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Inactivation of the Sas2 histone acetyltransferase delays senescence driven by telomere dysfunction

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Changes in telomere chromatin have been linked to cellular senescence, but the underlying mechanisms and impact on lifespan are unclear. We found that inactivation of the Sas2 histone acetyltransferase delays senescence in *Saccharomyces cerevisiae* telomerase (*tlc1*) mutants through a homologous recombination-dependent mechanism. Sas2 acetylates histone H4 lysine 16 (H4K16), and telomere shortening in *tlc1* mutants was accompanied by a selective and Sas2-dependent increase in subtelomeric H4K16 acetylation. Further, mutation of H4 lysine 16 to arginine, which mimics constitutively deacetylated H4K16, delayed senescence and was epistatic to *sas2* deletion, indicating that deacetylated H4K16 mediates the delay caused by *sas2* deletion. Sas2 normally prevents the Sir2/3/4 heterochromatin complex from leaving the telomere and spreading to internal euchromatic loci. Senescence was delayed by *sir3* deletion, but not *sir2* deletion, indicating that senescence delay is mediated by release of Sir3 specifically from the telomere repeats. In contrast, *sir4* deletion sped senescence and blocked the delay conferred by *sas2* or *sir3* deletion. We thus show that manipulation of telomere chromatin modulates senescence caused by telomere shortening.

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Introduction

Ageing in humans is accompanied by the shortening of telomeres, the nucleoprotein complexes that cap the ends of chromosomes, and several lines of evidence indicate that such shortening contributes to age-related pathology (Aubert and Lansdorp, 2008). Telomeres are generally maintained in a heterochromatic state, which in mammals is mediated by the posttranslational modification of histones and by DNA methylation (Baur *et al*, 2001; Garcia-Cao *et al*, 2004; Gonzalo *et al*, 2006; Blasco, 2007). In *Saccharomyces cerevisiae*, telomere heterochromatin is mediated principally by the silent information regulator (Sir) complex, composed of Sir2, 3 and 4 (Grunstein, 1997). Yeast telomeres are composed of irregular DNA repeats having the consensus $5'$-$[(TG)_{0-6}TGGGTGTG(G)]_n$-$3'$ and are non-nucleosomal. The repeats are instead bound by Rap1, to which Sir3 and Sir4 can bind independently or cooperatively (Wright *et al*, 1992; Moretti and Shore, 2001; Luo *et al*, 2002; Liaw and Lustig, 2006; Feeser and Wolberger, 2008). Sir4, which is also bound to Ku at the telomere terminus, recruits Sir2 to the complex, but Sir2 is not required for the binding of Sir3 or Sir4 to Rap1 (Hoppe *et al*, 2002; Luo *et al*, 2002; Roy *et al*, 2004). The histone deacetylase activity of Sir2 targets lysine 16 within the N-terminal histone H4 tails (H4K16) of nearby subtelomeric nucleosomes, and because the unacetylated H4 tail is a principal binding site for Sir3, Sir2-mediated deacetylation enables propagation of the Sir complex from the telomere repeats to subtelomeric sequences (Imai *et al*, 2000; Carmen *et al*, 2002; Hoppe *et al*, 2002; Luo *et al*, 2002). In contrast, acetylation of histone H4K16 by the Sas2 acetyltransferase limits this propagation by interfering with the binding of Sir3, thus setting a boundary separating telomeric and subtelomeric heterochromatin from internal euchromatin (Kimura *et al*, 2002; Suka *et al*, 2002; Shia *et al*, 2006; Xu *et al*, 2006; Altaf *et al*, 2007; Lafon *et al*, 2007). Although acetylation of the histone H4 tail opens chromatin and thus generally activates gene expression, Sas2 enforces the silencing of reporter genes inserted at subtelomeric loci or at the silent mating loci HML and HMR (Xu *et al*, 1999b, 2006; Shogren-Knaak *et al*, 2006; Zou and Bi, 2008). At least in the case of the telomere, this is because the Sas2-enforced boundary prevents loss of Sir proteins (which are at a limiting cellular concentration, particularly in the case of Sir3 (Wiley and Zakian, 1995; Lustig *et al*, 1996; Maillet *et al*, 1996)) from telomeric and subtelomeric sites (Kimura *et al*, 2002; Suka *et al*, 2002).

Telomere length is normally maintained by the constitutive expression of the telomerase enzyme in yeast, but *tlc1* mutants, lacking the RNA template component of telomerase, lose telomere length with cell division and thus gradually lose replicative potential (Lundblad and Szostak, 1989; Singer and Gottschling, 1994). Extensive shortening causes telomeres to uncap and signal cell cycle checkpoint-mediated arrest, termed senescence. This provides a model to study senescence caused by telomere shortening, and is distinct from other models of ageing in yeast, including those based on the number of daughters budded from each mother cell ('replicative ageing') or based on the time cells can survive in

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stationary phase ('chronological ageing'). Although most cells lacking telomerase senesce, rare survivors escape and maintain their telomeres using homologous recombination (HR)-based break-induced replication mechanisms (Lundblad and Blackburn, 1993; Le *et al*, 1999; Enomoto *et al*, 2002; IJpma and Greider, 2003; Lydeard *et al*, 2007). HR not only enables the growth of survivors, but also helps slow the rate of senescence before survivor formation, apparently by aiding the repair of replication-associated telomere damage (Lundblad and Blackburn, 1993; Le *et al*, 1999; Azam *et al*, 2006; Lee *et al*, 2007; Abdallah *et al*, 2009). Notably, the HR mechanisms that slow senescence and those that support survivor formation are not identical, with mutations such as the deletion of *POL32* (encoding a non-essential subunit of DNA polymerase δ) having no effect on the rate of senescence, but completely blocking the formation of survivors (Lydeard *et al*, 2007).

Recent evidence indicates an important interplay between telomere length and chromatin state. As telomeres shorten in mice lacking telomerase (*mTerc*$^{-/-}$ mutants), they lose DNA and histone modifications characteristic of heterochromatin (cytosine methylation and trimethylation of histone H3K9 and histone H4K20) and accumulate marks characteristic of open chromatin (acetylation of H3K9 and H4K20) (Benetti *et al*, 2007). Deletion of *Sirt6*, encoding a mammalian Sir2 homologue that targets H3K9, in mice causes both premature age-related pathology and telomere loss events (Michishita *et al*, 2008). Human senescent cells develop extensive heterochromatic regions on a genome-wide scale, visualized as senescence-associated heterochromatin foci (SAHF), but telomeres seem to be excluded from SAHF indicating possible loss of telomere heterochromatin at senescence (Ye *et al*, 2007). However, the mechanisms underlying these telomere length-related chromatin changes are unclear, as are the prospects for extending telomere-limited lifespan on the basis of their manipulation.

Here, we provide the first demonstration that senescence driven by telomere shortening can be delayed through modulation of telomere chromatin. Our findings show that disruption of histone acetylation through loss of Sas2 promotes loss of Sir3 from the telomere and thus enables HR-dependent telomere maintenance and delay of senescence.

Results

Loss of the SAS-I complex results in delayed senescence caused by telomere shortening

We used *S. cerevisiae* cells lacking telomerase to begin to identify chromatin factors modulating the rate of senescence caused by telomere shortening. Diploid cells heterozygous for wild type and deletion alleles of *TLC1* and for wild type and deletion alleles of a candidate chromatin regulatory factor were sporulated, and the senescence rates were compared between haploid progeny lacking *TLC1* alone or also lacking the chromatin factor. This allowed for controlled comparisons of senescence rates because all mutants had inherited telomeres of similar size and from the same epigenetic environment. Senescence was monitored by plotting the extent of growth after each day of serial reculturing versus population doublings (PDs) from spore germination, as carried out earlier (Johnson *et al*, 2001; Lee *et al*, 2007). PD rather than time was used as our metric because telomere shortening caused by the end-replication problem and other replication-related events is related to cell division and not time, and moreover, the use of PD prevents mutations that merely alter the rate of cell division from being mistakenly interpreted as modulating the rate of senescence of *tlc1* mutants. As observed earlier, the absolute lifespan of a strain of any given genotype varied somewhat between experiments, perhaps reflecting slightly different growth conditions, the different combinations of heterozygous alleles in the parental diploids or the natural variation in steady-state telomere lengths among different *TLC1*$^{+}$ cells. However, this variability does not affect our interpretations because all of our comparisons involved cells descended from the same diploid parent and compared within the same experiment. For these reasons, we emphasize that reliable comparisons of senescence rates cannot be made between experiments.

The results of our screen will be published separately (AC, MLK and FBJ, in preparation), but early in the course of screening, we found that although the deletions of 13 of 14 loci investigated either had no effect or were deleterious, deletion of *SAS2* delayed senescence by an average of 10 PDs (Figure 1A). As mutations that slow age-related processes identify mechanisms that are rate limiting for longevity more readily than do mutations that speed such processes, we decided to investigate how *sas2* deletion slows senescence. Sas2 is a member of the conserved MYST family of histone

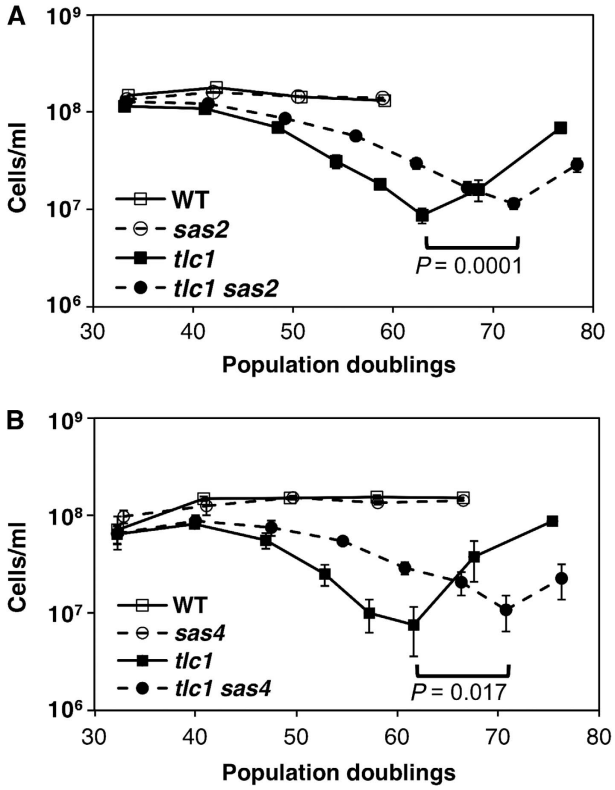


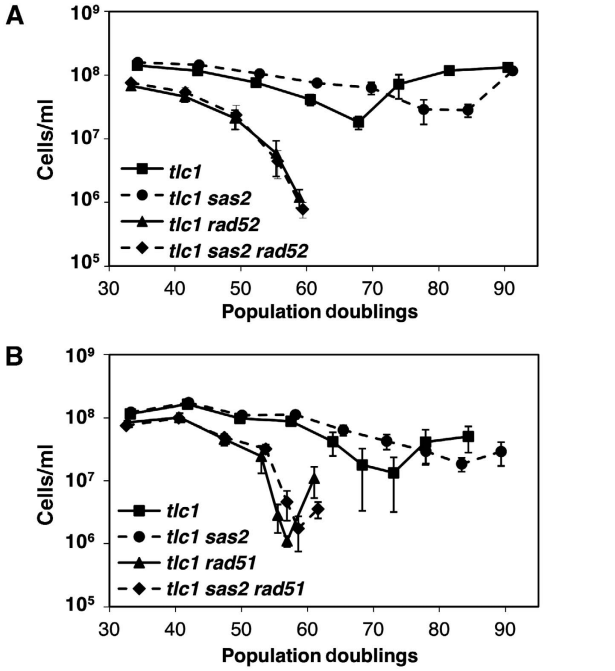
Figure 1 Loss of the SAS-I complex delays senescence of *tlc1* mutants. (**A**) Senescence rates were measured in liquid culture by serially passaging spore products of the indicated genotypes derived from a *TLC1/tlc1 SAS2/sas2* diploid. The means and s.e.m.s for 14 independent spore products per genotype are shown. (**B**) Effect of *sas4* deletion on replicative senescence was determined as in (A), except that four spore products per genotype were used.

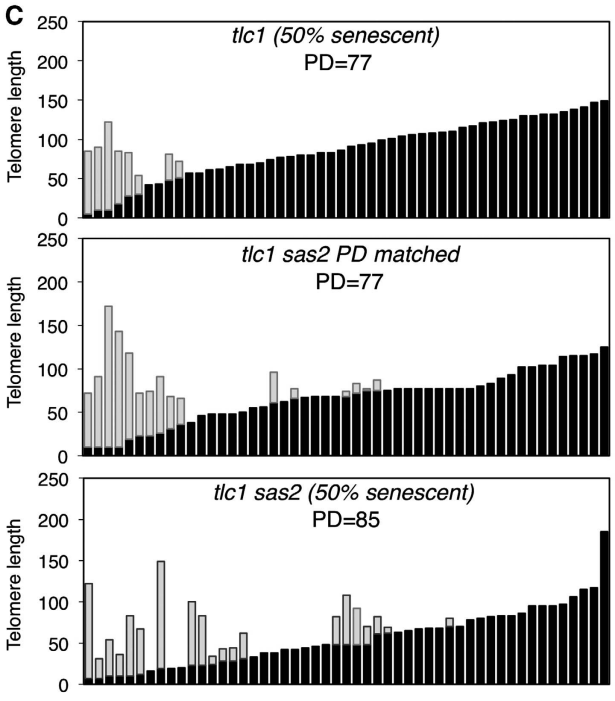
acetyltransferases and is the catalytic component of the SAS-I complex, comprising Sas2, 4 and 5, which functions principally to acetylate H4K16 (Xu *et al*, 1999a, b; Shia *et al*, 2005). Senescence was also delayed in *tlc1 sas4* mutants (Figure 1B), indicating that loss of the SAS-I complex explains the slowed senescence. Importantly, the delay did not result from a slowed growth rate because *sas2* mutants grow similarly to wild type (Supplementary Figure 1A) and because senescence rates are plotted versus PD instead of time (as an aside, when plotted versus time, the extension is still apparent; Supplementary Figure 1B). Further, the extension did not seem to be caused by the early appearance of survivors because the growth rate of *tlc1 sas2* mutants still reached a nadir similar to *tlc1* mutants (but at a later PD) and because Southern analysis of telomere length and structure revealed no evidence of early survivors (see Figure 3A; Supplementary Figure 2). Thus, extension corresponded to a shift in both senescence and survivor formation to later PDs.

Deletion of SAS2 delays senescence through an HR-dependent pathway

Earlier studies have shown that HR has a critical function in the maintenance of telomeres during senescence as well as in survivors, although the type of HR mechanisms are distinct in the two settings (Lundblad and Blackburn, 1993; Le *et al*, 1999; Teng and Zakian, 1999; Lee *et al*, 2007; Lydeard *et al*, 2007). We, therefore, tested whether extension by *sas2* deletion depends on HR by examining the rate of senescence in *tlc1 sas2* compared with *tlc1 sas2 rad52* or *tlc1 sas2 rad51* mutants. Rad52 is required generally for HR and functions in strand annealing and invasion into homologous targets, whereas Rad51 has more restricted functions in HR, principally involving strand invasion. As found earlier, *tlc1 rad52* and *tlc1 rad51* mutants senesced rapidly, and *tlc1 rad52* mutants did not form survivors (Figure 2A and B). The delay of senescence by *sas2* deletion was lost in the absence of Rad52 or Rad51 suggesting that loss of Sas2 slows senescence through an HR-based mechanism. Sas2 has a modest function in repressing the silent mating type locus *HML* (which carries the α mating type allele), and a minor function in repressing *HMR* (Xu *et al*, 2006). It was thus conceivable that *sas2* mutant *MAT a* haploids might coexpress levels of a and α genes sufficient to indirectly stimulate an HR-dependent pathway because the co-expression of a and α genes in haploids upregulates HR (Lee *et al*, 1999). However, the same delay of senescence by *sas2* deletion was observed in *MAT a* and *MAT* α haploids (not shown) and, moreover, was also observed in a different strain background (JKM111), in which both silent mating loci are deleted (Supplementary Figure 1C). Therefore, such indirect upregulation of HR does not explain delayed senescence by *sas2* deletion.

To test more directly whether *sas2* deletion stimulates telomere HR, examples of telomere I-L from *tlc1* and *tlc1*





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	Recombined	Normal	Total	%	*P*-value
tlc1 PD=77	8	44	52	15	
tlc1 sas2 PD=77	16	35	51	31	0.045
tlc1 sas2 PD=85	20	31	51	39	0.005

Figure 2 Delayed senescence through *sas2* deletion depends on HR. (**A**) *TLC1/tlc1 RAD52/rad52 SAS2/sas2* diploids were sporulated and the senescence of *tlc1, tlc1 sas2, tlc1 rad52* and *tlc1 sas2 rad52* mutants were compared. (**B**) Same as in (A) except that *rad51* deletion was tested in place of *rad52* deletion. The means and s.e.m.s for five (A) and six (B), respectively, independent spore products per genotype are shown. *P*-values for the differences between *tlc1* and *tlc1 sas2* mutants in the absence of Rad52 (A) and Rad51 (B) were 0.844 and 0.302, respectively. (**C**) Analysis of sequenced telomeres. Chromosome I-L telomeres from *tlc1* and *tlc1 sas2* mutants matched for PD or extent of senescence were PCR amplified, cloned and sequenced. Telomeres are sorted by length with the non-recombined tracks in black and the recombinant tracts in grey. (**D**) Summary of the data in (C), showing the percent of telomeres that were recombined and *P*-values compared with *tlc1* cells, calculated using a one-tailed Fisher's exact test. A full-colour version of this figure is available at *The EMBO Journal* Online.

sas2 mutants were cloned and sequenced. Yeast telomere repeats are imperfect and their sequences differ among telomeres. In a clone of senescing *tlc1* cells, the sequence of a shortening telomere is typically fixed, but recombination events can occasionally append new sequences to its terminus (Teixeira *et al*, 2004). The *tlc1* mutants had significantly lower levels of recombinant telomeres than *tlc1 sas2* mutants matched either for PD or extent of senescence (Figure 2C and D; Supplementary Figure 3), indicating that *sas2* deletion increases telomere recombination.

Telomere attrition rates and checkpoint responses are not affected by sas2 deletion

To evaluate the effect of *sas2* deletion on the global rate of telomere attrition, we used Southern analysis to measure telomere lengths during senescence of 14 pairs of *tlc1* or *tlc1 sas2* mutants (each pair derived from the same tetrad). Bulk telomere lengths shortened at the same rate in *tlc1* and PD matched *tlc1 sas2* mutants (Figure 3A). However, overall telomere lengths continued to shorten in *tlc1 sas2* mutants and reached shorter lengths at senescence than in *tlc1* mutants (Figure 3A; Supplementary Figure 2; $P=0.035$). Therefore, *sas2* deletion does not delay senescence by simply slowing the overall rate of telomere shortening, but rather allows cells to continue dividing to the point at which overall telomere lengths become shorter.

The greater extent of telomere shortening in *tlc1 sas2* mutants could be explained by a diminished sensitivity of *sas2* mutants to telomere uncapping, thus allowing cells to divide longer with uncapped telomeres and continue to shorten their telomeres. Although Sas2 has not been directly implicated in DNA damage response (DDR) pathways, several recent findings indicate that it might have such a function. These include (1) acetylation of H4K16 at short telomeres (see Figure 4B below) and at DNA double strand breaks (DSBs) during repair by HR (Tamburini and Tyler, 2005), (2) a function for hMOF, the human orthologue of Sas2, in ATM activation after ionizing radiation (Gupta *et al*, 2005) and (3) a function for the histone H3K79 methylase Dot1 (which cooperates with Sas2 to establish the boundary separating telomere heterochromatin from internal euchromatic loci) in activating checkpoint responses at uncapped telomeres (Altaf *et al*, 2007; Lazzaro *et al*, 2008). To investigate the contribution of Sas2 to DDR, *sas2* mutants were tested for growth inhibition caused by genotoxic agents including hydroxyurea (HU), methane methylsulfonate (MMS), 4-nitroquinoline-1-oxide (4NQO) or UV or γ irradiation. Deletion of *SAS2* did not confer increased sensitivity to these agents, indicating that DDRs are not affected globally (Figure 3B; Supplementary Figure 4), consistent with earlier findings (Osada *et al*, 2001).

To test the contribution of Sas2 to telomere-specific damage, we first compared its effects during senescence to

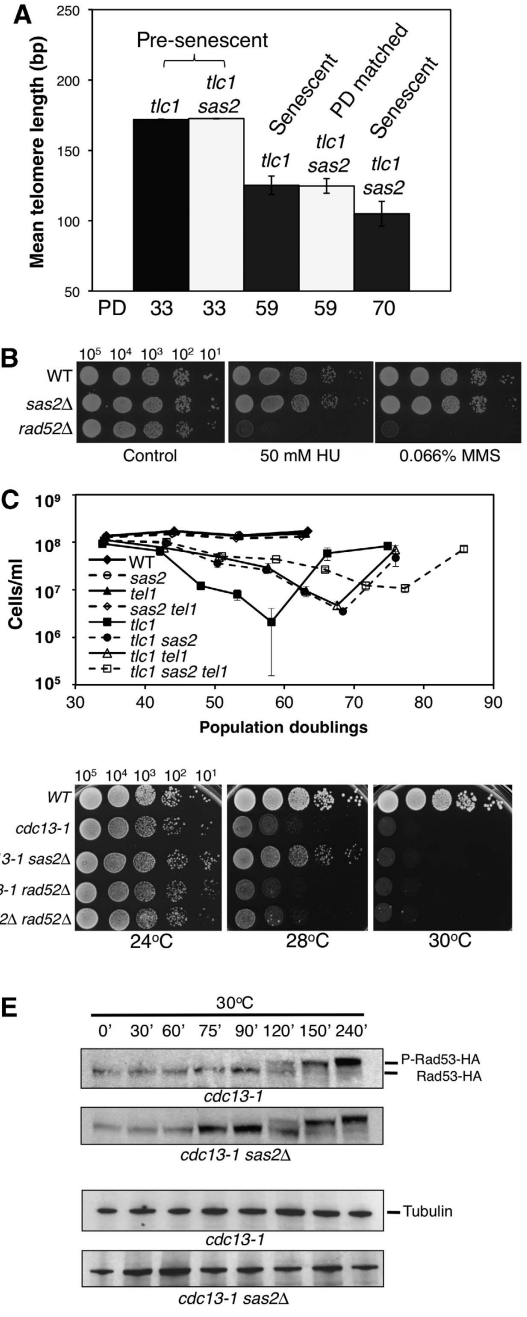

Figure 3 Deletion of *SAS2* does not change the overall rate of telomere attrition or the checkpoint responses to uncapped telomeres. (**A**) Detailed in Materials and methods, mean telomere lengths and s.e.m.s were calculated from Phosphorimager scans. Fourteen pairs of *tlc1* and *tlc1 sas2* mutants (each set from one tetrad; samples from Figure 1A) were senesced in liquid culture and samples at the indicated PDs were analysed. Note that senescent *tlc1* and *tlc1 sas2* mutants were harvested approximately two PDs before lowest point of growth (i.e. maximal senescence) to avoid contribution of survivors to the mean telomere length. The *y* axis represents the average telomere repeat tract length, calculated by subtracting the approximate length of Y' sequences between the XhoI site and the base of the telomere repeats (900 bp), and the *x* axis represents the number of PDs at which cells were harvested. (**B**) Effect of DNA damage agents (MMS and HU) on growth at 30°C was compared by spot assay of the indicated numbers of wild type and *sas2* mutant cells. A sensitive strain, *rad52*, was used as a control. (**C**) *TLC1/tlc1 TEL1/tel1 SAS2/sas2* diploids were sporulated and the senescence of *tlc1, tlc1 sas2, tlc1 tel1* and *tlc1 sas2 tel1* mutants were compared. (**D**) The effect of *sas2* deletion on telomere uncapping was determined in spot assays of *cdc13-1* mutants at permissive (24°C), semi-permissive (28°C) and non-permissive (30°C) temperatures. (**E**) Rad53 phosphorylation levels were compared in *cdc13-1* and *cdc13-1 sas2* mutants shifted for the indicated times to the non-permissive temperature (30°C). An HA-tag was fused at the 3′ end of the native *RAD53* ORF, and immunoblots were probed with anti-HA antibodies. Tubulin was used as a loading control. A full-colour version of this figure is available at *The EMBO Journal* Online.

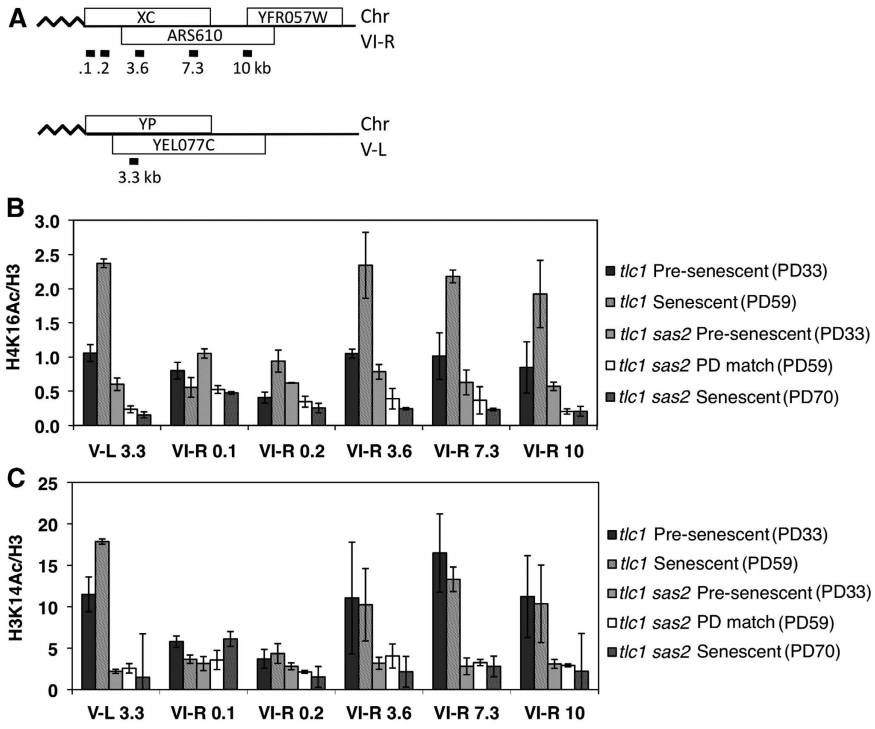

Figure 4 Telomere shortening results in increased *SAS2*-dependent H4K16 acetylation at subtelomeric regions. (**A**) Maps of telomeres VI-R and V-L showing probes used to interrogate ChIPed samples; the distances of the probes from the base of the telomere repeats are indicated. (**B**, **C**) ChIPs were performed on the following mutants: pre-senescent *tlc1* and *tlc1 sas2* senescent *tlc1*, PD-matched *tlc1 sas2* or senescent *tlc1 sas2*; PDs for each sample are indicated in *parentheses*. Chromatin was precipitated using H4K16Ac, H3K14Ac or H3 antibodies. The *y* axis represents ratios of H4K16Ac (B) or H3K14Ac (C) levels over total H3 levels. The means and s.e.m.s for three independent quantitative PCR (Q-PCR) experiments are shown, and similar results were obtained in four other experiments using independent biological replicates.

those of Tel1, a homologue of the mammalian ATM checkpoint kinase. Deletion of *TEL1* has been found to slow senescence, and Tel1 is thought to enhance recognition of a critically shortened telomere by the Mec1 kinase, a homologue of mammalian ATR, causing arrest (Ritchie *et al*, 1999; Abdallah *et al*, 2009). *sas2* and *tel1* deletions each slowed senescence of *tlc1* cells by about 10 PDs, and their effects were additive, with senescence slowed by ~20 PDs in *tlc1 sas2 tel1* mutants (Figure 3C). Thus, Sas2 and Tel1 most likely affect different processes, consistent with a checkpoint-independent effect of *sas2* deletion. As a further test, we used *cdc13-1* temperature-sensitive mutants, which at non-permissive temperature (30°C) lose functional Cdc13; normally Cdc13 caps telomeres by protecting the C-rich strand from exonucleolytic attack (Garvik *et al*, 1995; Lin and Zakian, 1996; Nugent *et al*, 1996; Zubko *et al*, 2004). Loss of Sas2 did not affect growth rates of *cdc13-1* mutants at permissive and non-permissive temperatures (Figure 3D, 24°C versus 30°C) indicating that Sas2 does not have a significant function in cell cycle arrest after telomere uncapping. Further, Sas2 did not affect the kinetics and extent of Rad53 phosphorylation, a key indicator of checkpoint responses to uncapped telomeres (Jia *et al*, 2004), after instantaneous telomere uncapping in *cdc13-1* mutants shifted to 30°C (Figure 3E; Supplementary Figure 5). However, loss of Sas2 at semi-permissive temperature (28°C) significantly improved *cdc13-1* growth (Figure 3D), similar to its contribution to slowed senescence in *tlc1* mutants. Importantly, just as in *tlc1* mutants, the improved growth that we observed in *cdc13-1* mutants was *RAD52* dependent. We, therefore, interpret these findings as indicating that Sas2 does not signal the telomere DDR, but under conditions in which cells are at risk for telomere dysfunction and are still replicating their DNA (i.e. *tlc1* mutants undergoing senescence or *cdc13-1* mutants growing at semi-permissive temperature), *sas2* deletion can facilitate HR-dependent telomere maintenance to prevent or repair critical telomere loss events.

Loss of H4K16 acetylation mediates delayed senescence caused by loss of Sas2

The main acetylation target of Sas2 is H4K16 at the boundary between telomeric heterochromatin and internal euchromatin (Kimura *et al*, 2002; Suka *et al*, 2002; Shia *et al*, 2006), but it also weakly acetylates H3K14 and has some functions at other heterochromatic regions, including the silent mating loci and ribosomal DNA (rDNA) (Meijsing and Ehrenhofer-Murray, 2001; Sutton *et al*, 2003; Oki *et al*, 2004; Xu *et al*, 2006). To identify Sas2 targets affected at senescence, we performed chromatin immunoprecipitation (ChIP) on pre-senescent and senescent *tlc1* mutants as well as pre-senescent, PD-matched and senescent *tlc1 sas2* mutants. Subtelomeric H4K16 acetylation increased in senescent *tlc1* mutants at the two different chromosomes examined compared with their pre-senescent counterparts, peaking around 3–10 kb inwards from the telomere repeats (Figure 4A and B). The increased acetylation depended on Sas2 and was not apparent immediately internal (0.1 kb) to the telomere repeats, perhaps because association of the Sir complex with telomere repeats stabilizes adjacent nucleosome-bound Sir complexes. Subtelomeric H3K14 acetylation levels, although

also dependent on Sas2, did not change with senescence in *tlc1* mutants (Figure 4C). There was no clear trend in H4K16 acetylation within the silent mating loci and rDNA at senescence (Supplementary Figure 6).

To test whether abrogation of the increased H4K16 acetylation explained the senescence delay conferred by *sas2* deletion, we examined senescence in cells in which the native H4 locus was replaced by plasmid-based H4 or H4 in which K16 was substituted with arginine (16R), a constitutive mimic of unacetylated lysine (Figure 5A and B). In 16R cells, *sas2* deletion no longer slowed senescence, indicating that extension depends on loss of H4K16 acetylation. In contrast, *sas2* deletion conferred full delay on cells with an H3K14R allele (Supplementary Figure 7). To determine whether the H4K16R allele is sufficient to confer slowed senescence, a *TLC1/tlc1* diploid carrying both 16K and 16R plasmids was sporulated and senescence of *tlc1* haploids carrying 16K or 16R was compared. Remarkably, the 16R allele delayed senescence significantly and by about five PD (Figure 5C), albeit not to the same extent as *sas2* deletion (cf. Figure 5A). However, two factors may explain the diminished effectiveness of 16R in the experiment. First, 16R is not chemically identical to unacetylated 16K, cannot be interconverted between unacetylated and acetylated states and would replace 16K genome wide instead of only at Sas2-targeted regions; together these might be suboptimal for slowed senescence. Second, the haploids inherit a mixture of 16K and 16R histones, which are gradually replaced by newly synthesized H4 encoded by the plasmid-based allele they carry, and this might diminish the apparent difference between the strains. Overall, these findings support H4K16 as the key target of Sas2 for setting the pace of senescence.

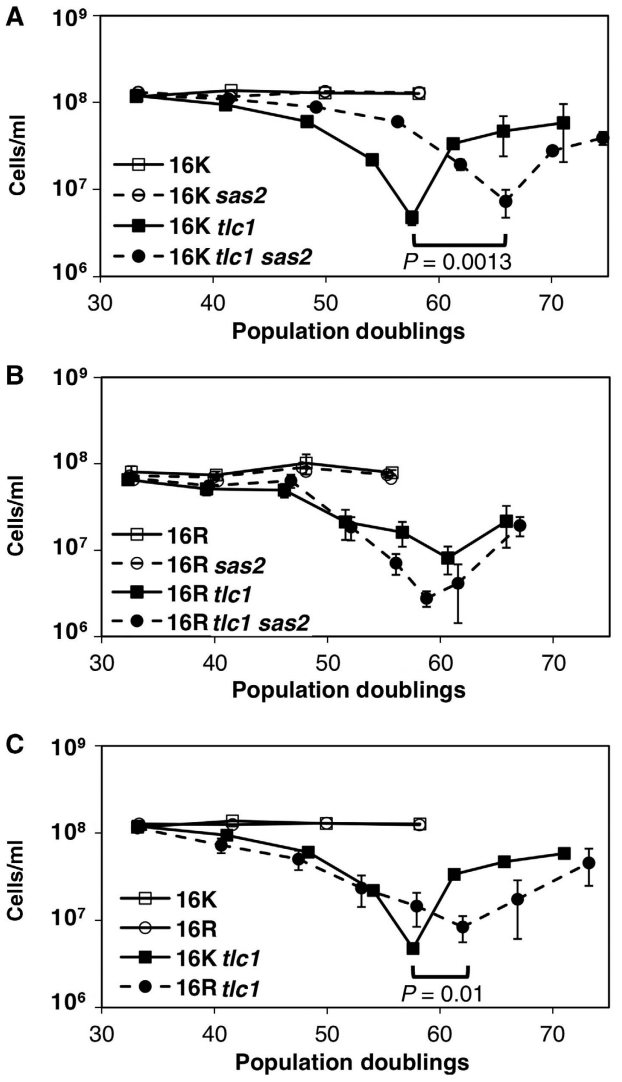

Figure 5 Loss of acetylation at H4K16 is epistatic to *sas2* mutation. (**A**) *sas2* delays senescence in the presence of a plasmid-based wild-type copy of H4K16. *TLC1/tlc1 SAS2/sas2* diploids carrying plasmid-based H4K16 (16K) as their only source of histone H4 were sporulated and growth monitored as in Figure 1. (**B**) *sas2* deletion does not slow senescence in *tlc1* mutants carrying H4K16R (16R) as their only source of histone H4. (**C**) Senescence rates of the indicated *tlc1* mutants from a *TLC1/tlc1 SAS2/sas2* diploid carrying both plasmid-based 16K and 16R H4 alleles were compared.

Deletion of SIR3 delays senescence and is epistatic to sas2 deletion and the H4K16R allele

Given the function of Sas2 in retaining the Sir complex at telomere ends (Kimura *et al*, 2002; Suka *et al*, 2002), we wondered whether modulation of Sir complex activities had a function in delayed senescence. Association of the Sir complex with subtelomeric nucleosomes through Sir3 binding to the H4 tail is normally strongly blocked by Sas2-dependent acetylation of H4K16 (Altaf *et al*, 2007; Onishi *et al*, 2007). Although Sas2 activity prevents Sir3 binding to nucleosomes, the TG repeats of the telomere are non-nucleosomal and Sir3 instead associates indirectly with the repeats through the telomere repeat-binding protein, Rap1, and through Sir4, which itself binds Rap1 as well as Ku at the telomere terminus (Moretti *et al*, 1994; Moretti and Shore, 2001; Roy *et al*, 2004). Further, cellular Sir3 levels are limiting (Wiley and Zakian, 1995; Hecht *et al*, 1996; Lustig *et al*, 1996; Maillet *et al*, 1996). Therefore, *sas2* deletion has two relevant consequences. First, the absence of acetylation on H4K16 opens binding sites for Sir3 at internal loci, and second, by doing so it depletes Sir3 from the telomere repeats and subtelomere. Thus, *sas2* deletion might delay senescence by enabling Sir3 to occupy beneficial internal chromosomal targets or by relieving a lifespan-inhibitory effect of Sir3 at the telomere or it could even be independent of Sir3. The first, second and third models predict that *sir3* deletion should speed, slow or have no effect on the senescence of *tlc1* mutants, respectively. We found that *sir3* deletion delayed senescence (Figure 6A), supporting the second model that loss of Sir3 from the telomere might explain the delay by *sas2* deletion. Indeed, the senescence profiles of *tlc1 sir3*, *tlc1 sas2* and *tlc1 sas2 sir3* mutants were nearly identical (Figure 6A), indicating that *sas2* and *sir3* deletions are equivalent for this phenotype.

To further explore whether *sas2* deletion and loss of H4K16 acetylation delay senescence by depleting Sir3 from the telomere, we performed the following experiments. First, we examined Sir3 binding through ChIP and confirmed that it is depleted by *sas2* deletion at telomeres in both *TLC1*+ and senescent *tlc1* cells (Figure 6B; Supplementary Figure 8). Second, we determined the epistatic relationship of delayed senescence by *sir3* deletion to the H4K16R allele. As histone H4K16R binds Sir3 avidly (Onishi *et al*, 2007), cells expressing only this form of H4 should enhance Sir3 binding at non-telomere sites and thus deplete Sir3 from the telomere

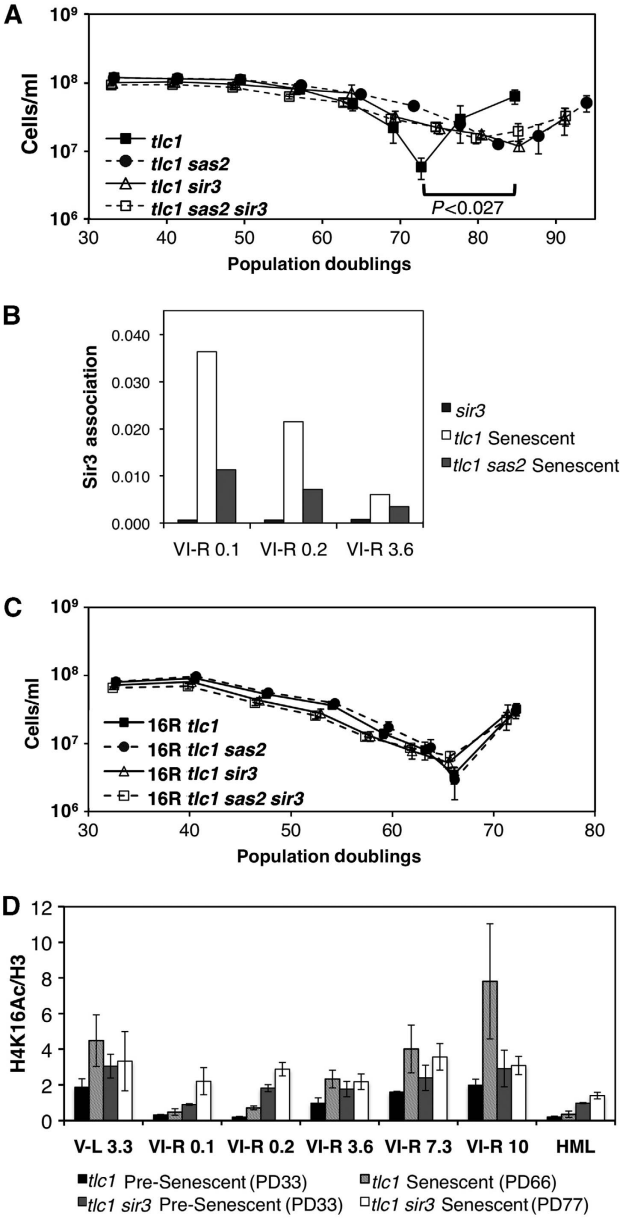

Figure 6 Loss of Sir3 delays senescence in the same manner as loss of Sas2. (**A**) *TLC1/tlc1 SAS2/sas2 SIR3/sir3* diploids were sporulated and the growth of six independent spore products per genotype was measured. Note that individual or combined deletion of *sas2* and *sir3* had no effect on the growth rate of *TLC1*$^+$ cells. The *P*-value for the difference between *tlc1* and *tlc1 sas2* ($P=0.026$), *tlc1 sir3* ($P=0.0024$) or *tlc1 sas2 sir3* ($P=0.027$) at maximal senescence is indicated by the least significant value. (**B**) Sir3 ChIP showing that deletion of *SAS2* depletes Sir3 from telomere proximal sites in senescent *tlc1* mutants. The *y* axis shows ChIP levels normalized to input. *sir3* null cells were used to control for non-specific antibody binding, and probes were as in Figure 4A. (**C**) *sir3* deletion does not delay senescence of *tlc1* mutants carrying H4K16R. *TLC1/tlc1 SIR3/sir3 SAS2/sas2* diploids, carrying plasmid-based *H4K16R* as their only source of histone H4, were sporulated and the growth of the indicated spore products ($N=6$ per genotype) were monitored as in Figure 1. (**D**) H4K16 acetylation remains high in *sir3* mutants. ChIP experiment as in Figure 4A comparing H4K16 acetylation between *tlc1* and *tlc1 sir3* mutants at early PDs or senescence. The means and s.e.m.s for three independent Q-PCR experiments are shown. A full-colour version of this figure is available at *The EMBO Journal* Online.

repeats. Similar to *sas2* deletion, *sir3* deletion had no effect on senescence in cells with H4K16R and indeed combining *sas2 sir3* deletions has no effect on senescence in *tlc1* mutants carrying H4K16R (Figure 6C). Therefore, *sas2* and *sir3* deletions likely delay senescence because they relieve an inhibitory activity of Sir3 at the telomere and not at other nucleosome-containing loci. Third, we compared H4K16Ac levels by ChIP in *tlc1* and *tlc1 sir3* mutants early after loss of telomerase or just before maximal senescence. Deletion of *SIR3* caused a general increase in H4K16Ac levels early after loss of telomerase, and moreover, these high levels were generally maintained at senescence (Figure 6D). Therefore, in cells lacking Sir3, slowed senescence does not require decreased H4K16Ac. Interestingly, H4K16Ac levels actually increased near the base of the telomere repeats in *tlc1 sir3* mutants at senescence, consistent with our ChIP data indicating that Sir3 remains bound at the distal telomere in senescent *tlc1* mutants. Lastly, we examined the effect of deletion of the Dot1 histone methyltransferase on senescence. Dot1 cooperates with Sas2 to establish the boundary separating telomeric heterochromatin from internal euchromatin by methylation of H3K79, which inhibits binding by Sir3 (Onishi *et al*, 2007; Buchberger *et al*, 2008). The *tlc1 dot1* mutants senesced approximately five PDs later than *tlc1* mutants (Supplementary Figure 9). This delay is smaller than that conferred by *sas2* deletion, but this difference is explained by the well-established fact that Dot1 has a less prominent function than Sas2 in preventing the spread of Sir3 past the boundary (Katan-Khaykovich and Struhl, 2005; Onishi *et al*, 2007; Yang *et al*, 2008). Together, these observations indicate that the mechanism by which *sas2* deletion and diminished subtelomeric H4K16Ac levels slow senescence is through release of Sir3 from the telomere and not through effects independent of Sir3.

Sir2, Sir3 and Sir4 have distinct functions during senescence

The Sir2 H4K16 deacetylase enables the propagation of the Sir2/3/4 complex from the Rap1-bound telomere repeats into subtelomeric nucleosomes by deacetylating H4K16, and thus providing a binding site for Sir3 (Onishi *et al*, 2007). If delayed senescence by *sas2* or *sir3* deletion is caused by the depletion of Sir3 from the telomere repeats, deletion of *SIR2* should have no significant effect on senescence because Sir2 is not required for the binding of Sir3 at the telomere repeats, where Sir3 is bound to Rap1 (Hoppe *et al*, 2002; Luo *et al*, 2002; Rusche *et al*, 2002). Indeed, we found that *tlc1 sir2* mutants have the same senescence profile as *tlc1* mutants (Figure 7A, and data not shown). Furthermore, *tlc1 sas2 sir2* mutants had slowed senescence similar to *tlc1 sas2* mutants, indicating that delay of senescence by *sas2* deletion does not depend on Sir2. The lack of effect of *sir2* deletion on the senescence rates of *tlc1* and *tlc1 sas2* mutants points to a function for Sir3 at the telomere repeats themselves, rather than at the subtelomeric nucleosomes immediately adjacent to the telomere repeats.

We next examined Sir4 and found that *sir4* deletion actually sped the senescence of *tlc1* mutants (Figure 7B and C). Further, *sir4* deletion effectively blocked the slowing of senescence conferred by *sas2* or *sir3* deletion. Thus, each protein of the Sir2/3/4 complex functions differently during senescence, and extension through loss of Sas2 or Sir3 depends on the presence of Sir4.

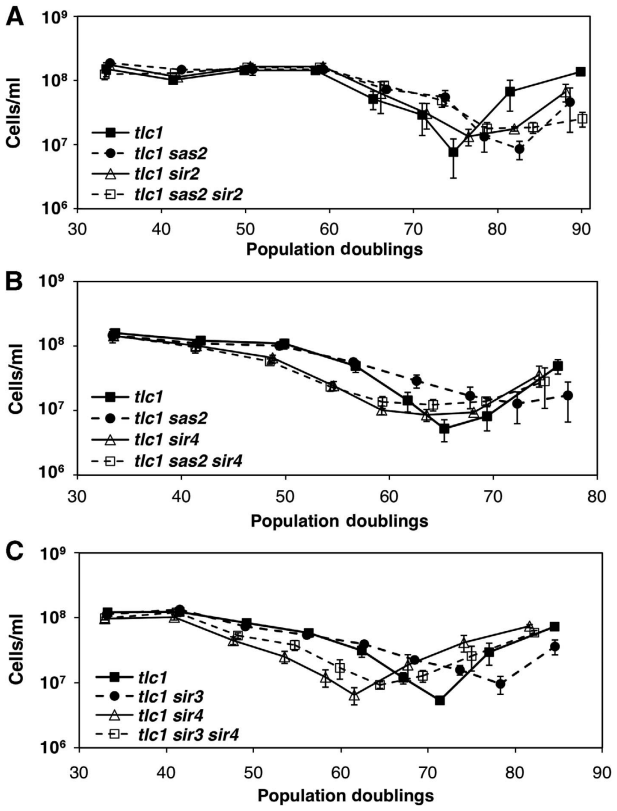

Figure 7 Contribution of Sir2 and Sir4 to delayed senescence by *sas2* deletion. (**A**) *TLC1/tlc1 SAS2/sas2 SIR2/sir2* diploids were sporulated and the growth of four independent spore products per genotype was measured. *P*-values for the difference between *tlc1* and *tlc1 sir2* mutants were not significant ($P = 0.15$). (**B**, **C**) Delayed senescence depends on *SIR4*. Senescence rates were examined similarly to (A) except with *sir4* in the absence of *SAS2* (B) or *SIR3* (C). The difference in PDs at senescence was significant for *tlc1* versus *tlc1 sir4* ($P = 0.0015$) and for *tlc1* versus *tlc1 sir3 sir4* ($P = 0.0385$), but not for *tlc1 sir4* versus *tlc1 sir3 sir4* ($P = 0.35$). A full-colour version of this figure is available at *The EMBO Journal* Online.

Discussion

Here, we show for the first time that modulation of chromatin can delay senescence driven by telomere dysfunction. Deletion of *SAS2*, encoding a histone H4K16 acetyltransferase, delayed both senescence and the onset of survivor formation. Telomeres in senescent *tlc1* mutants seemed to become less heterochromatic, because the acetylation of subtelomeric H4K16 increased at senescence. This acetylation was Sas2 dependent, and the senescence delay conferred by *sas2* deletion depended on the deacetylation of H4K16. Nonetheless, lifespan extension was not a direct effect of H4K16 deacetylation, but rather seemed to be a consequence of removal of Sir3 from the telomere repeats, thus facilitating an HR-dependent mechanism of telomere repair.

We suggest the following model for the interplay of Sas2, Sir3, Sir4 and H4K16 during the senescence of *tlc1* mutants (Figure 8) and detail our reasoning in the sections below. Telomere shortening is accompanied by Sas2-dependent acetylation of H4K16 and loss of Sir proteins from subtelomeric locations, but retention of Sir proteins at the shortened telomere repeats (Figure 8A). In *tlc1 sas2* mutants, subtelomeric H4K16 acetylation is lost, allowing Sir3 to leave the telomere repeats, which allows for efficient HR-based maintenance of telomeres and thus delays senescence (Figure 8B). In *tlc1 sir3* mutants, there is no Sir3 at the telomere repeats to begin with, and senescence is similarly delayed (Figure 8C). Sir4 binds to Rap1 (and Ku) at the telomere repeats in a Sir2 and Sir3-independent manner, and facilitates the HR mechanism.

Loss of Sas2 leads to delayed senescence through the activation of HR-dependent mechanisms

The delay in senescence conferred by *sas2* deletion did not correlate with an overall slowing in the rate of telomere shortening. Rather, the extent of overall telomere shortening was greater in senescent *tlc1 sas2* than in *tlc1* mutants. Delayed senescence was apparently not a consequence of a blunted DDR, because *sas2* deletion did not affect growth

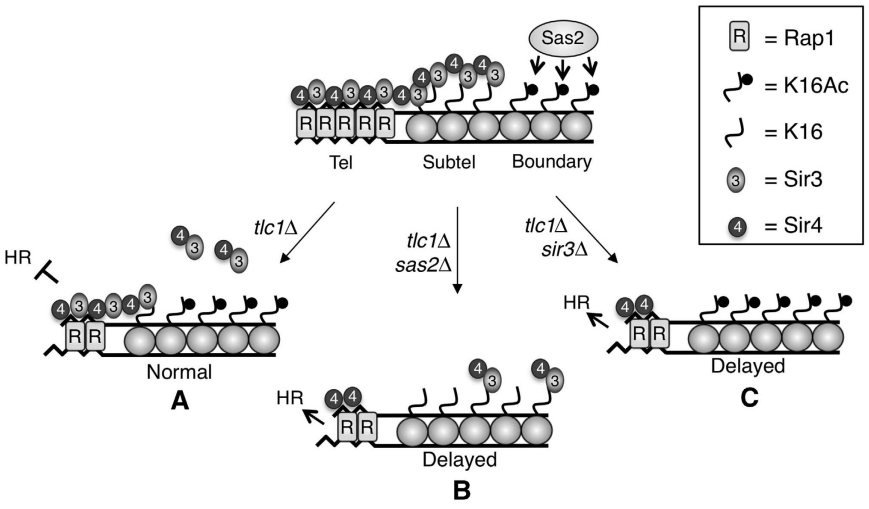

Figure 8 Model for the interactions among telomere length, H4K16 acetylation, Sas2, Sir3 and Sir4 during senescence in (**A**) *tlc1* mutants, (**B**) *tlc1 sas2* mutants and (**C**) *tlc1 sir3* mutants. Indicated are Rap1, Sas2, Sir3, Sir4, the acetylation state of H4K16, the receptivity of the telomere to HR and the rate of senescence (*normal* or *delayed*). Sir2 is omitted for simplicity. See text for details. A full-colour version of this figure is available at *The EMBO Journal* Online.

inhibition caused by DNA damaging agents, was not epistatic to *tel1* deletion during senescence and did not affect the kinetics of Rad53 phosphorylation or growth after uncapping of telomeres in *cdc13-1* mutants when shifted to non-permissive temperature. Delayed senescence depended on Rad52 and Rad51, which have central functions in HR and have been earlier shown to slow senescence (Le *et al*, 1999), and *sas2* deletion caused higher levels of telomere recombination during senescence. We found earlier that replication-associated telomere HR, apparently between sister telomeres, increases as telomeres shorten. Cells in which telomere HR had occurred gave rise to viable progeny, indicating that the telomere HR may contribute to the repair of telomere damage (possibly stalled replication forks, by allowing template switch events that bypass impediments to replication), and thus slow senescence by minimizing rare, but dramatic telomere loss events (Lee *et al*, 2007). Loss of a single telomere is sufficient to cause checkpoint arrest in yeast (Sandell and Zakian, 1993), and although such loss would not have a significant effect of the overall length profile given the contribution of the other 31 telomeres, it would prevent the continued shortening of other telomeres despite sufficient reserve length in a senescing *tlc1* mutant cell. Avoidance of rare loss events would prevent defects at a single telomere from halting cell division, allow cells to continue dividing and thus lead to greater shortening among all telomeres at senescence. This model is in line with the greater extent of telomere shortening seen within senescent *tlc1 sas2* cells.

As they are replicated unidirectionally, yeast telomeres are particularly reliant on HR-dependent mechanisms that rescue damaged or stalled replication forks (Marians, 2000; Branzei and Foiani, 2007), especially in cells lacking telomerase (Chavez *et al*, 2009). Although a damaged fork at an internal genomic site can be rescued by convergence with a neighbouring fork, no such neighbour exists for the fork that replicates the telomere terminus. In cells with telomerase, collapse and breakage of the damaged fork would lead to a dramatically shortened telomere that could be repaired by telomerase; in the absence of telomerase, the only recourse for avoiding dramatic telomere shortening would be to repair the fork through HR (Supplementary Figure 10). This explains how mutations that impair HR (e.g. *rad52*Δ or *sgs1*Δ) accelerate senescence, and how a mutation that improves HR-dependent repair (e.g. *sas2*Δ) delays senescence. Interestingly, *sas2* deletion also improved the growth of *cdc13-1* mutants at semi-permissive temperature in a Rad52-dependent manner. Addinall *et al* (2008) recently observed similar bypass of *cdc13-1* mutant arrest by deletion of SAS-I complex factors, but did not probe Rad52 dependence. As these conditions of partial telomere uncapping in dividing cells might lead to telomere replication difficulties (Miller *et al*, 2006), we suggest that telomere HR might similarly explain the rescue by *sas2* deletion in this setting. The association of the SAS-I complex with the CAF-I and Asf1 histone chaperones, which assemble nucleosomes onto replicating DNA, supports a function for Sas2 in the regulation of replication-associated processes (Meijsing and Ehrenhofer-Murray, 2001). Notably, rare but dramatic telomere loss events occur in fibroblasts obtained from people with Werner syndrome (Crabbe *et al*, 2004), which is characterized by premature features of ageing and is caused by lack of the WRN RecQ-family DNA helicase, which functions in HR pathways (Saintigny *et al*, 2002). An increase in these rare loss events also seems to explain the rapid senescence of *tlc1* mutants when the Sgs1 RecQ helicase is inactivated (Lee *et al*, 2007). Similar telomere loss events occur in human cells lacking the Sirt6 histone H3K9 deacetylase, which is required to recruit WRN to telomeres (Michishita *et al*, 2008). Thus, chromatin and RecQ-family helicases can apparently cooperate to maintain telomeres, and it will be interesting to determine whether a similar relationship exists between H4K16 acetylation and telomere maintenance by these helicases and other HR factors.

We emphasize that the HR mechanisms that slow senescence in *tlc1* mutants, and which are augmented in *tlc1 sas2* mutants, may be different from those operating to allow survivor formation. Indeed, *sas2* deletion did not simply cause early survivor formation, in contrast to the early survivor formation reported earlier for *tlc1* mutants lacking the histone H1-like protein encoded by *HHO1* (Downs *et al*, 2003). Rad52 was recently proposed to have a function in telomere protection during senescence that is inhibited by Tel1 and does not involve synthesis of new telomere DNA (Abdallah *et al*, 2009), for example through stabilization of stalled telomere replication forks. Our finding that deletions of *TEL1* and *SAS2* delay senescence in a non-epistatic manner suggests that each deletion impacts a different pathway, and thus Rad52 might function differently in each pathway. The increased telomere recombination in *tlc1 sas2* cells suggests that the Sas2-inhibited pathway does involve the acquisition of new telomere repeat tracts at shortened telomeres. We favour the idea that the increased telomere recombination explains the slowed senescence, but it is possible that it is merely correlated with it. Further, the Rad51 and Rad52 dependence of delayed senescence might reflect senescence in *tlc1 rad51* and *tlc1 rad52* mutants that is so rapid as to preclude benefit from *sas2* deletion, rather than reflecting a *bona fide* function for HR in *sas2*Δ cells. It is difficult to completely rule out this alternative interpretation, but we note that the defect in *tlc1 rad51/52* mutants is mild enough to permit over 55 PD before senescence. In addition, in the *cdc13-1* model, in which *sas2* deletion also confers a Rad52-dependent growth benefit at semi-permissive temperature, the growth defect of *cdc13-1 rad52* mutants is no worse than that of *cdc13-1* mutants (Figure 3C), and thus the Rad52 dependence for improved telomere maintenance need not reflect a growth defect.

We also note that our results are compatible with either a telomere specific or with a genome-wide function for Sas2 in the regulation of HR. However, we suspect that Sas2 preferentially affect HR near telomeres because (1) acetylation of H4K16 by Sas2 occurs predominantly at subtelomeric sites (Shia *et al*, 2006), (2) *sas2* mutants had normal sensitivity to several DNA damaging agents and (3) the mechanism by which *sas2* deletion slows senescence seems to be through removal of Sir3 from the telomere repeats. As Sir3 has no apparent direct function in global HR (Lee *et al*, 1999), if Sas2 does affect global HR, then it likely does so using mechanisms that are different from those that delay senescence in *tlc1 sas2* mutants.

Delayed senescence by sas2 deletion functions through the loss of acetylation at H4K16

Sas2 can acetylate K16 of histone H4 and, more weakly, K14 of histone H3 (Sutton *et al*, 2003). Analysis by ChIP revealed

that H4K16 levels increase selectively at subtelomeres during senescence of *tlc1* mutants and that the increases were Sas2 dependent, suggesting that *sas2* deletion slowed senescence through the loss of acetylation at H4K16. The reasons for increased subtelomeric H4K16 acetylation at senescence are not yet clear. It is possible that the shortening of the telomere repeats and the consequent loss of Rap1 nucleation sites causes a decrease in the local concentration of Sir proteins at telomeres and subsequently leads to fewer Sir proteins spreading into the subtelomeric region, thus opening sites on subtelomeric nucleosomes to acetylation by Sas2. Consistent with this view, at senescence, Rap1, Sir3 and Sir2 were each found to lose their normal localization in foci at the nuclear periphery and to become more diffusely nuclear (Straatman and Louis, 2007). Alternatively, shortened telomeres, which are sensed as DSBs, might increase H4K16 acetylation in the same manner as generic DSBs during HR-mediated repair (Tamburini and Tyler, 2005), and thus loss of Sir protein binding would be a consequence rather than a cause of increased acetylation. A third possibility is that because senescence activates a stress response (Nautiyal *et al*, 2002), and because other stresses induce phosphorylation of Sir3 and promote its loss from subtelomeric regions (Martin *et al*, 1999; Mills *et al*, 1999; Ai *et al*, 2002), Sir3 might be phosphorylated during senescence and be similarly lost. Regardless, our findings that an H4K16R mutation delays senescence and is epistatic to *sas2* deletion indicate that H4K16 is the key target of Sas2 for setting the pace of senescence.

Differential activities of Sir2, 3 and 4 during senescence

Deletion of Sas2 or the H4K16R mutation causes loss of the Sir complex from the telomere and relocalization to internal loci (Kimura *et al*, 2002; Suka *et al*, 2002). We, therefore, examined functions for Sir proteins during senescence in *tlc1* mutants. Sir3 deletion slowed senescence in a manner epistatic with *sas2* deletion, indicating that *sas2* deletion delays senescence through the loss of Sir3 from the telomere and not through translocation of Sir3 to internal loci because such a mechanism would be dependent on Sir3 rather than being inhibited by it. Of note, the delayed senescence conferred by *sir3* deletion, in the setting of functional Sas2 and fully acetylated H4K16, indicates that loss of H4K16 acetylation through *sas2* deletion does not lead to delayed senescence *per se*, but is rather the means by which Sir3 is depleted from the telomere. The epistasis of *sir3* deletion and H4K16R mutation for senescence rate supports this view. Thus, modulation of senescence through manipulation of subtelomeric nucleosomal chromatin occurs through effects on the non-classical (i.e. non-nucleosomal) heterochromatin of the telomere repeats. In addition, the fact that *sir3* and *sas2* deletions are equivalent is consistent with the model that *sas2* deletion maximally disrupts the heterochromatin boundary and thus depletes Sir3 from the telomere.

Sir4 had a different function than Sir3 during senescence and was required to prevent rapid senescence and for deletion of *SIR3* or *SAS2* to slow senescence. Sir4 can bind Rap1 and Ku at the telomere repeats independent of Sir2 and Sir3, and among the Sir proteins is uniquely capable of anchoring telomeres to the nuclear envelope (NE) (Hediger *et al*, 2002; Taddei *et al*, 2004). This involves interactions with Esc1 and Mps3, each of which localizes outside of nuclear pore complexes (NPCs) (Taddei *et al*, 2004; Bupp *et al*, 2007; Schober *et al*, 2009). Telomeres were recently found to translocate to NPCs at senescence (Khadaroo *et al*, 2009). The relationship of subnuclear localization to choice of HR repair pathway seems to be complex and is incompletely understood (Nagai *et al*, 2008; Bystricky *et al*, 2009; Oza *et al*, 2009; Schober *et al*, 2009), but we speculate that the critical function of Sir4 during senescence may be to regulate telomere localization at the NE and thus the choice of an HR pathway that slows senescence but does not drive survivor formation. It may be that the beneficial effect of Sir3 loss from telomeres is to facilitate this function of Sir4.

Sir2 was neither required for a normal rate of senescence nor the delayed senescence conferred by *sas2* deletion. These findings indicate that Sir3 inhibits lifespan at the telomere repeats, in which its interactions with Rap1 and Sir4 do not require Sir2. As *sir2* deletion might be expected to increase free Sir3 levels (by increasing H4K16 acetylation and thus blocking Sir3 binding to nucleosomes), but has no effect on the rate of senescence, we infer that Sir3 is bound to terminal repeats at saturating levels and thus *sir2* deletion does not increase Sir3 levels at the telomere repeats. The Sir2 family of proteins regulates the pace of ageing in many organisms (Guarente, 2007). This regulation ranges from lifespan inhibition to extension, for example limiting lifespan during the chronological ageing of yeast, but extending the replicative lifespan of yeast mother cells (Kaeberlein *et al*, 1999; Fabrizio *et al*, 2005). Our findings further emphasize the importance of molecular context in determining the effects of this important family of lifespan regulators.

An earlier study of the function of the Sir proteins in telomerase mutants found that individual deletion of the different *SIR* genes each caused early survivor formation (Lowell *et al*, 2003). We have never observed this for *SIR2* or *SIR3*, but it is possible that the different strain background (W303) and experimental design might explain the different results. Strains used in the earlier study apparently generated a high rate of petite mutants and caused the authors to use rho^0 strains (lacking mitochondrial DNA) for most of their experiments, but we did not observe petite mutants in our experiments. Furthermore, comparisons were made between haploid SIR^+ and *sir*Δ strains from which telomerase was deleted, rather than making comparisons between spore products derived from the same parental diploid as we did in our study; because *sir3*Δ and *sir4*Δ mutations shorten steady-state telomere lengths in *TLC1*+ cells (Palladino *et al*, 1993), this would cause more rapid senescence and thus early survivor formation, and in addition differences in telomere chromatin preceding the loss of telomerase might also have affected the results.

In summary, we have identified two factors, Sas2 and Sir3, that impact telomere chromatin and when removed, delay the senescence of yeast lacking telomerase. Similar to murine cells lacking telomerase (Benetti *et al*, 2007), the nucleosomal regions of telomeres in yeast telomerase mutants seem to become less heterochromatic at senescence as indicated by the increased acetylation of subtelomeric H4K16. The status of telomere chromatin in senescent human cells is largely unexplored, but the fact that telomeres are excluded from SAHF suggests that they too might lose heterochromatin features (Ye *et al*, 2007). It will be informative to characterize telomere chromatin dynamics in senescent human cells as

well as determine the function of the mammalian Sas2 orthologue, hMOF (Lafon *et al*, 2007), in this setting.

Materials and methods

Yeast strains and plasmids

Yeast were cultured at 30°C in YPAD or SC-complete medium (Amberg and Burke, 2005). All senescence experiments were performed in the BY4741/4742 background (Brachmann *et al*, 1998), except for Supplementary Figure 1C, which was in JKM111 (Moore and Haber, 1996). Unless otherwise specified, all alleles are deletions. See Supplementary Table 1 for strain details. Double and triple mutants were constructed by deleting one copy of *TLC1* in diploids heterozygous for genes of interest or by crossing haploid mutants of interest with *tlc1* mutants at early PDs after loss of telomerase, followed by serial streaking to equilibrate telomere lengths. The *cdc13-1* allele was introduced through pop-in/pop-out recombination using pVL451 (kindly provided by V Lunblad, Salk Institute, through J Haber). All strains were genotyped using auxotrophy or drug resistance along with PCR-based verification. Plasmid pWD23 carrying the H4K16R mutation or pWD37 (H3K14R) was generated by QuickChange (Stratagene) site-directed mutagenesis. The haploid strains expressing the H4K16R or H3K14R mutant as the sole source of H4 or H3, respectively, were made by transforming the mutant plasmid into FY1716, followed by selection on synthetic-complete medium containing 5-fluoroorotic acid. An HA tag was inserted at the *RAD53* locus as described (Longtine *et al*, 1998).

Senescence assays

Each senescence assay was performed starting with freshly dissected spore products at approximately PD 25. Spores ($N=4$ per genotype, unless otherwise noted) were serially passaged in liquid culture (either YPAD or SC complete; held constant within any experiment) for 22 h. For each passage, cells were counted using a Coulter Counter and 1.4×10^6 cells were diluted into 7 ml of fresh medium. Strains carrying the wild type or the mutant histone H4 plasmids were cultured in SC-Ura or SC-Trp media, respectively, for plasmids maintenance. The total cell counts for each culture were used to determine the number of PDs ($PD=\log_2(\text{final/starting concentration})$). *P*-values for differences between PDs at maximal senescence (lowest point of growth) were calculated using an unpaired two-sample two-tailed *t*-test.

Chromatin immunoprecipitation

ChIP experiments were performed essentially as described earlier (Strahl-Bolsinger *et al*, 1997), and full details are provided in Supplementary Data. Briefly, 10^8 cells were harvested and disrupted mechanically. Chromatin was immunoprecipitated using rabbit anti-H4K16Ac or anti-H3K14Ac, rabbit anti-total H3 or rabbit anti-Sir3 (kind gift of D Moazed). Purified DNA was quantified using Q-PCR. Primers are listed in Supplementary Table 2. All signals were first normalized to input using standard curves.

Telomere PCR and sequencing

Telomere PCR was performed as described in Lee *et al*, 2007 with the following modifications; genomic DNA from *tlc1* mutants at 50% senescence or *tlc1 sas2* mutants matched by degree of senescence or number of PDs was denatured and C-tailed. The telomere ends of chromosome I-L were amplified in the presence of 0.5 M betaine and 50–600 bp fragments were cloned using the StrataClone PCR Cloning kit (Stratagene). Telomere sequences were aligned and compared using MegAlign software. Sequences are available on request.

Additional experimental details are described in Supplementary Data.

Supplementary data

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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