Chapter

Telomere Formation Systems in Budding and Fission Yeasts

Julien Audry and Kurt W. Runge

Abstract

Telomeres are specialized structures essential for genomic stability in eukaryotic cells. Inducible systems causing telomere shortening or telomere formation from short tracts of telomere repeats were developed in the late 1990s in *Saccharomyces cerevisiae* and have been adapted to investigate multiple aspects of telomere biology. In the formation system, an internal tract of telomere repeats is placed next to an inducible double-strand break. Inducing the break converts the telomere tract into a functional telomere whose fate can be followed kinetically and allows one to assay elongation, protein recruitment, and the DNA damage checkpoint activation. This work was extended to *Schizosaccharomyces pombe*, as it shares some features of telomeric chromatin with mammalian cells that are missing in *S. cerevisiae*. The *S. pombe* system has revealed novel aspects of telomeric chromatin formation and similarities with *S. cerevisiae*. This chapter will review these past discoveries in different yeast model organisms, and what they reveal about telomere physiology that may well be conserved in mammals.

Keywords: telomere formation, chromosome end, double-strand break, checkpoint, heterochromatin, yeast, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, I-SceI, HO, endonuclease, Cre, recombinase

1. Introduction

1.1 Context

Telomeres are the physical ends of linear eukaryotic chromosomes. The chromosomal end structure of most telomeres is composed of G-rich DNA repeats bound by specific proteins (**Table 1** and **Figure 1**). One of the major functions of this nucleoprotein complex is to prevent chromosome fusions and the recognition of the ends as a double-strand break (DSB) [1, 2]. Due to the semi-conservative replication and nuclease activities occurring at the chromosome ends, the telomeres shorten at each cell division, a process called the "end replication problem" [3, 4]. To counteract this gradual shortening, telomerase can elongate short telomeres at each division [5, 6]. Telomerase is expressed constitutively in unicellular eukaryotic organisms such as yeasts, but not in most of the somatic cells of humans [7]. The gradual erosion of telomere repeats leads to loss of telomere binding proteins and chromosome end deprotection, which activates the DNA damage response (DDR). The DDR can cause replicative senescence or apoptosis, acting as an anti-proliferative barrier [8, 9]. The dysfunction of this barrier can generate genomic instability, chromosomal aberrations and initiate tumorigenesis or oncogenic transformation of the cells [10].

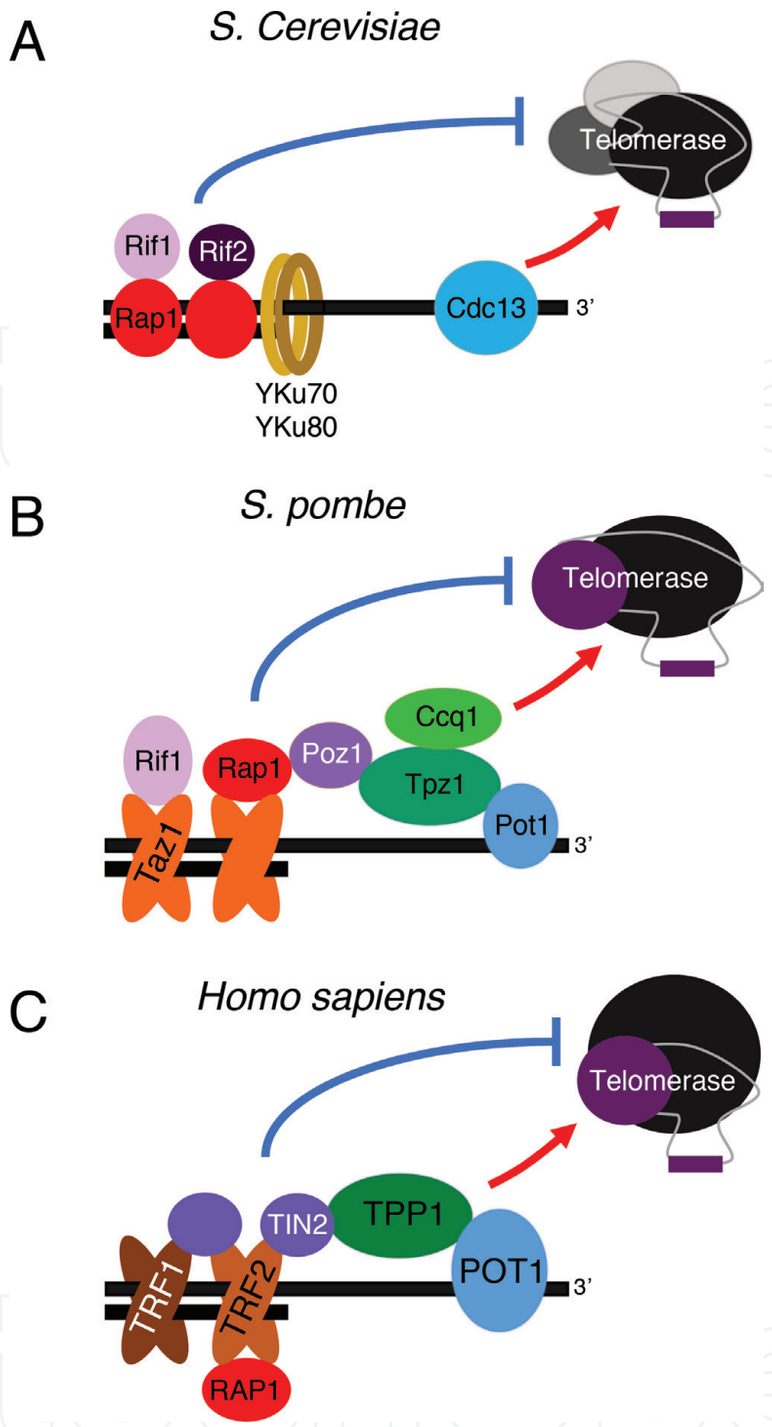

Figure 1.
Telomere complexes conservation from yeasts to humans (A) S. cerevisiae telomere complex. (B) S. pombe telomere complex. (C) Humans telomere complex (also named "shelterin").

Telomere length is defined as the number of short G-rich repeats at chromosome ends. Organisms have a required minimum number of repeats, or telomere repeat tracts, to recruit enough telomere binding proteins to accomplish telomere function [11–15]. Some proteins within the telomere protein complex recruit telomerase to allow elongation of the telomeres (positively regulating telomerase), while others prevent telomerase from continuously adding repeats (negatively regulating telomerase) (**Tables 1** and **2**).

Telomere length is linked to aging, as it was shown that late generation mice with limited telomerase (heterozygotes for telomerase RNA mTR+/−) have short telomeres and revealed a decrease of tissue renewal capacity [16]. In contrast, mice overexpressing telomerase have an extended lifespan [17]. In humans, a correlation

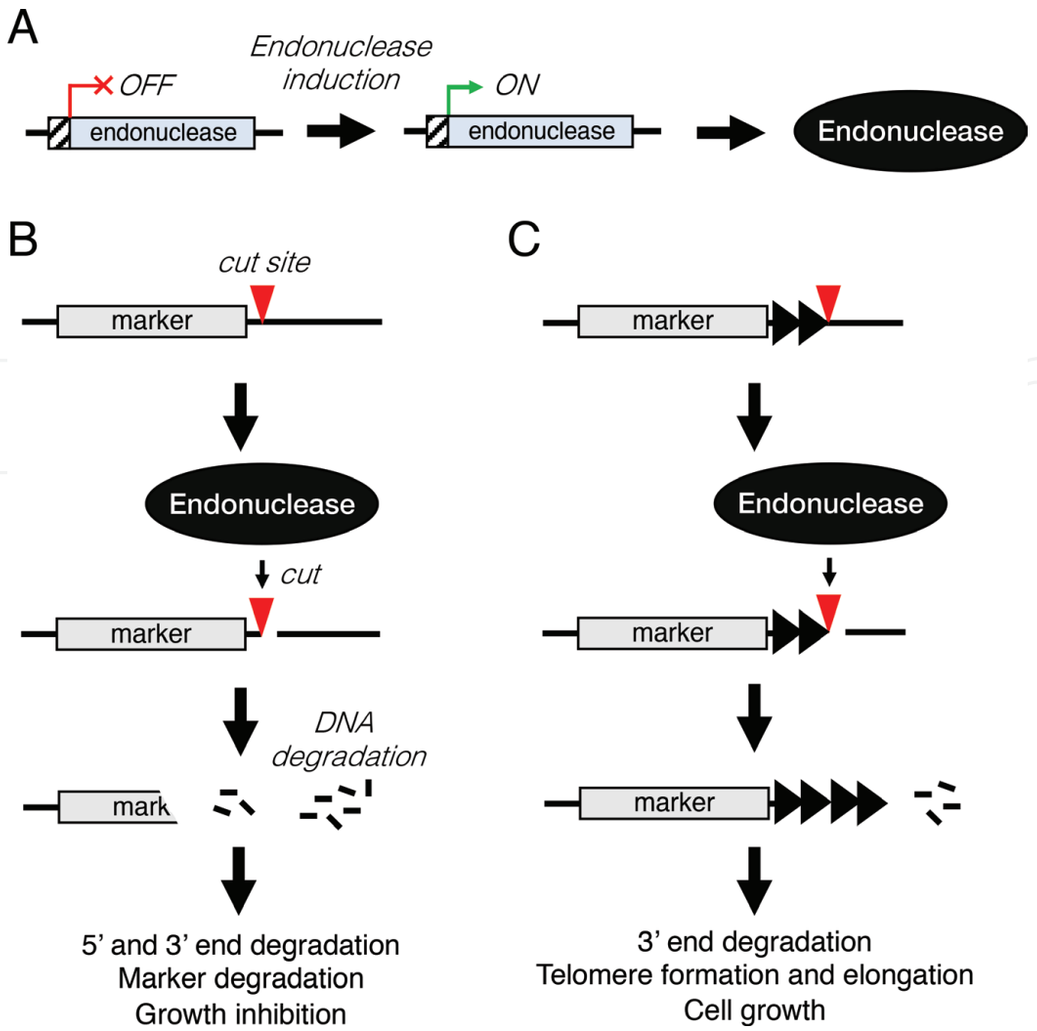

Figure 2.
Double-strand break (DSB) and telomere formation systems (A) restriction enzyme or endonuclease with no natural sites in the genome is produced in cells from a rapidly inducible promoter. (B) Inducible DSB system: a unique cut site (red triangle) is engineered next to a selectable marker gene. After induction, the endonuclease produces a DSB which leads to the degradation of both 5′ and 3′ strands on each side of the cut site (indicated by short black lines) and to the marker degradation, conducting to cell growth inhibition. (C) Inducible telomere formation system: the cut here exposes telomere repeats (black triangles) to form a new functional telomere that is stable and elongated. If the distal chromosomal DNA (3′ to the cut site) is dispensable, the new functional telomere allows normal cell growth. Modified and adapted from Wang et al. [107].

between telomere length, aging, and age-related diseases has also been shown (reviewed in [18, 19]). For instance, in dyskeratosis congenita or its most severe form: Hoyeraal-Hreidarsson syndrome, patients have abnormally short telomeres and shorter lifespan [20]. Most of the known mutations associated are found in telomere proteins (TIN2), telomerase (hTR and hTERT) or the telomerase-associated factor as dyskerin (DKC1), which stabilizes the telomerase RNA (hTR) (**Table 2**) [18]. Moreover, shortened telomere length was observed in other premature aging disorders such as Down's syndrome leading to accelerated aging, or Cockayne syndrome in which neurological degeneration is observed (aging-disorders linked to a telomere phenotype reviewed in [21]). In Down's syndrome, the genetic defect observed is mostly a trisomy of chromosome 21, which increased the biological age of tissues, whereas in Cockayne syndrome, mutations are described in the majority in Cockayne syndrome group B protein (CSB) involved in telomere length maintenance with TRF1 and TRF2 [21]. Prematurely shortened telomeres can also lead to loss of telomere capping function, which in turn can cause telomeres to behave as DNA breaks and undergo recombination to promote genomic instability leading to cell death or tumorigenesis [22]. Telomerase defects have also been associated with

S. cerevisiae	*S. pombe*	*H. sapiens*	Regulates telomerase	Major functions at telomeres	Refs
Rap1	Rap1	RAP1	–	Inhibits NHEJ, plays a role in telomere heterochromatin. ***Only in Sc***: binds telomeric dsDNA	[60, 61, 74–76]
Rif1	Rif1	RIF1	***Sc*** & ***Sp***: –	Only in ***Sc***: checkpoint inhibition at telomeres (with ***Rif2***). ***Hs***: not associated with wild-type telomeres (only at dysfunctional ends to promote NHEJ)	[60, 61, 77, 78]
-np-	Taz1	TRF1 TRF2	–	***Sp*** & ***Hs***: binds telomeric dsDNA, inhibits NHEJ, role in telomere heterochromatin	[58, 61, 79, 80]
YKu70/Yku80	Pku70/Pku80	KU70/KU86	Only in ***Sc***: +	Protects ends from being degraded. Affects subtelomeric heterochromatin	[81–84]
-np-	Poz1	TIN2	–	***Sp*** & ***Hs***: required for the "bridged organization"	[70, 71]
-np-	Tpz1	TPP1	+	***Hs***: telomerase recruitment & processivity. ***Sp*** & ***Hs***: telomerase activation	[68, 69, 85]
-np-	Ccq1	*-np-*	+	***Sp***: telomerase recruitment	[64–67]
-np-	Pot1	POT1	No role	Binds telomeric ssDNA, end protection	[62, 63]
Cdc13 (Est4)	*-np-*	*-np-*	+	Binds telomeric ssDNA, end protection and telomerase recruitment	[38, 86–88]
Stn1/Ten1	Stn1/Ten1	STN1/TEN1	–	End protection. Terminator of telomerase activity/ lagging-strand replication of telomere DNA. Form CST complex with Cdc13 in ***Sc*** and ***CTC1*** in ***Hs***	[69, 89, 90]

dsDNA: double-stranded DNA. -np-: not present. ssDNA: single-stranded DNA.
"+" means positively regulates telomerase. "–" means negatively regulates telomerase.

Table 1.
Major telomere proteins and functions of ***S. cerevisiae, S. pombe****, and* ***H. sapiens—Sc: S. cerevisiae, Sp: S. pombe, Hs: H. sapiens,*** *NHEJ: non-homologous-end-joining.*

cancer, and telomerase promoter mutations are prevalent in multiple cancer types [23], where the presence of telomerase activity allows extended growth of cancer cells [24].

S. cerevisiae	*S. pombe*	*H. sapiens*	
Est1	Est1	EST1	Regulatory subunit of the telomerase [91–93]
Est2	Trt1	hTERT	Catalytic subunit of the telomerase [93]
Est3	*-np-*	*-np-*	Regulatory subunit **only in *Sc*** [94]
Tlc1	TER1	hTR (hTERC)	Telomerase RNA [7, 95–97]

Table 2.
Major telomerase components of S. cerevisiae, S. pombe, and H. sapiens.

Telomeres are also able to convert adjacent chromatin into heterochromatin, a compact and transcriptionally silent form of chromatin with important roles in chromosome biology, for example, in development and mammalian female X-chromosome inactivation and development of cancers [25–27]. This function, the inhibition of the DDR, and how the cells distinguish a DSB from a telomere or heterochromatin regulation at the chromosome ends are still not fully understood. Elucidation of these mechanisms will provide an understanding of some of the molecular mechanisms associated with aging and the initiation of tumorigenesis.

1.2 Telomere complexes from yeasts to humans

The yeast model systems have the advantage of a small eukaryotic genome that can be easily altered due to high levels of homologous recombination [28–31]. The terminal telomere repeat tracts of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* follow the scale of these smaller genomes with lengths of ~0.3 kb [12, 32]. In contrast, telomeres size in humans is between 10 and 30 kb, but with measurement limitations due to a highly variable length either at the time of birth [33, 34], in different tissues [35] or among others between women and men [36]. Therefore, yeasts are significant model organisms for dissecting the molecular genetics of basic telomere biology (reviewed in [37, 38]).

Telomeres have also been studied in several budding yeasts somewhat related to *S. cerevisiae* [39, 40]. A striking difference between *S. cerevisiae* and these organisms is that while *S. cerevisiae* has a short heterogeneous telomere repeat sequence (abbreviated as $TG_{1–3}$), several other species of *Saccharomyces*, *Candida*, and *Kluyveromyces lactis* have longer, homogeneous repeats [39, 40]. These longer repeats allowed the cloning of the gene for telomerase RNA in *K. lactis* and *Saccharomyces castellii* (now referred to as *Naumovozyma castellii*) and the study of highly related telomere binding proteins that co-evolved with the different telomere sequences [39, 41–45]. Work in *N. castellii* showed that the Rap1 protein of this yeast can bind to both the double-stranded telomere repeats and the junction between the double and single strands DNA, suggesting a level of coordination between Rap1 and the single-strand telomere repeat binding protein Cdc13 [46, 47]. *N. castellii* may also provide insight into the regulation of telomerase synthesis of the 3′ overhang as this yeast synthesizes a specific 70 nucleotides 3′ overhang in late S-phase [48].

Mutation of the telomerase RNA in *K. lactis* allowed the synthesis of mutant telomere repeats which revealed surprising aspects of telomerase-dependent and telomerase-independent telomere elongation. Some mutant telomere repeats gave rise to hyper-extended telomeres in a phenomenon called “runaway telomere elongation,” which was thought to be related to the loss of telomere binding proteins that negatively regulate telomerase [42, 49]. Similar hyper-elongation phenotypes are known in the more “popular” yeasts, *S. cerevisiae* and *S. pombe*, caused by loss of

Rif1 and Rif2 from budding yeast or Taz1 or Rap1 from *S. pombe* (**Table 1**). *K. lactis* mutant telomere repeats have also been used to follow a telomerase-independent mode of recombinational telomere elongation (RTE). In this model, shortened telomeres become highly recombinogenic, and the formation of double-stranded circular DNAs made of telomere repeats (t-circles) allows a "rolling circle" replication mechanism to produce telomere repeats that can be incorporated into short chromosomal telomeres [45, 50] This model is supported by further work indicating that a single t-circle may be a sufficient source for the elongation of all chromosomal telomeres [51, 52]. Results from these studies indicate that *K. lactis* telomeres may become very short prior to RTE and provide a model for human cancers that replicate telomeres by a recombinational mechanism termed ALT for alternative lengthening of telomeres [53]. Therefore, these budding yeasts have provided insights into the mechanisms of telomerase-dependent and -independent telomere elongation. However, both of them lack a rapidly inducible telomere formation system, which has the potential to follow some of the unique processes observed in these yeasts in real time.

The fission yeast *S. pombe* is significantly different from *S. cerevisiae*, *K. lactis*, and *N. castellii*, having diverged from the budding yeasts early in fungal evolution [54]. A consequence of this divergence is that *S. pombe* has conserved many features of telomere protein and chromatin structure that were lost from the budding yeasts. *S. pombe* has the same genome size as *S. cerevisiae* but only three chromosomes compared to 16 for the same size genome, and early studies confirmed that *S. pombe* was amenable to the same molecular genetic manipulations as *S. cerevisiae* [28] including telomere formation [32, 55], but had differences in telomere repeat sequence and the structure of the repeated subtelomeric elements adjacent to chromosome ends [32]. The human telomere complex, also called "shelterin," is more similar to the telomere complex of *S. pombe* than to *S. cerevisiae* (**Tables 1** and **2**; **Figure 1**) (reviewed in [56, 57]). Rap1 does not bind directly to the double-strand telomeric DNA in humans or *S. pombe,* but is recruited to the telomeres by its interaction with double-stranded DNA binding proteins, that is, Taz1 in *S. pombe* and TRF1 and TRF2 in humans. In *S. pombe*, Taz1 and Rap1 inhibit NHEJ, playing a role in the silencing of subtelomere regions and negatively regulating telomerase [58–61]. At the single strand, Pot1 protects the 3′ overhang, as in humans, and is bound by Tpz1 [62, 63]. Tpz1, the ortholog of human TPP1, plays central roles in telomerase recruitment (via its interaction with Ccq1 [64–67]), telomerase activation (via its interaction with the catalytic subunit of the telomerase [68]) and the termination of telomere elongation (via its SUMOylation [69]). Poz1, the ortholog of human TIN2, links the double-strand DNA binding complex of Taz1-Rap1 and Rif1 to the single-strand DNA binding complex of Pot1-Tpz1-Ccq1 (**Figure 1**). The entire complex negatively regulates telomerase as loss of Poz1 or Rap1 results in elongation of the terminal telomere repeat sequence tracts [70, 71]. In addition to telomere proteins, *S. pombe* has also conserved heterochromatin-mediated transcriptional silencing via di- and tri-methylation of lysine 9 of histone H3 (H3K9me2 and H3K9me3), which is absent in *S. cerevisiae* [72, 73].

2. Telomere formation systems in yeasts

Inducible systems that form telomeres from short tracts of telomere repeats were first developed in the late 1990s in *S. cerevisiae* [98]. The system modified inducible DSB systems, which consists of the rapid expression of a restriction enzyme or endonuclease allowing a cut at a specific engineered locus (**Figure 2A** and **B**). These systems have variants in many organisms (reviewed in [99]) with relevance

for fundamental understanding of DSB repair mechanisms [100] and therapeutic response to DSBs, as in radiotherapies [101]. In yeast, two site-specific endonucleases, HO and I-SceI were used, with a similar kinetics of cleavage [102, 103]. However, I-SceI was preferred in early mammalian work [104].

The telomere formation system integrates telomeric DNA repeats next to an engineered cut site, and the newly exposed telomere tract is converted into a functional, stable telomere without causing growth inhibition (**Figure 2C**) [98, 105–107]. In contrast, an induced DSB in the middle of a chromosome leads to DNA degradation and growth inhibition [108]. A variant of this system involving an inducible recombinase and specific recognition sites was also made to target one specific telomere in budding yeast and artificially shorten it [89, 109–111].

The *S. cerevisiae* telomere formation system proved to be a highly useful tool, but was limited by the biology of this yeast species. By translating this system to *S. pombe,* some of these issues, such as the presence of H3K9me2 and 3 modifications and a high level of conservation of the telomeric complexes with humans were addressed (**Figure 1B** and **C**) [56, 70, 72, 73]. The recent development of the *S. pombe* telomere formation system thus opens new avenues to study telomeric chromatin regulation and telomere formation.

Telomere formation systems allow a real-time study of telomere formation, elongation or heterochromatin spreading from the newly induced end. It also gives us the opportunities to examine the effects of different mutations in telomere proteins or to test the protein requirements for how telomeres are distinguished from DSBs. This non-exhaustive list highlights the significant advantages of these systems compared to steady-state experiments, as studies introducing mutations into cells with existing telomeres can only monitor telomeres after they have reached their equilibrium state. Formation systems can also study the initiation of mechanisms associated with telomeres, such as heterochromatinization of the nearby sequences or DNA damage checkpoint inhibition. Technically, these systems allow multiple experiments such as ChIP for protein recruitment at the break, Southern blotting to follow the *de novo* telomere elongation or DNA degradation, cell morphology observation (as large budded cells in *S. cerevisiae* or elongated cells in *S. pombe*—characteristic of a G2/M arrest), or western blotting for Chk1 phosphorylation (checkpoint activation).

2.1 Inducible telomere systems in *S. cerevisiae*

The first inducible telomere formation system in *S. cerevisiae* used the HO endonuclease placed under the control of a galactose-inducible promoter (in a *MATa-inc* allele strain which contains a point mutation to avoid an unwanted HO cut at the *MAT* locus [112]) and to the HO recognition sequence placed next to an 80 bp tract of telomere repeats (**Figure 3A**) [98].

The telomere cassette containing the *ADE2* marker, the HO recognition site, and the *LYS2* marker, was inserted at the *ADH4* locus at the left arm of the chromosome VII (VII-L) at 20 kb from the telomere end (this 20 kb contains no essential genes) (**Figure 3A**). The *LYS2* gene here serves as a marker for loss of the non-essential distal DNA and indirectly measure the HO cut efficiency by comparing Lys+ and Lys− cells.

The Diede and Gottsheing studies [98, 113] highlighted the efficacy of the system: a rapid and efficient cutting exposed a new stable telomere end that was elongated by telomerase. They also described a cell cycle-regulated elongation and the requirement of the DNA primase and DNA polymerases α and δ for the *de novo* telomere elongation [98]. Additionally, they also revealed the involvement of the MRX (Mre11-Rad50-Xrs2) complex and its exonuclease activity at the new telomere end for its elongation and Cdc13 loading [113]. It is worth noting that short formed

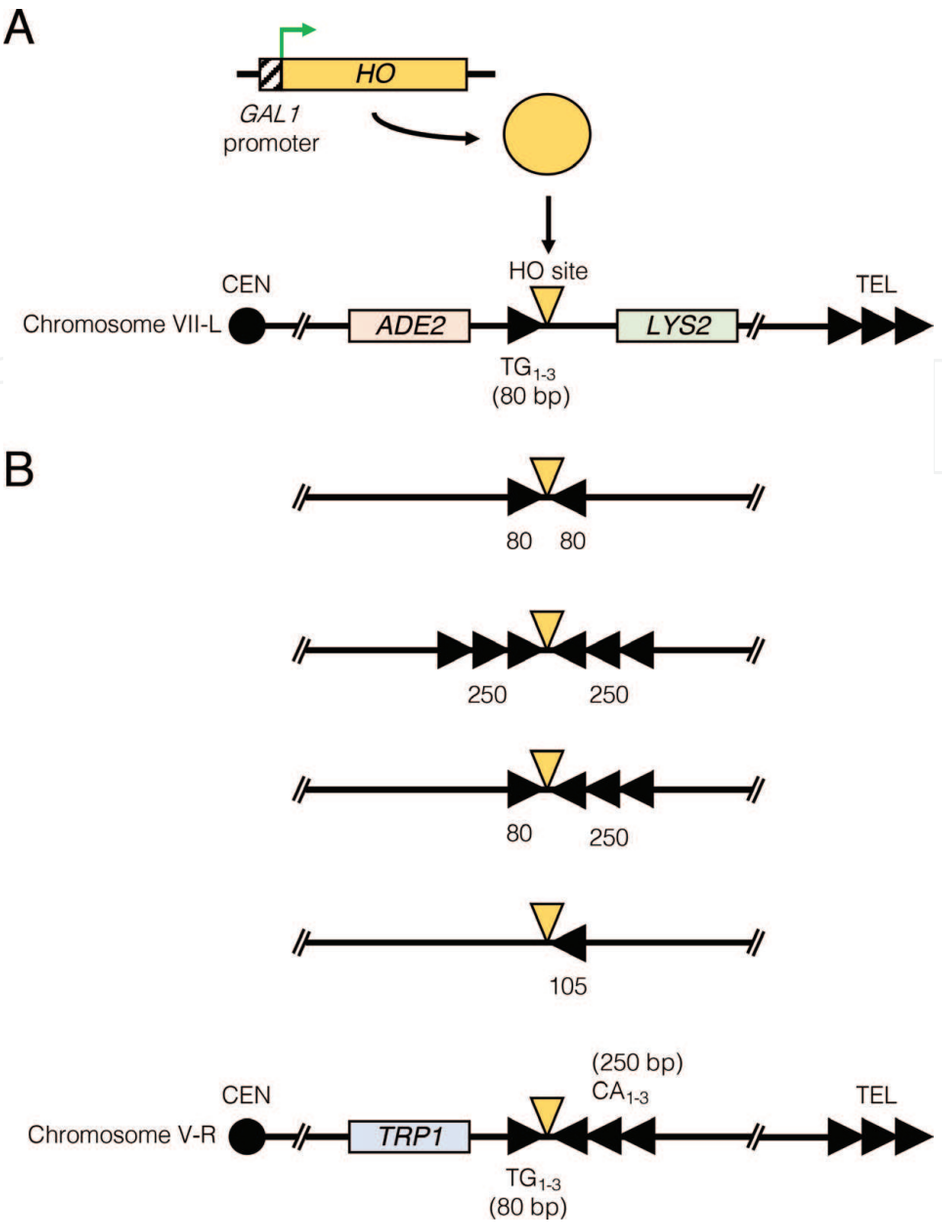

Figure 3.
Inducible telomere formation system in S. cerevisiae (A) schematic representation of the induction of the HO endonuclease (under the control of GAL1 promoter) in presence of galactose and modified chromosome VII-L containing the telomere cassette. The telomere cassette containing the ADE2 marker, the HO recognition site, and the LYS2 marker was inserted at the ADH4 locus at 20 kb from the telomere end (this 20 kb contains no essential genes) [98]. This insertion placed the HO recognition site at 13 kb from the distal telomere end. CEN: centromere. TEL: telomere (terminal black triangles). TG_{1-3}: telomere repeats sequence. (B) Variants of telomeres cassettes. The telomere cassette at the chromosome V-R containing the TRP1 marker was inserted at the YER188W locus (there are no essential genes from this locus to the chromosome end) [77]. Numbers represent the telomere tract length in base pairs (bp). CA_{1-3}: telomere repeats on the other side of the break in the opposite orientation represented in dark red. Modified and adapted from Ribeyre and Shore [77].

telomeres in different mutant strains that have a functional chromosome ends do not require MRX for elongation [114], highlighting how a broken end forming a telomere may differ from an existing end that requires elongation. Finally, they showed that the interaction of Ku (YKu70/YKu80) and telomerase RNA (*TLC1*) promotes telomere addition at the newly formed chromosome end [115].

Michelson et al. showed that even if the telomere formation system has the characteristics of a DSB, the cells respond differently when the DSB is next to a telomeric tract [116]. The *de novo* telomere end and degrading DNA fragment (**Figure 1C**) does not induce a normal checkpoint arrest, giving rise to a "telomere anti-checkpoint" activity.

The Shore lab followed up these results by creating new variants of the systems (**Figure 3B**) with some variations of the telomere tract length, or the addition of inverted telomere tract directly after the HO cut site (distal DNA fragment) [77]. Similarly, a telomere cassette was added at a different locus in the right arm of the chromosome V (V-R) with *TRP1* as the only proximal marker (**Figure 3B**) [77]. These variants were used to show that the telomere anti-checkpoint activity required the telomere proteins: Rif1 and Rif2. These results imply that a telomeric tract on one side of a break influences the recruitment of DNA damage checkpoint proteins on the other side [77].

An alternative inducible telomere elongation system has been constructed where an existing telomere with a functional chromosome end loses internal telomere repeats, so the resulting telomere tract is much shorter than normal [89, 109–111]. The first one used the site-specific recombinase Flp1 under a galactose-inducible promoter and two FRT (Flp1-Recognition Target) sites inserted at the *ADH4* locus on chromosome VII-L (**Figure 4A**) [109, 110, 117]. The system is based on the Flp1 recombinase excising a tract between two FRT sites and leaving only the distal telomere DNA sequence next to the terminal FRT site. Because the *FLP1* gene is encoded on the *S. cerevisiae* endogenous plasmid called the "2-micron circle," only cir° strains that lack this plasmid can be used with the FRT system. The system was later improved by using the loxP sites instead of FRT, and the Cre-recombinase, allowing its use in any strains, at the chromosome VII-L [111] or chromosome V-R, with an unchanged or short telomere induction (**Figure 4B** and **C**) [89, 118]. The constructs contain 16 inverted Rap1-binding sites, a loxP site, and a terminal telomere tract. Arrays of Rap1 binding sites at this position are considered by the cell to be a part of the terminal telomere tract, so the internal repeat tract distal to the loxP site is shorter than a normal telomere [119–121]. In these systems, after recombination, the remaining terminal tract is about 90 bp or 1/3 of the normal telomere length (**Figure 4B**) [89, 109–111, 117, 118]. These constructs were used to show a cell cycle restriction of telomere elongation in late S phase and a progressive telomerase inhibition upon telomere elongation [109, 110]. Additionally, an early replication of a short telomere was linked to an increase association of telomerase [111, 117] and highlighted two distinct roles of Stn1 in telomere capping and telomerase inhibition [89].

2.2 Inducible telomere systems in *S. pombe*

More recently, the telomere formation system was extended to *S. pombe*. The telomeric complex of the fission yeast has a high level of conservation with humans, both structurally with a "bridged organization" of telomere proteins [70] and in how telomerase is regulated (**Figure 1B** and **C**) (reviewed in [56]). This yeast also exhibits the H3K9me2 and -3 modifications present in multicellular eukaryotes that is absent in *S. cerevisiae* opening new perspectives of translational studies in telomeric chromatin regulations [72, 73].

The building of an efficient system took more time in *S. pombe*, as a rapidly inducible promoter was not available in this system. A system with the HO endonuclease using the *nmt1* promoter had been used but required 24 hours for full induction that would complicate kinetic analysis [122]. Two systems that could rapidly induce a site-specific nuclease that did not cut in the *S. pombe* genome were published in 2011 and 2012, but were inappropriate for a telomere formation system because they either prevented the use of the *ura4*$^{+}$ marker, which is important for monitoring telomere-associated heterochromatin [123–125], or special strains [126]. We, therefore, developed a telomere system that was using the anhydrotetracycline (ahTET)-inducible promoter and an I-SceI enzyme with preferred *S. pombe* codons [127] coupled to two NLS (Nuclear Localization Signals) to ensure a rapid

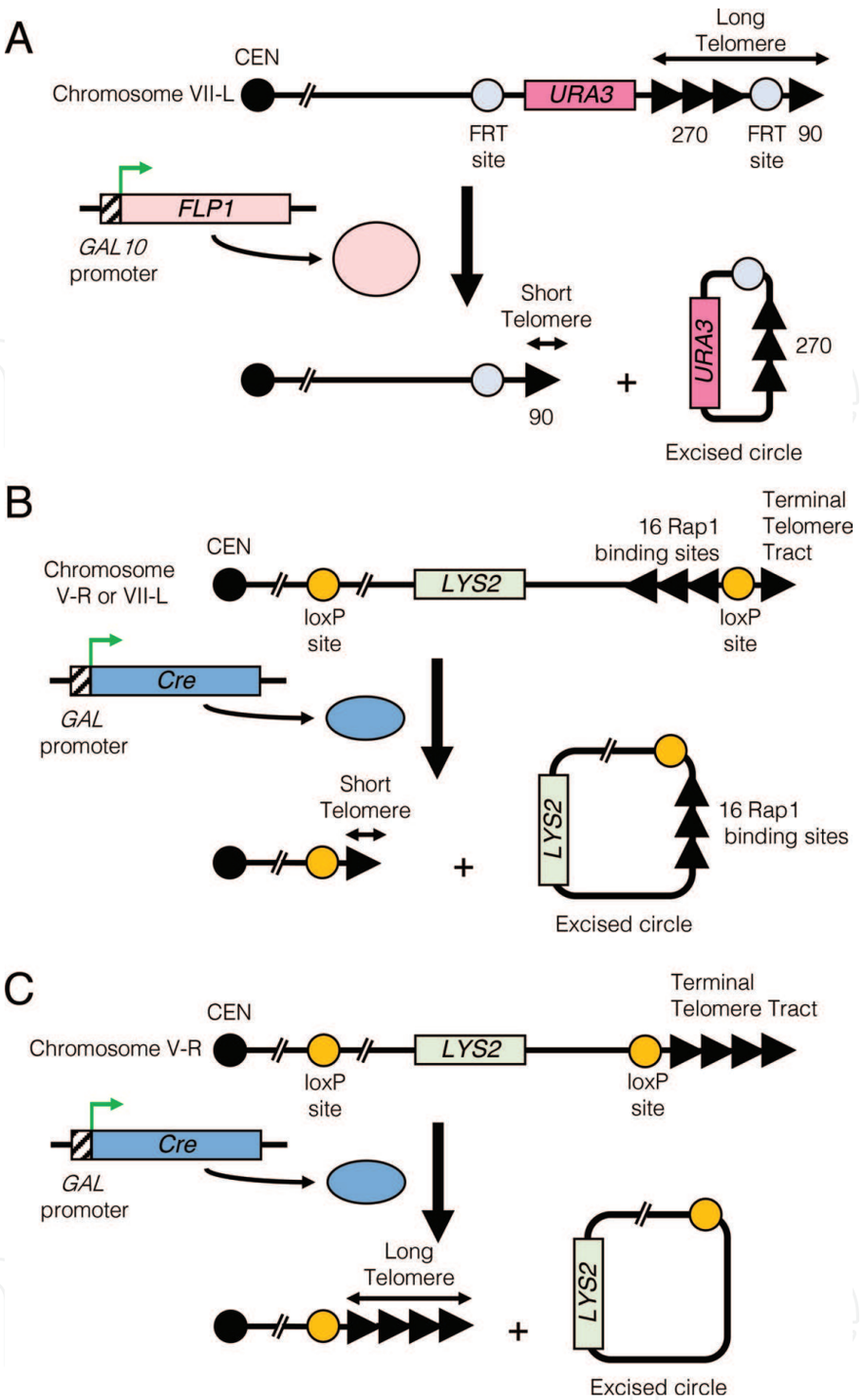

Figure 4.
S. cerevisiae systems to generate a single shortened telomere (A) schematic representation of the inducible recombinase-based system to generate a single shortened telomere at the chromosome VII-L (ADH4 locus). FRT: Flp1-recognition target site. The galactose-mediated induction allows the expression of Flp1 which induced homologous recombination through the FRT sites to generate an excised circle (containing the indicated elements) and a short telomere. Numbers represent the telomere tract length in base pairs (bp). Note that the terminal tract length is variable at individual telomeres due to lengthening and shortening at each cell division, and the numbers presented represent the average of the population [109, 110, 117]. (B) Variant system using loxP site and galactose inducible Cre-recombinase expression at the same locus (ADH4, chromosome VII-L) [111] or at the chromosome V-R end (YER188W locus) [89, 118]. (C) The control chromosome V-R construct that does not excise telomere repeats [89]. Modified and adapted from Marcand et al. and Puglisi et al. [89, 109].

and efficient cut [107] (**Figure 5A**). I-SceI has no endogenous site in the fission yeast genome [128], so the only DSB induced is at the engineered I-SceI site in the telomere formation system (called the proto-telomere cassette in *S. pombe*).

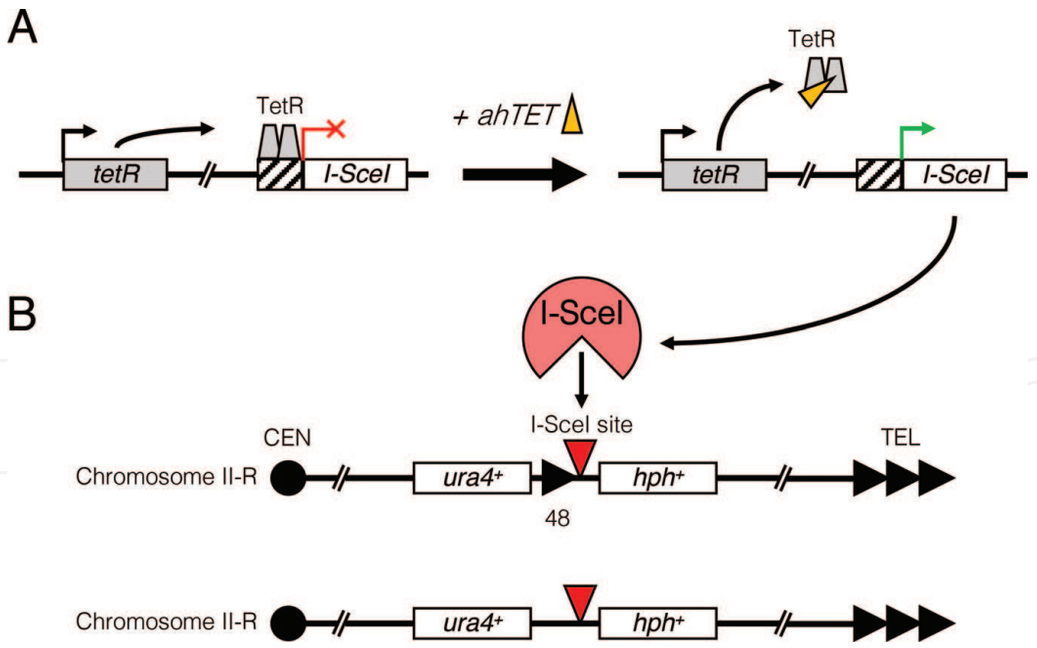

Figure 5.
The S. pombe inducible telomere formation system (A) the I-SceI endonuclease was expressed from a TetR controlled promoter (CaMV35S). In the absence of ahTET (left part), TetR represses the expression of I-SceI, and the addition of ahTET (9uM) into the medium induces expression (right part). (B) The 48 and 0 bp proto-telomeres cassettes are presented here and consist of a ura4$^+$ gene followed by either a 48 bp of telomere tract (black triangles) or no telomere sequences for the 0 bp version, the I-SceI cut site (red triangle) and the hygromycin resistance gene (hph$^+$) at the distal part of the construct. The cassettes were introduced at the chromosome II-R, 3′ to the gal1$^+$ locus. CEN: centromere. TEL: telomere (terminal black triangles). The number represents the telomere tract length in base pairs (bp). Modified and adapted from Wang et al. [107].

Two constructs were then created containing either 48 or 0 bp of telomere repeat sequence, an I-SceI cut site, and two flanking selectable markers: the *ura4*$^+$ gene and the hygromycin resistance gene (*hph*$^+$) (**Figure 5B**) [107]. The cassettes were placed at the right arm of the chromosome II (II-R), 3′ to the *gal1*$^+$ locus and, as in the *S. cerevisiae* systems, the region between this locus and the chromosome end was dispensable (without essential genes).

The *S. pombe* telomere formation system revealed several novel aspects of telomere function [107]. First, the DSB next to the telomere repeat tract immediately acquires telomere function: the end is immediately stable and not degraded (even in a strain lacking telomerase activity), does not undergo recombination, and the DNA damage response is somehow blocked. Second, the heterochromatin domain associated with telomeres formed in two distinct phases. The first consists of heterochromatin spreading from the telomere toward the interior of the chromosome over ~3 kb in the first cell division (about 5 hours). The newly formed telomere is elongated to wild type lengths during this time by telomerase. Heterochromatin spreading over ~10 kb continues for the next eight generations (population doublings) after the terminal telomere repeat tracts are at their equilibrium lengths. It is worth noting that the *clr4* deletion, which blocks heterochromatin formation, had no effect on telomere elongation [107], indicating that heterochromatin is independent of telomerase-mediated elongation as it is for chromosome end stability as observed in *S. cerevisiae* [129, 130]. In a second phase studied 34–87 population doublings after telomere formation, the established heterochromatin domain was surprisingly dynamic, with significant expansions and contraction of the heterochromatin mark H3K9me2 over the 35 kb domain that was monitored. Thus, different lineages from the same telomere formation event had different heterochromatin domains at different times after formation. These lineages were monitored at the single cell level by integrating the *ade6*$^+$ colony color marker at different distances from the

proto-telomere and plating single cells on inducing medium to follow the spreading of heterochromatin to silence the color marker at different stages of colony growth [107]. A marker close to the newly formed telomere initially showed expression before it was extinguished after several population doublings and remained extinguished as cells continued to grow. In contrast, markers placed further away from the telomere switched between the transcribed and repressed state in subsequent population doublings. These relative kinetics of telomere-associated functions would not have been observable using a telomere formed by cellular transformation, which requires ~30 generations of growth between telomere formation of a single cell to the generation of a sufficient number of cells to analyze the heterochromatin domain.

It is worth noting that this work and many other *S. pombe* heterochromatin studies follow the histone modification H3K9me2, but the H3K9me**3** mark is also associated with heterochromatin. Recent work by the Moazed lab showed that the H3K9me2 mark is associated with very low gene activity, and its conversion to H3K9me3 extinguished detectable transcription [131]. This model can explain the level of H3K9me2 near the newly formed telomere, which peaked about 3–13 kb from the new telomere in different experiments [107]. Presumably, H3K9me3 modification replaced H3K9me2 closer to the new telomere where expression of the *ura4*$^{+}$ and *ade6*$^{+}$ genes was undetectable [107]. Thus, the inducible telomere formation system may also prove useful in studying how the transition between these chromatin marks is regulated.

3. Conclusion

The inducible telomere formation system first developed in *S. cerevisiae* has provided several important results in the understanding of telomere elongation, in the cell cycle regulation of telomerase, in DNA checkpoint inhibition induced by a telomere end, and in the role of specific telomere proteins (**Table 3**) [77, 89, 98, 109, 110, 113, 115–118]. The new *S. pombe* system has allowed one to follow the establishment of telomere-end protection functions and the formation and dynamics of heterochromatin (**Table 3**) [107].

The ability to monitor the relative kinetics of different telomere-associated processes of replication, end protection, and chromatin domain formation has provided insights that could not be obtained using steady-state experiments with established telomeres. As telomere dysfunctions contribute to genomic instability or chromosome aberrations in human cancers, aging disorders, or specific pathologies such as dyskeratosis congenita [10, 19–21, 23, 24], telomere formation studies have the potential to identify defects related to these diseases. Heterochromatin also plays an important role in chromosome biology [25–27], and inducible telomeres and heterochromatin domains may shed light on these metazoan processes.

A key component of these telomere formation systems is the rapidly inducible DSB to expose the new physical chromosome end with adjacent telomere repeats. Inducible DSB systems using I-SceI or CRISPR/Cas9 have been used in mammalian cells to reveal differences in DNA repair at internal sites compared to subtelomeric ones [132–137]. An issue with the CRISPR/Cas9 approach is that it is a two-component system consisting of a guide RNA to target the endonuclease to a precise site, so how rapidly inducible this system is compared to the single component I-SceI nuclease needs to be carefully tested. Using I-SceI, an inducible telomere system in mouse cells to measure telomere repeat addition over 48 hours was developed [105]. While not used kinetically, the system allowed the assay of the effect of the DNA damage kinase ATM on the addition of new telomere repeats by telomerase.

Organism	System description	Used to show
S. cerevisiae	• HO endonuclease • Galactose inducible promoter • Specific cut site at chromosome VII-L or V-R to create a new telomere end	• New telomere stable and elongation by telomerase [98]. • Telomere elongation is cell cycle regulated [98]. • DNA primase and DNA polymerases α and δ are required of the *de novo* telomere elongation [98]. • Involvement of the MRX complex and its exonuclease activity at the new telomere end for its elongation and the Cdc13 loading [113]. • Ku complex interacts with *TLC1* (telomerase RNA) to promote telomere addition at the newly formed chromosome end [115]. • The *de novo* telomere end and degrading DNA fragment does not induce a checkpoint arrest [116].
S. cerevisiae	• FLP1 recombinase • Galactose inducible promoter • FRT sites inserted at chromosome VII-L to artificially shorten a telomere end	• Progressive telomerase inhibition upon telomere elongation [109]. • Cell cycle restriction of telomere elongation in late S phase [110]. • Telomerase is preferentially associated with short telomeres [117].
S. cerevisiae	• Cre recombinase • Galactose inducible promoter • LoxP sites inserted at chromosome VII-L or V-R to artificially shorten a telomere end	• Early replication of a short telomere linked to an increased association of telomerase [111]. • Two distinct roles of Stn1 in telomere capping and telomerase inhibition [89].
S. pombe	• I-SceI endonuclease • Tetracycline (TetR) controlled promoter (CaMV35S) • Specific cut site at chromosome II-R to create a new telomere end	• New telomere-end stable and elongated by telomerase [107]. • Gradual heterochromatin formation which remains dynamic after the new end reaches its equilibrium length [107].

Table 3.
Summary of telomere formation systems in different yeast species.

The events discovered in the *S. cerevisiae* and *S. pombe* telomere formation systems will provide important models for testing in this and other mammalian telomere formation systems.

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Conflict of interest

The authors declare no conflicts of interest.

Author details

Julien Audry[1*] and Kurt W. Runge[1,2]

1 Department of Inflammation and Immunity, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio, USA

2 Department of Genetics and Genome Sciences, Case Western Reserve University, Cleveland, Ohio, USA

*Address all correspondence to: audryj@ccf.org

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