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Telomere tales: chromatin, telomerase and telomere function in *Saccharomyces cerevisiae*

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Abstract. Unusual chromatin structures underlie epigenetic effects at the silent mating-type loci and telomeres in yeast. Many of the same genes appear to function in transcriptional silencing observed at both the silent mating-type loci and at telomeres. The observation that these loci are united by a requirement for shared factors suggests that the structure of chromatin at these regions is similar. Alteration of telomeric chromatin components affects regulation of transcription, telomeric length, recombination and chromosomal stability. Mutations in *TLC1* and *EST2*, which both encode components of telomerase, cause identical phenotypes: progressive shortening of telomeric DNA, increased chromosome loss and eventually cell death. In this review, we examine the relationship between telomeric chromatin and telomere replication and discuss the possibility that telomerase itself is an integral part of telomeric chromatin structure.

Key words. *Saccharomyces cerevisiae*; telomeres; silencing; chromatin; telomerase; recombination.

Introduction

Over half a century ago, independent studies by Hermann Muller and Barbara McClintock first described the importance of telomeres, the physical ends of chromosomes (reviewed in ref. 1). Muller demonstrated that upon X-ray irradiation, *Drosophila* chromosomes were subject to rearrangement, deletion and inversion events. He noted, however, that terminal deletions and inversions were absent from his mutant collection, suggesting that alterations of terminal chromosomal regions were incompatible with viability. Further insight into the function of telomeres came from McClintock's analysis of the behaviour of broken ends of dicentric chromosomes. She observed that dicentric chromosomes in maize were ripped apart during mitosis and that the resulting newly formed ends subsequently either fused together or were 'healed'. Taken together, Muller and McClintock's experiments argue that telomeres are required to prevent chromosome fusion, degradation and loss.

Telomeres are composed of unique chromatin and in most organisms share common features. Generally, telomeric DNA consists of short, tandemly arrayed repeats that are flanked by middle-repetitive subtelomeric sequences [2, 3]. Telomeric chromatin is non-nucleosomal, and in many organisms DNA binding proteins specific for telomeres have been identified (reviewed in ref. 4). Such proteins are proposed to act as 'caps' that may be necessary to facilitate telomere replication and to prevent chromosome fusion and degradation by nucleases. Like other heterochromatic regions, telomeres replicate late in the S phase of the cell cycle [5, 6]. Complete telomere replication requires both conventional 5′ to 3′ DNA replication enzymes and the ribonucleoprotein telomerase, a specialized reverse tran-

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scriptase containing an RNA that functions as an internal template for DNA synthesis onto the very ends of telomeres (reviewed in ref. 7).

Telomeric DNA length has been linked to both ageing and cancer (reviewed in refs 8–12). Telomeres in human germline cells are long (10–12 kb), and telomerase is active [11]. In contrast, telomeres are short (2–4 kb) and lack telomerase activity in nondividing, senescent cells of somatic tissues [11]. In most malignant tumours assayed, telomerase activity is detected, suggesting a reactivation of telomerase during proliferation [13]. These observations led to the telomere hypothesis of ageing and immortalization (reviewed in refs 8–12). According to this model, somatic cells normally have downregulated telomerase activity and shortening telomeres and upon telomere loss or sufficient shortening, stop dividing and become senescent. In contrast, upon transformation, a small proportion of cells overcome senescence and become immortalized by reactivating telomerase. A causal relationship between telomere shortening and cellular senescence or telomerase reactivation and immortality has yet to be demonstrated. Indeed, some cancer cells have long telomeres despite the absence of detectable telomerase activity (reviewed in refs 11, 12), and cells isolated from telomerase-deficient mice remain capable of transformation [14]. However, the recent identification of human telomerase-components [15–19] upholds telomerase as an important potential target for the development of antitumour agents.

The budding yeast *Saccharomyces cerevisiae* has emerged as a model organism for studying telomere biology in part because telomeric chromatin has epigenetic transcriptional properties. In yeast, genes positioned either proximal to telomeres or at the silent mating-type loci *HMR* and *HML* (*HM* loci) are subject to silencing, a form of transcriptional repression that is associated with special chromatin structure (reviewed in refs 20–22). Silencing at the *HM* loci is well characterized and requires a number of *cis*-acting sequence elements and *trans*-acting proteins. The position-dependent silencing that occurs at telomeres is known as telomere position effect (TPE) and shares many of the same components as silencing at *HM* loci [20, 23]. Yet inherent features of telomeres distinguish them from the *HM* loci, including a telomerase requirement for replication and increasing examples of genes which when mutated perturb silencing only at the telomeres.

Recent reviews have focused on yeast telomeres [24–26], telomerase [7, 27–29], telomeric chromatin structure [20, 30] and the components of silenced chromatin, Rap1p, Sir2p and Sir3p [31–33]. Here, we focus on research defining telomeric chromatin structure, replication, and recombination in *S. cerevisiae*. In particular, we consider the ideas that telomerase subunits may be structural components of chromatin rather than transient interactors and that recombination is important for telomere length regulation.

Telomeric DNA and subtelomeric repeats

Telomeric DNA is composed of short sequence repeats. In *S. cerevisiae*, the repeat consensus sequence is $C_{2\text{-}3}A(CA)_{1\text{-}5}/(TG)_{1\text{-}5}TG_{2\text{-}3}$ (abbreviated as $C_{1\text{-}3}A/TG_{1\text{-}3}$) and extends on average 300 ± 75 bp [34, 35]. Two classes of middle-repetitive sequences, Y′ and X elements, immediately flank the telomeric $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats (fig. 1) (reviewed in ref. 3). These sequences were initially identified in searches for autonomously replicating sequence (ARS) elements [36, 37]. Both X and Y′ sequences contain ARSs that function episomally as origins of replication [37], and ARSs in Y′s can act as origins in vivo [38]. The highly conserved Y′s are described in two size classes, 5.2 kb (short) or 6.7 kb (long), and appear in tandem arrays of up to four copies, each separated by ~ 50–130 bp of $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats [3, 37, 39–42]. Recent analyses have revealed that X′s are quite complex, containing a ~ 560 bp core sequence and often multiple shorter elements [43]. X elements appear to be a common chromosomal feature, as they appear at nearly all chromosomal termini [3].

The structural or functional significance of Y′ and X sequences is not understood, and because not all telomeres have Y′ elements, they cannot be essential. The primary structure of Y′s reveals that they may have originated as transposable elements; an ORF within these elements is similar to yeast and mammalian RNA helicases, a feature shared with many mobile elements [41]. Although Y′s are transcribed at low levels in mitotic cells, there is no evidence that they are currently active transposable elements [41]. Indeed, Y′s have been proposed to aid in chromosome stability, influence the spreading of transcriptional silencing and facilitate recombination-dependent mechanisms of telomere maintenance [3, 40].

Figure 1. Diagram of a yeast telomere. Telomeric DNA is composed of 300 ± 75 bp of $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats. Flanking the repeats are the middle repetitive Y′ and X sequence elements. Y′ elements are either 5.2 or 6.7 kb in size and are present in zero to four copies. X sequences are more heterogeneous in size, containing a 560-bp core and often multiple, shorter elements. A single copy of the X element resides at most telomeres. Short runs of $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats are found between Y′ elements and occasionally between X and Y′ elements. The centromere is represented by the circled C.

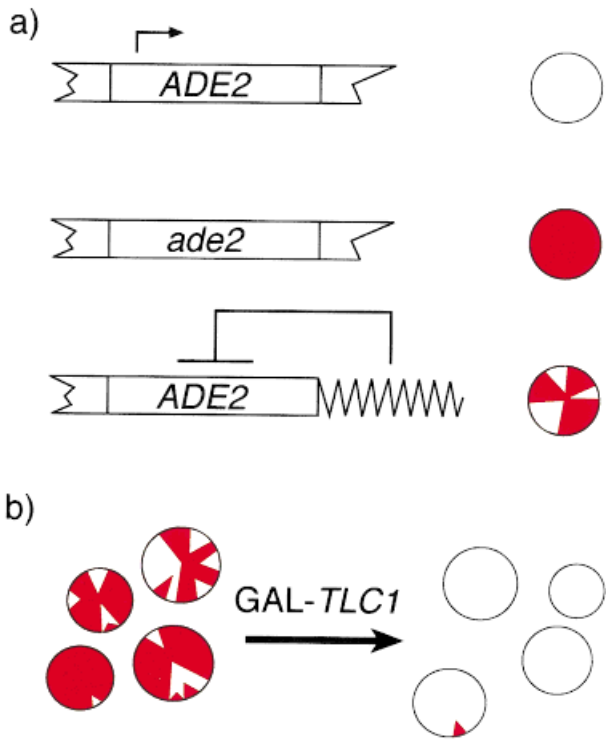

Figure 2. Genes positioned proximal to telomeres are subject to transcriptional silencing. (*a*) At its wild-type locus, the *ADE2* gene is expressed, resulting in white colonies. In an *ade2* mutant or when *ADE2* is transcriptionally silenced, a pigmented biosynthetic precursor of adenine accumulates within cells and red colonies result. When *ADE2* is positioned adjacent to a telomere, its expression is ~50% silenced. Telomeric silencing is stably inherited through many cell divisions, but can be reversed. Thus, a single cell harbouring an *ADE2*-marked telomere grows into a red-and-white sectored colony. (*b*) Overexpression of the *TLC1* RNA by *GAL* induction leads to a loss of transcriptional silencing of an *ADE2*-marked telomere [125]. This observation suggests a link may exist between telomerase and transcriptional silencing at telomeres.

Telomeric silencing and the telosome

TPE was first observed when an RNA Pol II-transcribed gene such as *ADE2* was positioned next to $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric DNA (*ADE2*-TEL), and its expression appeared unusual (fig. 2a) [44]. Any randomly selected single colony was estimated to have an ~50% chance of repression of the *ADE2*-TEL gene. However, the repressed state was epigenetic in that it could be stably inherited for many cell divisions, but was reversible, and switches could be detected during the growth of the colony [44]. Furthermore, studies using different marked telomeres revealed that the fraction of repressed cells was inversely proportional to the reporter gene's distance from the telomere [44, 45]. Transcriptional silencing was observed when the reporter gene's promoter was ~1–3 kb from the $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats, but was relieved at distances greater than 3.5 kb [45]. Thus, telomeric silencing, like silencing at the *HM* loci, was demonstrated to be position-dependent, but neither gene-specific nor telomere-specific [44]. Telomeric effects on transcription have also been observed in *Schizosaccharomyces pombe*, *Drosophila* and *Trypanosoma brucei* [46–49].

The relationship between the subtelomeric Y′ sequences and TPE is unclear. In fact, Y′ elements have been proposed to act variously as either boosters of or buffers against the spread of telomeric silencing [3, 43, 45, 50]. For example, a *URA3* reporter gene is silenced at greater distances from the telomere in the presence of a single Y′ element [45], but is not subject to TPE when positioned within a Y′ element [3, 43, 50, 51]. Can these seemingly contradictory results be reconciled? There may be fundamental differences between inserting a gene proximal to a Y′ element compared to within a Y′. For instance, internal stretches of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA can silence neighbouring genes [52], and short stretches of $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats positioned centromere-proximal to Y′ elements may assist in the silencing of *URA3* when it is positioned adjacent to a Y′ element [3]. In contrast, perhaps the normal, low level of Y′ transcription stimulates *URA3* expression when it is placed within the Y′ element, thereby eliminating TPE.

The nature of telomere-associated chromatin was examined by analysing micrococcal nuclease (MNase) and DNase I digestion patterns of a single *URA3*-marked telomere and of X elements, Y′ elements and $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats from normal, unmarked telomeres [35]. MNase analysis revealed that *URA3* and X and Y′ elements were packaged in nucleosomes. In contrast, MNase digestion of the $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats from both the *URA3*-TEL and natural telomeres produced a large, heterogeneous smear rather than discrete bands characteristic of nucleosomal packaging. This nonnucleosomal pattern suggested that $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeat DNA bound by protein other than or in addition to histones. Indeed, as discussed below, a nonnucleosomal telomeric DNA/protein complex exists in vivo and is defined as the telosome. Furthermore, the region between the $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats and the *URA3* gene was hypersensitive to DNase I, pointing to the existence of a boundary between the two types of telomere-proximal chromatin [35].

The observation that the *URA3*-TEL construct used in the experiment described above was subject to transcriptional silencing, despite being packaged in a nucleosomal configuration shared with transcriptionally active genes, seems paradoxical. To explore the chromatin further, *dam* methyltransferase marking studies were performed to compare distinct alleles of *URA3* at its natural locus and at a telomeric position [53]. 100% of the chromosomal locus was methylated, whereas only 30% of telomeric *URA3* was methylated. Muta-

tions in genes required for TPE restore full *dam* methylation of *URA3*-TEL [53]. Since transcriptionally inactive DNA is generally much less accessible to *dam* than is actively transcribed DNA [54], the reduced methylation of telomeric *URA3* suggests that the access of transcriptional machinery to this locus is limited.

Does telomeric chromatin modulate the expression of endogenous telomere-proximal *S. cerevisiae* genes? One report suggests that transcription of a defective, inactive member of the Ty5 retrotransposon family occurs in silencing defective backgrounds [55]. However, for many genes in proximity to telomeres (such as *MAL*, *SUC* and *PHO* gene family members), alterations in the level of expression of wild-type genes at their natural loci due to TPE have not been reported. Thus, it seems likely that in *S. cerevisiae* silencing of telomeric reporter genes is a reflection of the unique chromatin structure found at telomeres rather than a mechanism by which the expression of endogenous genes is regulated.

Protein components of telomeric chromatin

Over 30 genes that affect the chromatin structure and/or length of telomeres have been identified, often on the basis of a defect in telomeric silencing (reviewed in refs 3, 26). These genes encode either proteins that are components of telomeric chromatin or factors that affect telomeric chromatin indirectly, for instance, through post-translational modification. Many genes that perturb TPE when mutant or increased in dosage also influence transcriptional silencing at the *HM* loci, suggesting that the chromatin in these regions is similar [20]. Below is a summary of *RAP1*, *RIF1*, *RIF2*, *SIR2*, *SIR3*, *SIR4*, and the genes encoding histones H3 and H4. All of these genes affect TPE and *HM* silencing [20, 56], although not necessarily to the same extent, and our emphasis is on the roles these genes play in maintaining telomere integrity. Multiple criteria define Rap1p, Sir2p, Sir3p, Sir4p, and histones H3 and H4 as components of a telomeric chromatin complex. Although Rif1p and Rif2p have not yet been shown to directly associate with this complex, their mutant and dosage phenotypes make a compelling argument for their presence in telomeric chromatin (fig. 3).

The repressor/activator protein, Rap1p

RAP1 is essential and encodes an 827-amino acid protein required for both repression and activation of a number of genes (reviewed in refs 28, 31). Rap1p binds DNA at the *HM* loci both in vitro [57–59] and in vivo [60]. Permutations of the 13-bp Rap1p consensus binding site are also found on average every 18 bp within the $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric DNA repeats [61], and Rap1p binds approximately every 18 bp of double-stranded telomeric DNA in vitro [61, 62]. The co-crystal structure of the Rap1p DNA binding domain (amino acids 361–596) and an 18-bp telomeric DNA fragment reveals that Rap1p contains structural similarity to the proto-oncogene Myb [63]. Interestingly, Myb-like motifs are predicted in three other proteins that bind telomeres, human TRF1 [64], human TRF2 [65, 66] and fission yeast *S. pombe* Taz1p [67]. Although Rap1p shows little sequence identity with either TRF1, TRF2 or Taz1p [64–67], and the telomeric DNA repeats differ significantly among these organisms, the conservation of a Myb-like structural domain suggests that Rap1p, TRF1, TRF2 and Taz1p may bind telomeric DNA similarly.

Rap1p binds telomeric DNA in vivo [35, 60, 68], and the extent of binding has been characterized through in situ formaldehyde cross-linking, followed by immunoprecipitation, and polymerase chain reaction (PCR) using an array of primers designed to detect DNA

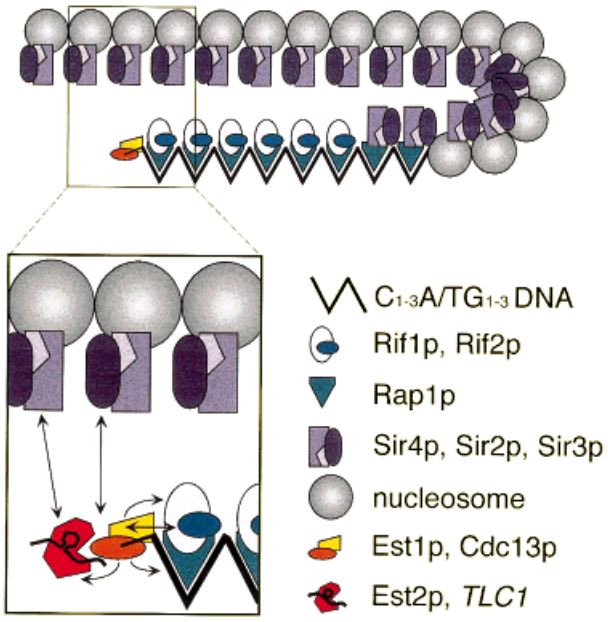

Figure 3. Model of telomeric chromatin. Rap1p binds double-stranded telomeric DNA repeats, and subtelomeric DNA is packaged into nucleosomes. Rif1p and Rif2p are proposed to associate with Rap1p at sites most distal from the centromere [30, 56]. Sir2p, Sir3p and Sir4p form complexes with both Rap1p and histones. The telomere is proposed to fold back or loop, potentially facilitating interactions between chromatin components bound to telomeric and subtelomeric DNA [30, 60]. For simplicity, nucleosomal DNA is not illustrated. (Inset) Est1p and Cdc13p are proposed to bind to single-stranded telomeric DNA 3′ overhangs in vivo and may serve as docking sites for the catalytic components of telomerase, Est2p and *TLC1* RNA. As indicated by arrows, Est1p, Cdc13p, Est2p and *TLC1* RNA may interact with other telomeric chromatin components such as Rap1p, Rif1p, Rif2p, Sir2p, Sir3p and Sir4p.

sequences at various distances from a telomere [60]. From these studies, the presence of Rap1p was inferred in chromatin that extended 0.5–4.0 kb from the telomere [60]. Additionally, isolated foci of Rap1p were observed from 10–30 kb away at two different telomeres. These results were unexpected because previous experiments were consistent with the idea that Rap1p bound telomeres exclusively at the $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats, whereas nucleosomes packaged subtelomeric DNA [35]. One possible explanation for this observation is that Rap1p does not directly bind to the DNA in these subtelomeric regions. Rather, the telomere may naturally fold or loop to form higher-order structure and facilitate Rap1p association with chromatin components found at subtelomeric sites (fig. 3) [30, 60].

Distinct classes of *RAP1* mutants have revealed a requirement for *RAP1* function in silencing at telomeres and the *HM* loci, and in telomere length control [69–73]. For instance, *rap1-17* encodes a truncated protein missing its C-terminal 165 amino acids [71]. Although rap1-17p binds to telomeric DNA in vitro, *rap1-17* mutants grow about twice as slowly as wild-type yeast, display a 20-fold increase in chromosome loss and, most remarkably, have heterogeneous, radically elongated telomeres (>4 kb in some instances compared with the usual ~300 bp) [71]. Moreover, *rap1-17* mutant strains have silencing defects at both *HML* and telomeres [72].

Overexpression of Rap1p increases telomere length heterogeneity and causes cells to grow more slowly than wild type [68]. By pedigree analysis, a 12% death rate per cell division was observed for cells overexpressing Rap1p, and this increased death rate appeared to be due, at least in part, to an increase in the rate of chromosome loss [68] much like that observed for *rap1-17* mutants. An in-frame deletion allele of *RAP1*, *rap1ΔBB*, is incapable of supporting cellular viability, because it encodes a protein lacking amino acids 19–479, a region including part of the Rap1p DNA binding domain [68, 74]. Overexpression of *rap1ΔBB* in a wild-type strain results in a loss of TPE and causes telomeres to elongate up to 200 bp, but has no effect on chromosome stability, suggesting that telomere lengthening per se does not cause chromosome instability [68, 75]. Instead, overexpression of the C-terminus of Rap1p may titrate out factors that are required for TPE and that normally limit telomere length.

Rap1p interacting factors, Rif1p and Rif2p

Rif1p and Rif2p were identified as proteins that interact with the C-terminus of Rap1p in two-hybrid assays [56, 76]. Neither *RIF1* nor *RIF2* is essential, and the proteins they encode, 1916 and 395 amino acids, respectively, do not contain informative sequence motifs [56, 76]. Interestingly, Rif1p and Rif2p themselves interact suggesting that Rif1p, Rif2p and Rap1p may form a complex in vivo [56]. *rif1Δ* and *rif2Δ* mutants have similar phenotypes, consistent with the idea that they have related functions [56, 76]. The mutant strains have telomeres that are 100–300 bp longer than wild type, display enhanced TPE and exhibit a 3.5- to 7.5-fold increased frequency of chromosome loss [56, 72, 76]. *rif1Δ rif2Δ* double mutants exaggerate each of these phenotypes, demonstrating that the mutations act synergistically [56]. Normal binding of Rif1p and Rif2p to the C-terminus of Rap1p may assist in preventing telomere elongation.

The *rap1-17* allele is predicted to encode a protein incapable of binding either Rif1p or Rif2p because it lacks the C-terminal interaction region [56, 71, 76]. In fact, the telomere length phenotype of *rap1-17 rif1Δ rif2Δ* triple mutants is similar to either a *rap1-17* mutant or a *rif1Δ rif2Δ* double mutant, consistent with a model in which Rap1p, Rif1p and Rif2p interact in vivo [56]. The observation that overexpression of the C-terminal 97 amino acids of Rap1p results in telomere elongation can be explained if Rif1p and Rif2p are titrated away from full-length Rap1p in the presence of extra Rap1p C-terminus molecules [56]. In support of this hypothesis, overexpression of either Rif1p or Rif2p is able to suppress such an imbalance and reduce telomere length [56].

The silent information regulator proteins: Sir2p, Sir3p and Sir4p

SIR2, *SIR3* and *SIR4* are absolutely required for silencing at both the *HM* loci and telomeres, yet none of these genes is essential for viability ([23], reviewed in refs 20–22). Mutations in *SIR2*, *SIR3* and *SIR4* correlate with hyperacetylation of histone H4 present at both the *HM* loci and the subtelomeric Y′ elements [77]. A large body of evidence from diverse experimental approaches suggests that chromatin structure at these loci is fundamentally different from bulk chromatin. The Sir proteins are likely to function in establishing and maintaining these distinct chromatin structures.

SIR2 encodes an evolutionarily conserved 562-amino acid protein [32, 78–80]. Homologues of Sir2p have been identified from bacteria to humans, and four *HST* genes (*H*omologues of *S*ir *T*wo) exist in *S. cerevisiae*, two of which are implicated in telomeric silencing [32, 80]. Unique amongst the *SIR* genes, *sir2* mutants affect both silencing and recombination within the repetitive rDNA in addition to its functions at the *HM* loci and telomeres [81–83]. Based on the observation that histones H3, H4 and H2B at *HM* loci and telomeres are hypoacetylated when Sir2p is overexpressed, while silencing itself is unaffected, Sir2p is proposed to function as either a histone deacetylase or a protein that modulates histone acetylation [77]. However, as yet, the

acetylation status of rDNA chromatin or of the extended silent telomere proximal chromatin produced upon *SIR3* overexpression (see below) is not known.

SIR3 encodes a 973-amino acid protein that shares 50% sequence identity in its N-terminal 214 amino acids with the Origin Replication Complex component, Orc1p [78, 79, 84]. Orc1p, together with the five other subunits, binds to ARS elements in vivo, and the resulting complex is required to initiate DNA replication [85–87]. Despite the long-held view that there may be links between DNA replication and silencing, it is not known whether the Sir3p/Orc1p homology reflects interaction of the two proteins with common partners or has other functional significance (reviewed in refs 33, 88, 89).

SIR4 encodes a 1358-amino acid protein that shares limited sequence similarity with nuclear lamins [78, 90, 91]. Based on this homology, Sir4p might be enmeshed directly in the nuclear envelope. This association has not been reported, although like Rap1p and Sir3p, Sir4p may localize near the nuclear periphery [92, 93].

SIR2, *SIR3* and *SIR4* mutant and dosage studies indicate that Sir proteins play important roles in maintaining telomere and chromosome structure and function. *sir3Δ* and *sir4Δ* mutants have shorter telomeric DNA, approximately 50 bp and 100–150 bp, respectively, than wild-type yeast. The effect is additive, as *sir3Δ sir4Δ* double mutants have up to 200 bp less telomeric DNA than wild-type [92]. *sir2Δ* mutants have not been reported to affect telomere length. *sir3Δ* and *sir4Δ* mutants display modest increases in the rate of mitotic recombination and chromosome loss [92], and *sir2Δ*, *sir3Δ* and *sir4Δ* mutants are defective in illegitimate recombination and DNA end joining [94]. Inducible, high levels of expression of Sir2p or Sir3p, but not Sir4p, are toxic and cause dramatic defects in chromosome stability [95]. Combined overexpression of Sir2p and Sir3p results in a 90% loss rate per cell division of a 125-kb linear chromosomal fragment. Neither the chromosome loss nor toxicity phenotypes of Sir2p, Sir3p or Sir2p/Sir3p co-overexpression appear to result from global repression of transcription [95]. The possibility that the toxicity associated with Sir2p/Sir3p overexpression is the result of the titration of an essential factor from a critical genomic locus remains to be determined. The C-terminus of Sir4p is implicated in interactions with other silencing factors based on its 'anti-sir' effect. This effect was defined because overexpression of either full-length or a C-terminal fragment of Sir4p disrupts silencing at both the *HM* loci and telomeres, suggesting the C-terminus of Sir4p titrates other silencing proteins from these loci [45, 90, 96].

Sir3p is a limiting component of telomeric chromatin [45]. By increasing the gene dosage of *SIR3*, TPE was observed to extend or 'spread' from the telomere toward the centromere [45]. Whereas a gene positioned greater than ~3 kb from a telomere is not silenced ordinarily, increasing *SIR3* gene dosage resulted in improved silencing at distances up to 16 kb. A direct demonstration that Sir3p spreads along chromosomes was made using formaldehyde cross-linking mapping. Increased *SIR3* gene dosage was shown to result in the recovery of Sir3p from ~0.8–15.0 kb along the chromosome as compared with ~0.8–2.5 kb under normal dosage conditions [97]. Using this same cross-linking technique, this extended silenced chromatin was further examined by asking whether Rap1p, Sir2p and Sir4p spread with Sir3p upon increased *SIR3* dosage. The distribution of Rap1p was not reported to change in response to Sir3p overexpression [60]. In contrast, the amount of Sir2p and Sir4p at telomere proximal sites was reduced, and Sir4p, but not Sir2p, spread modestly in the presence of overexpressed Sir3p. These observations led to the proposal that telomeric chromatin in Sir3p overexpressing strains is not simply an extension of the telomeric chromatin found in cells expressing wild-type levels of Sir3p, but rather differs in the composition and distribution of Sir2p and Sir4p ([60], reviewed in ref. 30).

Histones H3 and H4

All histones have 'tail' regions containing multiple conserved lysine residues that are subject to acetylation (reviewed in refs 98, 99). The acetylation state of the histones appears to influence their interaction with DNA. Thus, actively transcribed genes are associated with acetylated histones, whereas transcriptional repression is correlated with histone hypoacetylation [100, 101]. Point mutations and deletions within the N-terminal tail of H4 (amino acids 4–28) reduce silencing at the *HM* loci and telomeres [23, 102]. Mutations in the N-terminus of H3 cause a complete loss of silencing at telomeres, and a defect at *HMR* in combination with H4 mutants [103]. Histone H4 present at telomeres and the *HM* loci is hypoacetylated relative to actively transcribed genes and is acetylated comparably to wild-type in *sir* mutants [77, 104, 105]. At the *HM* loci, three out of the four lysines in the tail region of H4 are hypoacetylated, mimicking the acetylation pattern of H4 observed in the centric heterochromatin of *Drosophila* [104, 106]. These observations support the idea that modifying the acetylation state of histones may be an important general method of regulating silenced regions.

The silent chromatin complex at telomeres

Genetic evidence suggests that silencing proteins and histones H3 and H4 are essential for maintaining wild-type telomeric chromatin structure and that they function as a complex (fig. 3). Indeed, a variety of experiments confirm that many of these proteins can

interact in vitro and do form complexes in vivo. For instance, Sir3p coimmunoprecipitates with Sir2p, Sir4p, Rap1p and histones H2A, H2B, H3 and H4 [60, 95, 97]. Sir3p interacts with the C-termini of Sir4p and Rap1p in vitro [107–109] and in two-hybrid experiments [107]. Furthermore, Sir3p interacts in vitro with Sir2p and the N-termini of histones H3 and H4 [97, 109]. Additionally, the C-terminal 154 amino acids of Sir4p can mediate interactions with Sir4p itself and with the C-terminus of Rap1p by two-hybrid analysis [107, 110], and Sir4p can bind to H4 and H3 tails in vitro [97].

Much has been learned about Rap1p/Sir2p/Sir3p/Sir4p complex formation at telomeres by immunofluorescence microscopy. Rap1p was first shown to localize to the ends of pachytene-arrested meiotic chromosomes [111]. More recently, through a combination of fluorescence in situ hybridization (FISH) and immunofluorescence microscopy, punctate foci of Rap1p, Sir3p and Sir4p were shown to coincide with subtelomeric Y′ elements [92, 93]. The staining pattern of Sir2p appeared more complex since it colocalized with Rap1p at telomeres and with Nop1p in the nucleolus [112]. All of these observations are consistent with the formaldehyde cross-linking data showing that Rap1p, Sir3p, Sir4p and Sir2p are present in telomeric chromatin, and that Sir2p is also present at rDNA [60, 97, 112].

Although there are 64 telomeres in a diploid yeast, only 7–8 anti-Rap1p staining foci are observed in most cells [92, 93]. The number of anti-Rap1p foci increased to an average of 16 if cells were treated with nonionic detergent [113]. These observations suggest that in wild-type cells telomeres cluster, possibly through protein-protein interactions, and raise the question of whether or not mutations that disrupt telomeric silencing also perturb telomere clustering. In *sir2Δ*, *sir3Δ*, and *sir4Δ* mutants, anti-Rap1p staining foci become both more numerous and more diffuse [92, 112]. Surprisingly, *sir3Δ* and *sir4Δ* mutants show only subtle, qualitative differences in FISH localization of Y′ sequences relative to wild type, suggesting that telomeric clustering is not *SIR*-dependent and is not sufficient for silencing [92, 93]. Telomeric localization of Sir3p and Sir4p is also disrupted in silencing-defective mutants. For example, deletion of *SIR2*, or mutations in the C-terminus of Rap1p or the N-termini of histones H3 and H4, lead to diffuse anti-Sir3p and/or anti-Sir4p staining [96, 112, 114]. Likewise, a *sir3Δ* mutation results in dispersion of anti-Sir4p staining, and Sir3p and Sir4p are delocalized when overexpressed [115].

Finally, studies suggest that Sir2p, and under certain circumstances Sir3p and Sir4p, also localize at the nucleolus [112, 116, 117]. The observation that distinct silenced loci share overlapping subsets of chromatin components suggests that there may be a dynamic interplay between the loci contributing to the regulation of both silencing and chromatin structure.

Yeast telomerase and the *EST* genes

Twenty-five years ago, Watson noted that conventional DNA replication enzymes would be incapable of fully replicating linear chromosomes, thus defining the 'end-replication problem' ([118], reviewed in ref. 119). More specifically, removal of the RNA primer required to initiate DNA synthesis would leave an unreplicated gap in the lagging strand. Initially through the study of ciliates, but more recently through studies of yeast and mammalian cells, much has been discovered about how this replication problem is solved. In most organisms, a specialized ribonucleoenzyme called telomerase is required. Telomerase is composed of both RNA and protein components. The RNA moiety serves as an internal template for the 5′ to 3′ addition of new nucleotides to the G-rich strand of the telomere, making telomerase a reverse transcriptase. Following telomerase-dependent addition of a single-stranded DNA overhang to the G-rich strand of the telomere, conventional DNA synthesis of the C-rich strand proceeds. Recent reviews detail the biochemistry of telomerase and telomere replication [7, 29].

The mechanism of telomerase-dependent elongation appears to be an evolutionarily conserved process based on the remarkable observation that yeast cells have the capacity to use ciliate telomeric DNA as 'seeds' from which fully functional telomeres can be formed [120–122]. On linear plasmids, as few as 28 bp of *Oxytricha* C_4A_4 repeats act as effective seeds [121]. Moreover, a circular plasmid containing ~300 bp of *Tetrahymena* repeats flanking both sides of a marker gene becomes stably linearized at low frequency. Linearization occurs through the addition of yeast telomeric DNA repeats to the *Tetrahymena* sequence, and these linearization events are detected by screening for loss of the marker gene [123]. Yeast mutants defective in telomere formation, replication and maintenance were identified based on their inability to stably form and maintain the linear version of this plasmid [123, 124].

How might the absence of telomerase activity affect *S. cerevisiae*? A mutation in a component of telomerase yielding a nonfunctional enzyme was predicted not to cause immediate inviability [123]. Rather, loss of telomerase activity would result in a gradual shortening of $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric DNA, and only after many cell divisions would the effects of this loss be manifested. Abolishing telomere function would ultimately be catastrophic, resulting in the inability to stably maintain chromosomes and cell death [123]. Five genes have been identified which when mutated result in the predicted phenotypes: *TLC1 (T*elomerase *C*omponent *1)*, *EST1*, *EST2*, *EST3* (*E*ver *S*horter *T*elomeres), and *CDC13*/*EST4* [123–125]. Yeast cells with *tlc1Δ*, *est1Δ*, *est2Δ* or *est3* mutations exhibit a loss of telomeric DNA estimated at ~3 base pairs per generation (fig. 4a) [123–

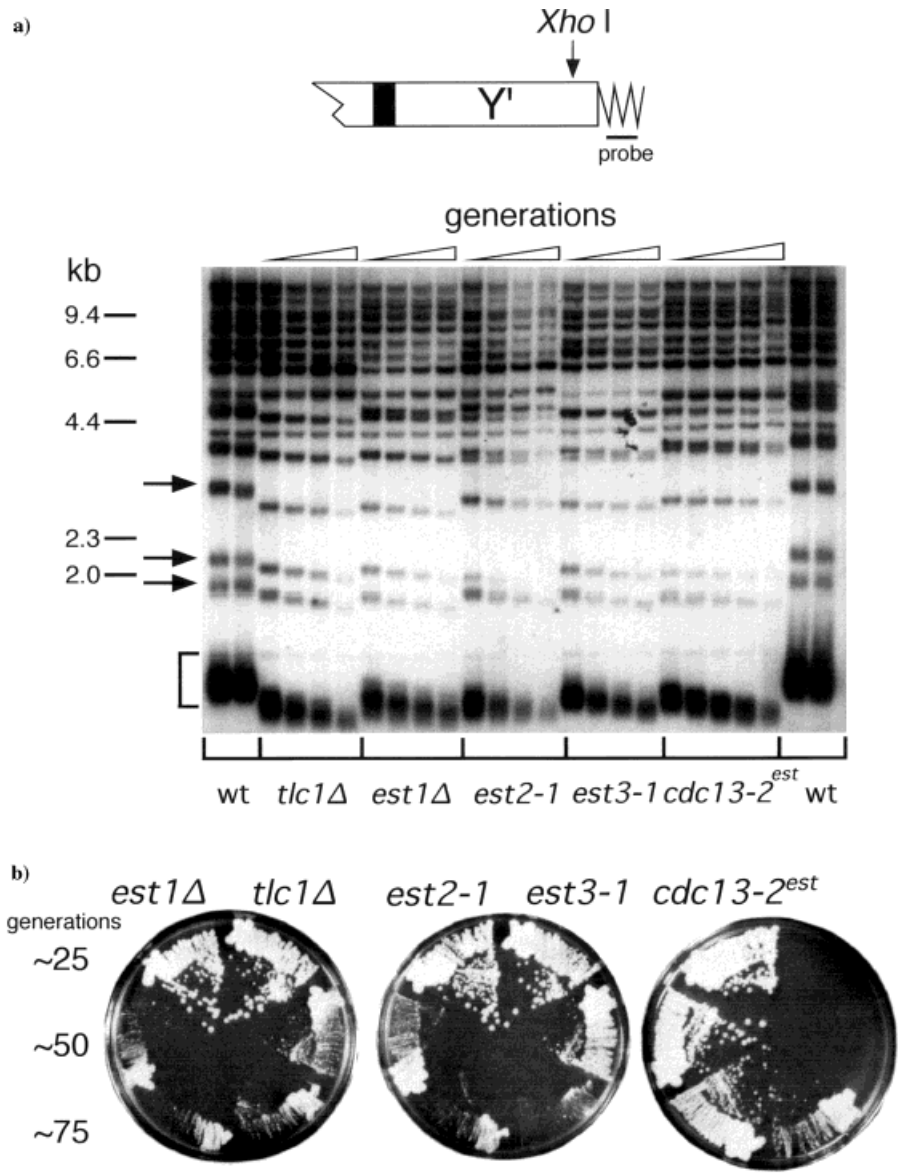

Figure 4. Mutations in *TLC1*, *EST1*, *EST2*, *EST3* and *CDC13* result in a progressive shortening of telomeric DNA and senescence. (*a*) Wild-type, *tlc1Δ*, *est1Δ*, *est2-1* and *est3-1* strains were serially subcultured four times representing ~65–70 total generations of growth. Due to the phenotypic lag exhibited by the *cdc13-2*^{est} mutant, this strain was grown for an extra ~15 generations. As diagrammed, a single *Xho* I site is present within Y′ elements. Genomic DNA harvested from each serial subculture was digested with *Xho* I and Southern hybridization was performed with a probe specific for $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats. The bracketed region represents the telomeric DNA of all Y′-containing telomeres. Non-Y′-containing telomeres are indicated by arrows. Wild-type samples flank mutant samples, which are ordered by increasing generations. (*b*) Comparisons of *est1Δ*, *est2-1*, *est3-1* and *cdc13*^{est} mutant cells after the indicated number of generations of growth reveal that a progressive loss of viability or senescence phenotype correlates with telomeric DNA shortening. Each restreak represents ~25 generations growth; an additional restreak is shown for *cdc13*^{est}. Note that the *est2-1* allele is phenotypically identical in these assays to *est2Δ* [124]. (Reprinted with permission from: Lendvay T. S., Morris D. K., Sah J., Balasubramanian B. and Lundblad V. (1996) Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional *EST* genes. Genetics **144:** 1399–1412, © 1997 Genetics Society of America.)

126]. Initially, loss of *TLC1*, *EST1*, *EST2* or *EST3* function has no detectable effect on the ability of mutants to form colonies similar in size and number to wild-type cells. By ~50 generations, *tlc1Δ*, *est1Δ*, *est2Δ* and *est3* mutants grow less heartily, and after ~75 generations, >225 bp of DNA have been lost, many cells are dead and those few that are viable grow markedly more slowly than wild-type yeast (fig. 4b)

[124, 125]. The limited replicative potential displayed by these mutants is referred to as senescence. A *cdc13-2^{est}* mutant is phenotypically identical to *tlc1Δ*, *est1Δ*, *est2Δ* and *est3* mutants except for a slight delay (~15 generations) in the onset of telomere shortening and senescence (fig. 4) [124, 126]. As discussed below, a small fraction of yeast cells escape the dire consequences of *tlc1Δ* and *estΔ* mutations through the formation of *RAD52* recombination-dependent survivors [124, 127].

The *tlc1Δ* and *estΔ* mutant phenotypes are indicative of an inability to fully replicate and maintain telomeres, suggesting that one or more of these genes encodes a component of telomerase. Indeed, *TLC1* encodes the RNA template and *EST2* encodes the catalytic protein component of yeast telomerase [125, 128, 129]. Because *tlc1Δ* and *estΔ* mutants have identical phenotypes and because double and quadruple mutant combinations show no enhanced or synthetic phenotypes, the products encoded by these genes are proposed to function in a single telomerase-mediated replication pathway [124, 126, 130, 131]. In vitro, Est1p and Cdc13p both bind single-stranded telomeric DNA [126, 130, 132]. Thus, Est1p, Est3p and Cdc13p may be noncatalytic subunits of a telomerase complex whose activity is required to recruit *TLC1* and Est2p to the telomere in vivo. *EST3* functions have not yet been described. Properties of *TLC1* RNA and the Est1, Est2 and Cdc13 proteins are summarized below.

TLC1 was discovered by an associated telomeric silencing defect [125]. Singer and Gottschling searched for cDNAs which when overexpressed led to derepression of an *ADE2*-marked telomere (fig. 2b). *TLC1* was one of several cDNAs meeting this criterion, and it was the only cDNA class that specifically derepressed telomeres but not the *HMR* silent mating-type locus. Overexpression of *TLC1* for ~60 generations of growth caused a loss of 90–220 bp of telomeric DNA [125]. Although none of the original *TLC1* isolates was full length, overexpression of a complete *TLC1* clone has the same silencing defect as the truncated isolates (M. Singer and D. Gottschling, unpublished observation). The implications of these observations for a role of *TLC1* RNA in chromatin structure are discussed below.

Upon sequencing *TLC1* cDNA clones, no predicted ORFs longer than 43 amino acids were found although they were observed to contain the sequence 5′ CACCACACCCACACAC 3′ [125]. From examination of de novo telomere formation, a subset of this motif was predicted previously to serve as a template for yeast telomere replication [122]. To test *TLC1*'s function, experiments were performed in which a region within the putative template domain was mutated to create an endonuclease restriction site. This new restriction site became incorporated into telomeric DNA in vivo via the mutated template, demonstrating that *TLC1* encodes the telomerase RNA component [125].

The *TLC1* RNA is ~1.3 kb, making it significantly longer than telomerase RNAs from many other organisms (e.g. ref. 133). Recent experiments revealed several other interesting properties of the *TLC1* RNA, including that it is a polyadenylated RNA Pol II transcript [134]. This contrasts with telomerase RNAs from ciliates that are nonpolyadenylated RNA polymerase III transcripts [133, 135], but explains the presence of *TLC1* in the cDNA library from which it was first identified [125]. Although the poly(A)$^+$ fraction is stable, it appears to be the precursor of a nonadenylated form and represents only 5–10% of the total *TLC1* RNA. An increase in the amount of poly(A)$^+$ *TLC1* is observed between the G1 and S phases of the cell cycle, suggesting that *TLC1* RNA level is regulated in a cell cycle-dependent manner. The importance of the *TLC1* RNA poly(A) tail is unknown, but is proposed to stabilize the molecule, perhaps during the assembly of the telomerase ribonucleoprotein. These results also raise the possibility that there may be distinct poly(A)$^+$ and poly(A)$^-$ telomerase complexes [134].

A variety of in vitro telomerase activity assays were developed in efforts to identify catalytic protein components of telomerase [7, 128, 136–138]. One approach has been to partially purify telomerase activity from whole cell extracts [128, 136]. When such extracts are supplied with an oligo primer and the nucleotides dGTP and dTTP, a *TLC1*-dependent extension of the primer is observed [128, 129, 136]. Following this general approach, telomerase activity was shown to be present in extracts prepared from *est1Δ*, *est3* and *cdc13-2^{est}* mutant strains, indicating that Est1p, Est3p and Cdc13p are not catalytic components of telomerase [131, 136]. In contrast, telomerase activity was not observed in extracts derived from *est2* mutant strains [128, 129]. Additionally, Est2p coimmunopreciptitates with *TLC1* RNA [128, 129] and telomerase activity [129]. These observations, in combination with sequence comparison data (see below), strongly suggest that Est2p is the catalytic subunit of telomerase [128]. However, PCR-based telomerase detection systems and immunoprecipitations of Est1p have identified Est1p-dependent and *TLC1*-independent, telomerase-like activities [138, 139]. These results have been interpreted to suggest the possibility that Est1p may increase the processivity of telomerase and that a second telomerase RNA template may exist [138, 139]. Thus, the roles of Est1p, Est3p and Cdc13p in telomerase activity are not yet defined, whereas a catalytic role for *TLC1*/Est2p is well established.

Telomeric DNA replicates late in the S phase of the cell cycle [5, 6], and during this time telomeres transiently acquire 3′ $TG_{1\text{-}3}$ overhangs estimated at >50 bp in length [140]. These overhangs form immediately after the replication fork reaches the telomeres [141] at both ends of a linear plasmid [142]. In the absence of *TLC1*, single-stranded overhangs still form in the same cell cycle-regulated manner as observed for wild-type cells [142, 143]. This finding reveals that these long G-rich

overhangs form independently of telomerase, and may instead result from nuclease processing of the $C_{1\text{-}3}A$ strand [142, 143]. These combined observations lead to the proposal that as a very late step in chromosome replication, $TG_{1\text{-}3}$ tails form at both ends of each chromosome by a strand-specific nuclease, thereby creating substrates for telomerase-dependent extension of $TG_{1\text{-}3}$ DNA. Following telomerase elongation, conventional DNA repair machinery presumably fills these overhangs, leaving short 3′ overhangs at both ends of each chromosome [119, 142]. Based on studies from ciliates [144], such short overhangs are likely to be present at each telomere throughout the cell cycle, possibly serving as binding sites for terminus-specific proteins [142].

EST2 encodes a basic, 884-amino acid protein that is similar to p123, a component of *Euplotes* telomerase [124, 128, 129]. Consistent with the notion that Est2p and p123 are catalytic components of telomerase, both Est2p and p123 contain reverse transcriptase motifs most commonly found in group II introns and non-LTR (long terminal repeat) retrotransposons [128]. Recently, human and *S. pombe* homologues of Est2p and p123 were identified, and their discovery revealed additional regions of conservation among telomerases [15, 16]. Point mutations made in any of three aspartates in Est2p that are absolutely conserved in telomerases and reverse transcriptases resulted in senescence and, in the two mutants examined, loss of telomerase activity [128, 129]. When these *est2* mutants were expressed in high copy in a strain containing wild-type *EST2*, telomeres were somewhat shortened but senescence was not observed [128]. This may reflect that telomeres shorten insufficiently to reach a crisis point for senescence. Thus, these mutant est2 proteins may partially interfere with in vivo telomerase activity by titrating other factors necessary for telomerase function such as *TLC1*, Est1p, Est3p or Cdc13p.

EST1 encodes a 699-amino acid protein with no known homologues, though it has been reported to share limited sequence identity with both DNA and RNA-dependent polymerases [123, 145]. In vitro, recombinant Est1p binds single-stranded but not double-stranded DNA. This binding corresponds to the G-rich strand of yeast telomeres and requires a free 3′ overhang [130]. The possibility that Est1p may bind the 3′ overhang of telomeres in vivo is particularly appealing considering that single-stranded G-rich telomeric DNA overhangs are present in vivo [140, 142, 143]. Additionally, gel-shift experiments reveal that two distinct DNA/Est1p complexes can be formed, suggesting that multimerization of Est1p occurs in vitro [130]. Deletion analysis identified amino acids 435–565 to be required for ssDNA binding in vitro and for Est1p function in vivo [130]. The dissociation constant of Est1p for single-stranded telomeric DNA is a modest 250 nM, suggesting that in vivo Est1p may require a binding partner to strengthen this interaction, as is observed for the α-subunit of the *Oxytricha* heterodimeric telomere-binding complex [146]. The possibility that another of the Est proteins such as Cdc13p might fulfill this role remains intriguing (see below).

Est1p was demonstrated to coimmunoprecipitate specifically with *TLC1* RNA, suggesting that it may associate with or be a component of telomerase in vivo [138, 139]. However, because only 1–5% of total *TLC1* was estimated to coimmunoprecipitate with Est1p, *TLC1* may exist in distinct bound and unbound pools [138, 139]. In vitro, recombinant Est1p has been shown to bind RNA nonspecifically [130]. A deletion derivative of Est1p abolishes binding to both RNA and single-stranded telomeric DNA. Through competition experiments, Est1p appears able to bind both RNA and telomeric DNA independently [130]. One possible interpretation of this observation is that in vivo Est1p binds both telomeric DNA and *TLC1* RNA, but the interaction with *TLC1* RNA requires an additional specificity factor. For instance, Est1p might associate physically with Est2p and through this interaction bind to a region of *TLC1* RNA.

CDC13 is an essential gene, making it unique among the *TLC/EST* genes [147]. *CDC13* encodes a 924-amino acid protein [147] that, like Est1p, binds to single-stranded $TG_{1\text{-}3}$ telomeric repeats in vitro suggesting it may also bind these repeats in vivo [126, 132]. Unlike Est1p, Cdc13p does not require a free 3′ overhang to bind telomeric DNA [126, 132]. The mutant phenotype of *cdc13-2^{est}* is suppressed by overexpression of *EST1* but not *TLC1* or *EST2*, hinting at an interaction between these proteins in vivo [126]. The cdc13^{est} mutant protein remains capable of binding $TG_{1\text{-}3}$ telomeric repeats in vitro, suggesting that the mutation may block functional interactions without interfering with its telomere binding [126].

Interestingly, *CDC13* was first characterized through analysis of a temperature-sensitive allele, *cdc13-1* [147]. The *cdc13-1* mutant cells exhibit a dramatic increase in mitotic recombination of telomere proximal DNA at semirestrictive temperatures, and an accumulation of single-stranded telomeric and subtelomeric DNA at restrictive temperatures [147]. These single-stranded tails extend for several kilobases, include the $TG_{1\text{-}3}$ telomeric repeats, and appear to trigger the *RAD9* DNA damage checkpoint, causing cells to arrest in the G2 phase of the cell cycle [147]. These observations suggest one function of Cdc13p may be to bind single-stranded telomeric DNA, thereby blocking exonuclease attack of the telomeric $C_{1\text{-}3}A$ strand and preventing G2 cell cycle arrest. In contrast to the *cdc13-2^{est}* mutant, the *cdc13-1* mutant does not senesce. Unlike the phenotype of a *cdc13-2^{est} tlc1Δ* double mutant, a *cdc13-1 tlc1Δ* double mutant senesces much more rapidly than a *tlc1Δ* single mutant [126]. Furthermore, the restrictive temperature

of *cdc13-1* is reduced in a *cdc13-1 tlc1Δ* double mutant [126]. These allele-specific interactions define the two proposed roles of Cdc13p in the cell: Cdc13p may be required both to prevent the formation of long, single-stranded telomere tails and to facilitate telomerase-dependent replication, perhaps through interactions with Est1p. Despite the different phenotypes of cells bearing the *cdc13-1* or *cdc13-2^{est}* alleles, both clearly point to Cdc13p as an important factor in telomere maintenance.

The accumulated data lead to the conclusion that *TLC1* RNA and Est2p are catalytic components of yeast telomerase. Whereas Est1p and Cdc13p may be required for in vivo telomerase function, they are not absolutely required for telomerase activity in vitro. Based on the observations that Est1p and Cdc13p bind to single-stranded telomeric DNA in vitro and that *TLC1* RNA and Est1p coimmunoprecipitate, it seems possible that Est1p and Cdc13p may function as non-catalytic components of telomerase that together recruit catalytic telomerase to its substrate (fig. 3, inset). Whether Est3p plays a direct or indirect role in any of these processes remains to be determined.

Both telomere length and topology can influence silencing [75, 148]. These observations derive from comparison of $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats on circular versus linear molecules and lead to the proposal that telomeric silencing requires a terminus-specific binding factor [75]. This idea is based on the finding that TPE is relieved when extra telomeres are introduced into yeast on linear plasmids. In contrast, the presence of extra internal $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats within circular plasmids does not affect TPE [75]. Because *TLC1* overexpression itself abolishes telomeric silencing, one possibility is that telomerase or an associated factor may be the terminus-specific binding component of telomeric chromatin. The *TLC1* overexpression silencing defect can be explained in several ways. For example, excess *TLC1* RNA may interact directly with the telomeric repeats, thereby preventing binding of chromatin components to the telomeric DNA. Alternatively, *TLC1* RNA may normally interact with a factor required for telomeric silencing, and the overexpression of *TLC1* may titrate this factor away, resulting in derepression. Such a factor might be identified genetically by seeking a multicopy suppressor of the *TLC1* overexpression telomeric silencing defect. Since overexpression of *TLC1* was reported to shorten telomeric DNA by 90 to 220 bp, a more trivial explanation is that a reduction of $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats might disrupt telomeric chromatin and result in the loss of telomeric silencing [125].

Preliminary studies have reported telomeric silencing defects associated with mutant high-copy versions of the *EST1* and *CDC13* genes [130, 132]. Overexpression of *EST1* does not affect telomeric silencing; however, overexpression of missense alleles of *EST1* in a wild-type strain produces telomeres 80–150 bp shorter than normal and a concomitant loss of telomeric silencing, reminiscent of that seen for *TLC1* overexpression [130]. Similarly, overexpression of *cdc13-1*, but not *CDC13*, has been reported to reduce telomeric silencing, although this observation is complicated somewhat by the fact that these experiments were performed at high temperatures [132]. Further overexpression studies with both wild-type and mutant *CDC13* should help resolve this issue. Whether *EST2* and *EST3* also affect telomeric silencing is not known.

Although telomeric silencing has not yet been evaluated fully in strains mutated for *EST1*, *EST2*, *EST3* and *CDC13* functions, if Est1p and Cdc13p are bound to telomeric DNA in vivo, they are by definition components of telomeric chromatin. Evidence available is consistent with this interpretation, but will be substantially strengthened if Est1p and Cdc13p are found to be present within the telosome and are shown to localize to telomeres by FISH and immunofluorescence microscopy. The localization of an Est2p/*TLC1* RNA complex to telomeres can be envisioned in at least two ways. First, telomerase might be recruited to telomeres in a transient, cell cycle-dependent manner. Following addition of $TG_{1\text{-}3}$ repeats, telomerase would either be released, inactivated or degraded. Alternatively, telomerase might be present at telomeres throughout the cell cycle, possibly complexed with Est1p and Cdc13p. Upon receiving the appropriate signal, telomerase would be activated to extend the $TG_{1\text{-}3}$ strand. In this model, telomerase would be a structural component of telomeric chromatin. If indeed Cdc13p, Est1p, Est3p, Est2p and *TLC1* RNA (or a subset of these factors) are found at telomeres in vivo, they might interact physically with other telomeric chromatin components such as Rap1p, Rif1p, Rif2p, Sir2p, Sir3p or Sir4p (fig. 3, inset). Perturbations of the proposed Est2p, *TLC1*, Est1p, Cdc13p telomerase complex might result in the disruption of these interactions, thereby accounting for the observed silencing defects.

Telomeric DNA length regulation through recombination and counting mechanisms

As described above, mutations and alterations in the dosage of telomeric chromatin components, telomerase components, and *EST1*, *EST3* and *CDC13* affect both telomere length and TPE, and in some cases also affect chromosome stability and recombination. Specific examples of recombination events that assist in telomere formation, telomeric DNA length regulation and ultimately the maintenance of chromosomes are discussed here. How telomeric chromatin components influence these recombination-dependent length control mechanisms is largely unknown. Rap1p, a major component

of telomeric chromatin, is likely to be involved, and a recent report suggests 'counting' of Rap1p bound at telomeres is responsible for regulation of telomere length [149].

In wild-type yeast cells, recombination at telomeres occurs infrequently [3]. This is surprising because the abundance of subtelomeric Y′ elements at many chromosomes in laboratory yeast strains suggests that recombination may have been an important means of distributing these sequences in the ancestors of modern yeast [3, 40]. Indisputably, however, both classes of Y′ elements can and do recombine [50]. In fact, marked Y′ elements are observed to undergo a variety of duplications and deletions through ectopic and unequal sister chromatid exchange mechanisms [50]. Interestingly, short and long Y′ elements recombine preferentially with members of their own size class [50]. Thus, under appropriate circumstances, Y′ elements might be expected to recombine and spread from telomere to telomere (see below).

Recombination also plays an important role in telomere formation. Seeding experiments with heterologous telomeric repeat sequences, as described above, revealed that recombination nearly always accompanies telomere formation on linear plasmids [121]. These recombination events, however, are independent of *RAD52*, a gene required for most forms of mitotic recombination involving double-strand breaks, suggesting this type of recombination must involve the very ends of telomeres.

What happens to a chromosome when it loses a telomere? This question was addressed by introducing a supernumerary chromosome, containing a cleavage site for the HO-endonuclease proximal to one of its telomeres, into haploid yeast [150]. When HO was induced, the telomere was cleaved and eliminated from the chromosome. Concomitantly, the *RAD9*-dependent DNA damage checkpoint was triggered and the cells were arrested. The arrest, however, was transient. An examination of cells after they had escaped the arrest revealed that the majority repaired the cleaved chromosome end by a *RAD52*-dependent gene conversion event. A minority of cells, surprisingly, were able to propagate the broken chromosome missing its telomere for several generations. These results suggest that a cell may be able to tolerate a chromosome without a telomere, although the chromosome cannot be stably or indefinitely maintained unless it is repaired through *RAD52*-dependent recombination.

Coincident with $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric DNA shortening, there is an increased frequency of chromosome loss and cell death in *tlc1Δ* and *estΔ* mutants [123–126]. Given the observation that a broken chromosome end can be healed through recombination [150], it might be imagined that a recombination-dependent pathway could allow cells to survive in the absence of functional telomerase. Indeed, a small fraction of *tlc1Δ* and *estΔ* mutant cells can escape senescence and grow at rates comparable to wild-type cells [124, 125, 127]. These cells, termed 'survivors', have been most carefully characterized in the *est1Δ* mutant background, although survivors in *est2Δ*, *est3*, *cdc13^{est}* and *tlc1Δ* strains appear to have similar properties [124, 125, 127]. Whereas the $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric DNA shortened in survivors as in early *est1Δ* cells, the subtelomeric Y′ elements were greatly amplified and radically rearranged (fig. 5). In fact, many telomeres lacking Y′ elements in wild-type cells and early *est1Δ* cultures were shown to have acquired them in survivors. Survivor formation appeared to be a dynamic process, because the extent of Y′ amplification was not identical among survivors. Furthermore, after additional time in culture, survivors themselves senesce, implying that the rescuing Y′ elements were subject to the same loss as the original telomeric repeats. In the absence of *RAD52*, survivors were never observed. Instead, *est1Δ rad52Δ* double mutant cells became senescent and died. Thus, *RAD52*-dependent recombination at telomeres may serve as a backup telomere maintenance pathway [127], and one role of Y′ elements may be to function in this alterna-

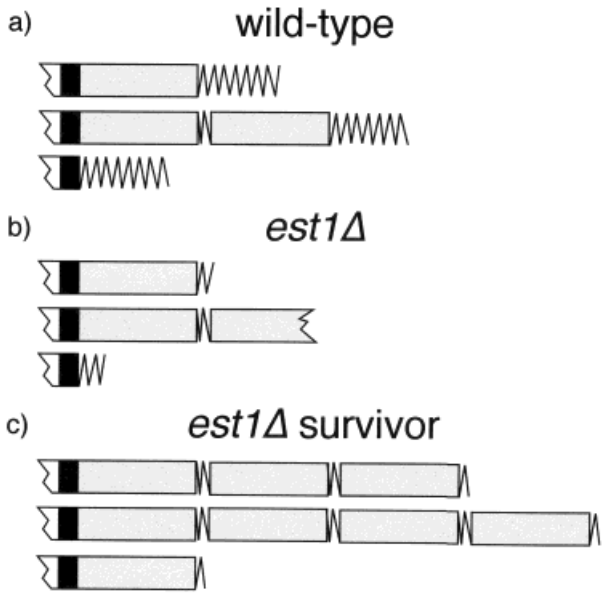

Figure 5. Y′ elements are amplified and rearranged in survivor strains. (*a*) In a wild-type strain, telomeres are fully replicated, telomeric DNA length is maintained at 300 ± 75 bp and Y′ elements are stable. (*b*) In senescing *est1Δ* cells, replication of telomeres is incomplete, and telomeric DNA shortens ~3 bp at each division, eventually resulting in cell death. (*c*) In *est1Δ* survivor strains, telomeric DNA is shortened, but Y′ elements are amplified and rearranged. For instance, telomeres that contain no Y′ elements in wild-type or *est1Δ* senescing strains often acquire them in survivors. Similar observations have been reported for *tlc1Δ*, *est2Δ*, *est3* and *cdc13^{est}* mutants [124, 125]. (Adapted from ref. 127.)

tive pathway [3]. A similar *RAD52*-dependent survivor pathway exists for the yeast *Kluyveromyces lactis* in strains lacking the *TER1* telomerase RNA [151]. The clear parallels between survivors in these two yeasts suggest that *RAD52*-dependent recombination may be an evolutionarily conserved mechanism for telomere maintenance in the absence of telomerase. When telomeres become sufficiently short or are lost, a recombination-dependent pathway can be activated to cap chromosome ends, and hence to maintain cell viability.

The converse problem of what happens to chromosomes when telomeric DNA becomes inordinately long has been examined and also appears to be mediated, at least in part, by recombination [71, 152]. As noted earlier, telomeric DNA is extremely heterogeneous and elongated in cells harbouring the *rap1-17* allele. However, this increase in length is unstable, and populations of elongated telomeres occasionally shorten rapidly [71]. This phenomenon, terminal rapid deletion (TRD), appears stochastic and is not specific to any one chromosome. Further, TRD is an inherent property of the mutant telomeres rather than a direct result of the C-terminally truncated rap1-17p [152]. This was demonstrated by crossing a *rap1-17* mutant strain with a marked telomere to a wild-type strain. Although in most cases, *RAP1* wild-type haploids that inherited the elongated, marked telomere underwent gradual shortening, TRD did occur, suggesting that it may be a normal mechanism of telomeric DNA length regulation.

TRD in wild-type cells is influenced by mutations that affect recombination and chromatin structure [152]. For instance, TRD is decreased $\sim$3-fold in a *rad52Δ* mutant, suggesting that double-strand break repair and gene conversion events contribute to TRD. In the presence of *hpr1Δ*, a mutation that specifically increases intrachromosomal recombination, a 10-fold increase in TRD is observed [152]. This increase in TRD is dependent upon *RAD52*, suggesting TRD even in *hpr1Δ* mutants also occurs through *RAD52*-dependent mechanisms [152]. Mutation of *SIR3*, but not *SIR2* or *SIR4*, also influenced TRD [152]. A high proportion of the *sir3Δ* isolates examined had aberrant TRD profiles, often failing to shorten their telomeric DNA to the same extent as wild-type cells [152]. In contrast, *sir3Δ hpr1Δ* double mutant strain had more rapid TRD than an *hpr1Δ* mutant alone [152]. These observations underscore the idea that Sir3p normally contributes to telomere length regulation, potentially with Hpr1p.

The heterogeneity of telomeric DNA lengths among nonhomologous chromosomes appears absolutely required for TRD [152]. This conclusion comes from studies in which a haploid *rap1Δ* mutant with a plasmid-borne copy of *rap1-17* was serially subcultured until all telomeres became similarly elongated [152]. A plasmid-borne wild-type *RAP1* gene was then exchanged for the *rap1-17* plasmid, so that the experiment started with chromosomes of comparable length and wild-type *RAP1*. TRD was not detected in any isolate derived from this strain even upon the introduction of an *hpr1Δ* mutation. This observation led to the speculation that in *RAP1* strains harbouring half elongated telomeres and half wild-type length telomeres, for example, telomeres are 'measured' against one another, and through intrachromatid recombination or other mechanisms, elongated telomeres become abruptly shortened. In contrast, when telomeres are of uniform length, TRD is not triggered.

The observation that mutations in the C-terminus of Rap1p induce defects in telomere length [69–71, 73] has led to the proposal that telomere length might be measured by 'counting' the number of Rap1p molecules bound at the $C_{1\text{-}3}A/TG_{1\text{-}3}$ tract [149]. This idea was tested in a strain with binding sites for the transcriptional activator Gal4p (UAS_G) positioned proximal to the $C_{1\text{-}3}A/TG_{1\text{-}3}$ tract at a *URA3*-marked telomere. The effects of expressing a construct encoding the DNA binding portion of Gal4p (Gbd) or a Gbd fusion to the C-terminus of Rap1p (Gbd/Rap1) were evaluated [149]. In the presence of Gbd/Rap1, but not Gbd alone, $C_{1\text{-}3}A/TG_{1\text{-}3}$ tract length shortened, presumably because Gbd/Rap1 was tethered to the UAS_G sites. Increasing the number of UAS_G sites present proximal to the telomere led to even greater reduction in $C_{1\text{-}3}A/TG_{1\text{-}3}$ tract length, but not in a simple arithmetic function, suggesting an additional level of complexity to this form of telomere length regulation. In control experiments, a Gbd/rap1-12 mutant fusion protein targeted to the UAS_G-containing telomere resulted in less shortening of the $C_{1\text{-}3}A/TG_{1\text{-}3}$ tract length than wild-type Gbd/Rap1, consistent with the observation that telomeres in strains harbouring the rap1-12 allele are aberrantly long [70]. Gbd/Rap1-dependent shortening was also observed in a *sir4Δ* mutant, demonstrating that telomeric silencing was not necessary for telomere shortening. Additionally, tethering of Gbd/Sir3 or Gbd/Sir4 fusions to the telomere could silence the adjacent *URA3* gene but did not result in telomere shortening. Thus, the establishment of silencing at telomeres is neither necessary nor sufficient to regulate $C_{1\text{-}3}A/TG_{1\text{-}3}$ tract length. Instead, the data suggest that telomeric DNA size may be regulated by a feedback mechanism involving the number of telomeric Rap1p molecules. More specifically, as telomeres shorten and the number of Rap1p molecules bound to telomeric DNA decreases, telomerase may be activated. Upon Rap1p binding to the newly synthesized telomeric DNA, the number of Rap1p molecules present at the telomere might then reach a threshold level that triggers a decline in telomerase activity [149].

The maintenance of telomeric length and structure is clearly a complicated process. Remarkably, yeast tolerate the loss of telomeric DNA by either abrupt (e.g. HO-cleavage) or incremental (e.g. *estΔ*) mechanisms. In

each case, the potentially disastrous consequences of telomere loss are remedied by activation of a recombination-dependent mechanism. Conversely, lengthening telomeric DNA has no apparent negative effects on otherwise wild-type yeast cells. However, in the presence of mixed-length telomeres, cells efficiently cleave the elongated telomeres, apparently at least in part by a recombination-dependent mechanism, suggesting that shorter telomeres may be more optimal. Even when growth conditions are not limiting, telomeric DNA in a wild-type population of cells is heterogeneous (300 ± 75 bp), and individual telomeres undergo stochastic changes in length [153]. These fluctuations in $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats imply that the length of telomeres may differ from mother to daughter cell or among telomeres within a single cell. Hence, the precise number of Rap1p molecules bound to the $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats may vary from one telomere to another, suggesting degrees of complexity beyond a uniform Rap1p-counting mechanism. Whereas the presence of Rap1p bound to telomeric DNA appears necessary for proper telomere length maintenance, it may not be the only chromatin component required for telomerase-dependent telomere length control. Instead, length may normally be influenced by a balance between the activity of telomerase, telomeric chromatin components such as Rap1p, Rif1p and Rif2p, *RAD52*-dependent and independent recombinational machinery, and other factors.

Conclusions

Telomeres are key to faithful replication and passage of DNA from mother to daughter cells because they help prevent detrimental events such as chromosome fusion, degradation and loss. Perhaps for these reasons, the repetitive nature of telomeric DNA and mechanisms for its replication are evolutionarily conserved. In many organisms including yeast, telomeres have an unusual structure resembling heterochromatin. The relationship between gene expression, telomere replication and recombination is gradually being elucidated as the components of telomeric chromatin are defined.

The observation that a reporter gene placed proximal to a telomere can be transcriptionally repressed led to the discovery that telomeric chromatin is strikingly similar to the chromatin found at the *HM* loci [23, 44]. Much evidence supports the idea that Rap1p, Sir2p, Sir3p, Sir4p and histones together form a complex extending for several thousand base pairs from the telomeric repeats into subtelomeric regions. The mechanisms by which the structure of telomeric chromatin is established and maintained are likely to be dynamic. For instance, dosage of Sir3p influences the distance telomeric chromatin extends from the telomere and the composition of the telomeric chromatin complex itself [45, 60]. Moreover, Sir3p has been characterized as a phosphoprotein whose phosphorylation is regulated by MAP (mitogen-activated protein) kinase cascade signalling [154]. Conditions such as starvation, heat shock and exposure to mating pheromone all result in hyperphosphorylation of Sir3p which is correlated with changes in telomeric silencing [154]. Thus, phosphorylation of Sir3p may be a control point for silenced chromatin and could be important in assembly of silent chromatin, much like phosphorylation of HP1 in *Drosophila* is correlated with developmentally regulated heterochromatin assembly [155].

Other enzymatic activities are likely to play roles in modifying components of telomeric chromatin. For example, the deubiquitinating enzyme Ubp3p interacts in vitro with Sir4p [108]. Loss of *UBP3* function results in a slight increase in transcriptional silencing, although whether this occurs directly through Sir4p or through ubiquitination is not yet established [108]. Mutations in either *NAT1* or *ARD1*, which encode subunits of an N-terminal acetyltransferase complex, or in *SAS2*, a putative acetyltransferase, completely relieve telomeric silencing [23, 156]. In addition to the genes described here, many others have been identified which when mutated or overexpressed alter TPE and telomeric DNA length [3, 26]. Sorting through these genes is likely to identify both additional structural components of telomeric chromatin as well as proteins required to modify the components of telomeric chromatin and regulate its assembly.

Est2p and *TLC1* RNA were identified as catalytic components of yeast telomerase [125, 128]. Neither Est1p, Cdc13p or Est3p is necessary for telomerase activity in vitro. However, they may be noncatalytic subunits of telomerase, because mutations in any one of the genes encoding these proteins result in phenotypes identical to those observed for *est2Δ* or *tlc1Δ* mutants [124, 126, 130, 131, 136]. In vivo, Est1p and Cdc13p likely bind to the single-stranded $TG_{1\text{-}3}$ overhangs at both ends of each chromosome [126, 130, 132]. Thus, Cdc13p and Est1p may serve to localize the catalytic subunits of telomerase to the telomere, raising the possibility that telomerase itself is a component of telomeric chromatin.

Structural components of chromatin with enzymatic activities are not without precedence. For instance, DNA topoisomerase II, the enzyme required to decatenate DNA following eplication localizes along the entire length of chromosomes as a metaphase 'scaffold' during mitosis (reviewed in ref. 157). Whether telomerase itself might have similar, structural interactions with the ends of chromosomes or other regions of the nucleus remains a fascinating possibility.

The effects of telomeric chromatin on telomeric recombination are not fully understood. *RAD52*-dependent mechanisms of repairing broken chromosomes, thereby enabling cells to survive in the absence of telomerase, have been identified [127, 150]. Whether the presence of

telomeric chromatin components facilitates or hinders gene conversion events that heal broken chromosomes remains to be determined.

In recent years, through a combination of genetics, biochemistry and cell biology, a wealth of information has been collected about the structural components of silenced chromatin, telomeres and telomerase. Not known are how telomeric chromatin is assembled, how telomerase-dependent telomere replication occurs, and the relationship of both processes to each other and to recombination. It has been proposed that telomeres may serve as reservoirs for the silencing components Sir2p, Sir3p and Sir4p [158]. More specifically, telomeres may serve as storage sites for these Sir proteins until environmental or other conditions dictate their movement to the *HM* loci, nucleolus or other genomic sites. Alternatively, the structure of the chromatin at telomeres itself may have an important, as yet unidentified, role in maintaining telomeres and hence viability.

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