. Several studies have reported that the number of Rap1p–Rif1p–Rif2p complex molecules at telomeres adversely influences telomere length in a telomerase-dependent manner (Marcand *et al.*, 1997, 1999; Wotton and Shore, 1997; Ray and Runge, 1999; Levy and Blackburn, 2004; Teixeira *et al.*, 2004). The *ogg1Δ* strain displays a reduced binding of Rap1p and Rif2p to telomere DNA, which could decrease their negative control over telomerase and disrupt the balance between telomere protein complex and telomerase in telomere length homeostasis. Furthermore, genetic and EMSA analyses suggest that increased oxidative stress can exacerbate telomere lengthening and that the level of oxidized guanines inversely correlates with the amount of Rap1p molecules at telomeres. Thus, it is possible that the quantity of oxidative base lesions determines the degree of telomere lengthening, for example, a telomere harbouring more base lesions may have fewer telomere-bound Rap1p–Rif1p–Rif2p complex molecules and thus allow increased access of telomerase to add more telomere repeats. This may explain the observed unequal telomere lengthening among telomeres, possibly derived from differential oxidative base damage across telomeres.

Telomere length can be maintained by recombination, although this event has been considered to be significant

only when telomerase is defective and telomeres become critically short (Lundblad and Blackburn, 1993; Singer and Gottschling, 1994; Lendvay *et al.*, 1996). This observation is further supported by the fact that *rad52Δ* strain does not show telomere length abnormality in telomerase-positive cells (Dunn *et al.*, 1984; Le *et al.*, 1999) and seems to elongate short telomeres at a similar rate as wild-type strain (Marcand *et al.*, 1999). In this study, we showed that telomere lengthening could occur through Rad52p-mediated recombination in *ogg1Δ* clones. Thus, deletion of *OGG1* may induce recombination in telomeres. We propose that base oxidation and its impact on Rap1p binding to telomeres may contribute to telomere recombination in *ogg1Δ* strain. It has been reported that regions with a high frequency of recombination are preferentially located within chromosomal regions with a high density of 8-oxoG (Ohno *et al.*, 2006), and that recombination rates are substantially increased in BER-deficient yeast cells harbouring mild oxidative DNA damage in the genome (Swanson *et al.*, 1999). It is possible that in *ogg1Δ* strain telomeric oxidative guanine lesions may promote Rad52-mediated illegitimate recombination that serves as a compensatory response to tolerate oxidative DNA damage. Furthermore, deletion of the telomere-binding protein, Taz1, leads to replication fork stalling in the telomere of *Schizosaccharomyces pombe* (Miller *et al.*, 2006), which may invoke recombination, and disruption of Rap1 results in elevated recombination in the telomere of *K. lactis* (Cesare *et al.*, 2008). As oxidative base lesions can attenuate Rap1's binding to telomeric DNA, it is possible that altered Rap1 binding may affect telomere recombination. Alternatively, Ogg1 can inhibit Rad52p strand annealing and exchange activity (de Souza-Pinto *et al.*, 2009), and removal of Ogg1 would, therefore, relieve this inhibition and activate the recombination pathway.

The observation that disruption of either telomerase or homologous recombination abolishes telomere lengthening in *ogg1Δ* strain suggests that one of the pathways is responsible for the telomere overelongation. However, it is important to point out that partial telomere lengthening can still happen in some *ogg1Δ* clones when either telomerase or homologous recombination is inactivated. Nevertheless,

inactivation of both pathways can fully rescue telomere lengthening in *ogg1Δ* mutants. These data suggest that in some *ogg1Δ* clones both pathways are used to elongate telomeres. Thus, telomerase and recombination pathways may independently or corporately participate in telomere length regulation in *ogg1Δ* strain. It remains unclear whether certain conditions (e.g. quantity and quality of genome and/or telomere lesions) may selectively trigger the activation of telomerase, recombination, or both pathways.

Repair of oxidative guanine lesions in telomeres

More than one DNA glycosylases are involved in oxidative base lesion repair in *S. cerevisiae*. DNA glycosylases, Ntg1 and Ntg2, are found to repair oxidative base lesions, but primarily oxidized pyrimidines (Memisoglu and Samson, 2000). In mammals, Neill DNA glycosylase has been reported to repair FapyG (Hu *et al*, 2005); however, a Neill orthologue is yet to be identified in *S. cerevisiae*. In this study, we have shown that higher levels of oxidative guanine lesions are detected in the telomeres of *ogg1Δ* strain. Furthermore, *OGG1* deficiency leads to telomere lengthening. Thus, Ogg1p may be a primary DNA glycosylase involved in repairing oxidized guanines in telomeres and engaging in telomere length regulation in *S. cerevisiae*.

Telomere lengthening in *ogg1Δ* strain could be exacerbated by increasing endogenous oxidative stress through the elimination of a peroxiredoxin, Tsa1p. This result suggests that telomere lengthening may be determined by the level of oxidative damage. In *ogg1Δ* strain, telomere lengthening eventually reaches a steady-state level, implying that endogenous oxidative damage on telomeric guanines may be limiting *in vivo*. In addition, other back-up repair machinery may also be active in the absence of Ogg1p and may partially repair oxidized base damage. Multiple pathways with overlapping specificities are involved in the removal of spontaneous DNA damage in *S. cerevisiae* (Swanson *et al*, 1999). The nucleotide excision repair (NER) pathway generally removes bulky DNA lesions, but has also been implicated in the repair of 8-oxoG (Huang *et al*, 1994; Scott *et al*, 1999; Boiteux *et al*, 2002). NER may serve a role in restricting telomeric oxidative guanine damage in *ogg1Δ* strain. Furthermore, *OGG1* deficiency may promote recombination (de Souza-Pinto *et al*, 2009) that could be involved in the processing of DNA lesions normally repaired by the BER pathway.

Biological consequence of oxidative guanine lesions in telomeres

Our studies demonstrate that oxidative damage can occur in the telomeric guanines in *S. cerevisiae*. Besides high content of guanine bases in the telomere sequence, telomere heterochromatin structure and telomere-associated proteins may mask oxidized guanines from being accessible to BER for efficient repair and contribute to guanine damage in telomeres. In addition, guanine itself is prone to oxidation compared with other bases and triple guanines are even more prone to oxidation (Saito *et al*, 1998; Yoshioka *et al*, 1999). Perhaps one biological importance of telomeric guanine oxidation is that its capacity to function as a 'sink' for oxidative damage may have a protective effect on other regions of the genome, protecting them from DNA damage.

Absence of Ogg1p results in moderate telomere lengthening and Rap1 dissociation with telomeres. These changes, however, do not significantly alter telomere silencing in *ogg1Δ* strain. Thus, endogenous oxidative base damage in telomeres is unable to exert significant effect on telomere position effect. In *S. cerevisiae*, telomerase deficiency, for example, *EST2* deletion, leads to telomere shortening and ultimately cell death. However, a small portion of the culture survives as a result of telomerase-independent telomere maintenance through recombination (Lundblad and Blackburn, 1993; Lendvay *et al*, 1996). Interestingly, deletion of *OGG1* delays loss of cell viability in some clones of *est2Δ* mutants, but it does not have such effect in *est2Δ rad52Δ* mutants. Thus, recombination may have had a role in the rescue of loss of cell viability in *est2Δ* mutant, when oxidized guanines are present in telomeres.

In summary, we report that oxidized guanines are increased in the telomeric region of *ogg1Δ* DNA glycosylase deletion strain and are associated with telomere lengthening in *S. cerevisiae*. In addition, we provide insight into genetic and molecular mechanisms underlying telomere lengthening. Our data demonstrate that Ogg1p is an important repair factor in telomeric guanine damage repair and telomere length regulation. Given the evolutionary conservation of Ogg1 proteins from yeast to mice and humans, we speculate that similar mechanisms may prevail to repair oxidatively damaged guanines in telomeres and impact telomere length equilibrium in mammals.

Materials and methods

Yeast strains and genetic procedures

Yeast transformation, media, genetic procedures, and cell viability assay have been described previously (Gietz *et al*, 1992; Singer and Gottschling, 1994; Lendvay *et al*, 1996; Burke *et al*, 2000). Yeast strains used in this study are described in the Supplementary data.

Detection of oxidative base lesions in the telomeric DNA

DNA isolation and identification of oxidative base lesions in telomeres were performed as previously described with minor modifications (Kruk *et al*, 1995). In brief, DNA was isolated from spheroplasts by precipitation with high concentration of salt. A total of 10 µg of DNA from wild-type and *ogg1Δ* strains was treated with *AluI*, *HaeI*, *HinfI*, and *MspI* at 37°C overnight. DNA was then treated with or without 8 U of *E. coli* formamidopyrimidine-DNA glycosylase (New England Biolabs) at 37°C for 15 min. Fpg was inactivated by heating at 60°C for 15 min. Single-stranded DNA fragments were separated on 1% alkaline agarose gel according to their sizes. Single-stranded telomere DNA fragments were detected by Southern blot analysis using ³²P-labelled polyTG₁₋₃ fragments and visualized by autoradiography. The extent of increased signals towards smaller DNA fragments is proportional to the amount of Fpg-sensitive lesions present within the telomeric DNA and can be extrapolated to estimate the number of lesions (Kruk *et al*, 1995) as described in Supplementary Figure S1.

RNA preparation and RT-PCR

Total RNA was prepared using TRIzol (Gibco BRL). The cDNA was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen) and gene-specific primers according to the manufacturer's instruction. PCR amplification of *PIF1* and *TUB1* cDNA was conducted using Taq DNA polymerase (New England Biolabs). Primers used for *PIF1* or *TUB1* genes were 5'-TCTGATTCCGATGATTGGGA-3' and 5'-TTCAACGCTGGTCTTAATGG-3' or 5'-AATCCAAGCTGGAATTGCG-3' and 5'-CCAAATTGGTTGAAATTCG-3', respectively.

DNA preparation and Southern blot analysis

DNA was isolated from exponentially growing cultures (Ausubel *et al*, 2001) and digested with *Xho*I. Southern blot analysis was performed using Dig-labelled polyUG_{1–3} fragments or radiolabelled polyTG_{1–3} fragments. DNA was quantified using ImageQuant software. The length of terminal restriction fragments (TRFs) containing Y' telomeres was estimated as described previously (Askree *et al*, 2004). The length of TRFs was subtracted by that of Y' element fragment (~875 bp) to yield Y'-containing telomere length (LeBel *et al*, 2006).

Electrophoretic mobility shift assay (EMSA)

About 10 µM of individual oligomer was mixed with same concentration of its complementary oligomer in polynucleotide kinase buffer, heated to 85°C for 5 min followed by cooling at room temperature for 1 h. Oligonucleotides without 8-oxoG were labelled using adenosine [γ -³²P]-triphosphate (Perkin Elmer) and polynucleotide kinase (New England Biolabs). Labelled oligonucleotides (5 pM) were mixed with different concentration of unlabelled competitor oligonucleotides and 50 pM purified Rap1 protein (Garbett *et al*, 2007) in BS buffer (50 mM Tris-Cl (pH 7.5), 250 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 5 mM MgCl₂, 250 mM KCl, 20% glycerol, and 1.25 mg/ml BSA) at room temperature for 25 min. Free and Rap1-bound oligonucleotides were separated on 4% native polyacrylamide gel and quantified by Phosphor-Imager analysis.

Chromatin immunoprecipitation (ChIP) and western blotting

Chromatin immunoprecipitation and dot blot were performed using anti-Rap1p or anti-HA antibodies as described previously (Ji *et al*, 2008; Hirano *et al*, 2009). Briefly, dot blot was probed with a random-labelled probe specific for the *ARO1* gene as control. The same blot was then stripped and probed with radiolabelled polyTG_{1–3} fragments. The ratio between immunoprecipitated and input telomeric repeats reflected the amount of telomere-bound Rap1 or Rif2 proteins. The value for *ogg1Δ* strain was then normalized to that for the wild type. Western blot was used to measure Rap1p and Rif2p level and ChIP efficiency. Whole cell

extracts and supernatants of immunoprecipitation were detected by western blot using anti-Rap1 or anti-HA antibodies (Ji *et al*, 2008; Hirano *et al*, 2009).

Telomere PCR and sequencing

Telomeres were amplified from genomic DNA using PCR (Teixeira *et al*, 2004; Hector *et al*, 2007). Briefly, polyC was added to a telomere using terminal transferase. A TEL06R or TEL15L telomere was then amplified using a polyG primer and a primer specific for these telomeres (Hector *et al*, 2007). The PCR product was gel purified and cloned into pCR2.1 vector (Invitrogen) and was sequenced by Genewiz Corporation using M13R or M13F primer.

Telomeric silencing assay

The *ogg1::HIS3MX* cassette was PCR amplified from pFA6a-*HIS3MX* and transformed into UMY2585 (*TELVIIL::URA3 TELVR::ADE2*). Transformants were selected on SC-His plates and deletion of the *OGG1* gene was confirmed by PCR. The *ogg1Δ* transformants were re-streaked three times on YEPD plates. Suppression of telomeric *URA3* and *ADE2* gene in UMY2585 and its *ogg1Δ* derivatives were scored as described previously (Kyrien *et al*, 1993).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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