MINIREVIEW / MINISYNTHÈSE

Protection and replication of telomeres in fission yeast[1]

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Abstract: Telomeres, the natural ends of linear chromosomes, must be protected and completely replicated to guarantee genomic stability in eukaryotic cells. However, the protected state of telomeres is not compatible with recruitment of telomerase, an enzyme responsible for extending telomeric G-rich repeats during S-phase; thus, telomeres must undergo switches from a protected state to an accessible state during the cell cycle. In this minireview, we will summarize recent advances in our understanding of proteins involved in the protection and replication of telomeres, and the way these factors are dynamically recruited to telomeres during the cell cycle. We will focus mainly on recent results from fission yeast *Schizosaccharomyces pombe*, and compare them with results from budding yeast *Saccharomyces cerevisiae* and mammalian cell studies. In addition, a model for the way in which fission yeast cells replicate telomeres will be presented.

Key words: telomere, telomerase, DNA replication, checkpoint, DNA repair.

Résumé : Les télomères, les extrémités naturelles des chromosomes linéaires, doivent être protégés et complètement répliqués pour garantir la stabilité génomique des cellules eucaryotes. Cependant, cette protection des télomères est incompatible avec le recrutement de la tolémérase, une enzyme responsable de l'extension des éléments télomériques répétés riches en G lors de la phase S, de telle sorte que les télomères doivent alterner d'un état protégé à un état accessible lors du cycle cellulaire. Dans cette mini-synthèse, nous résumerons les percées récentes permettant de mieux connaître les protéines impliquées dans la protection et la réplication des télomères, et comprendre comment ces facteurs sont recrutés de façon dynamique aux télomères lors du cycle cellulaire. Nous nous concentrerons principalement à discuter des résultats récents obtenus chez la levure à fission *Schizosaccharomyces pombe*, et à les comparer avec les résultats obtenus chez la levure à bourgeonnement *Saccharomyces cerevisiae* et chez les cellules mammifères. De plus, un modèle expliquant comment les levures à fission répliquent leurs télomères sera présenté.

Mots-clés : télomère, télomérase, réplication d'ADN, point de contrôle, réparation d'ADN.

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Introduction

To maintain a stable genome, telomeres must minimally fulfill two essential functions: the protection and complete replication of chromosome ends. Telomeres must be protected from degradation or fusion and should not induce permanent cell-cycle arrest if cells are to stay viable and to multiply. A simplistic view of telomere protection might predict that binding of telomere-specific proteins would completely exclude DNA repair and DNA damage checkpoint proteins from telomeres. However, studies have shown that DNA double-strand break repair proteins, including the Ku70-Ku80 and Mre11/Rad32-Rad50-Nbs1 (MRN) complexes, are normally bound to telomeres, and are necessary for normal telomere function in yeasts and human (d'Adda di Fagagna et al. 2001; Gravel et al. 1998; Nakamura et al. 2002; Viscardi et al. 2007; Zhu et al. 2000) (Table 1). Studies have also shown that the checkpoint sensor complexes Rad1-Rad9-Hus1, Rad17-Rfc2–5, Tel1 (ATM), and Rad3-Rad26 (ATR-ATRIP) associate with telomeres and contribute to the maintenance of normal telomere length (Ahmed and Hodgkin 2000; Bianchi and Shore 2007*b*; Longhese et al. 2000; Moser et al. 2009; Nakamura et al. 2002; Sabourin et al. 2007). However, these telomere-bound checkpoint sensor proteins do not arrest cell-cycle progression. Because deletion of other checkpoint proteins that work downstream of these sensor proteins in the checkpoint signaling cascade, such as fission yeast Crb2, Chk1, and Cds1 (mammalian homologs of 53BP1, CHK1, and CHK2, respectively), does not lead to any defect in telomere length maintenance (Dahlén et al. 1998; Matsuura et al. 1999; Nakamura et al. 2002), te-

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Table 1. Telomere-associated proteins.

Factors	Human	Fission yeast	Budding yeast	Function (Hs, human; Sp, fission yeast; Sc, budding yeast)
Telomerase catalytic core	TERT	Trt1	Est2	Reverse transcriptase subunit
	TR	TER1	TLC1	RNA template subunit
Telomerase accessory factors	EST1A/B	Est1	Est1	Associate with telomerase core enzyme; Hs Est1 proteins may be more involved in nonsense-mediated decay and processing of TERRA
			Est3	Found only in Sc; functionally related to HsTPP1?
	Dyskerin			Specific to higher eukaryotes (Hs)
G-tail binding proteins and their associated factors	POT1, TPP1, TIN2	Pot1, Tpz1, Poz1, Ccq1		POT1–TPP1–TIN2–TRF1–TRF2–RAP1 are part of shelterin complex (Hs); Pot1–Tpz1–Poz1–Ccq1 form a stable complex and Poz1 interacts with Rap1 (Sp)
			Cdc13	Cdc13–Stn1–Ten1 may function as telomere-specific RPA-like complex (Sc)
		Stn1, Ten1	Stn1, Ten1	Stn1–Ten1 form a telomere capping complex (Sp)
Telomere DB proteins	TRF1, TRF2	Taz1		Bind directly to GT-rich telomeric DNA by Myb-like domain; Sc appears to lack TRF1/TRF2 ortholog; SpTaz1 is related to both Hs TRF1 and TRF2
			Rap1	ScRap1 directly binds to telomeric DNA
Telomere DB protein associated factors	RAP1	Rap1		ScRap1 homolog, recruited to telomeres by TRF2 (Hs) or Taz1 (Sp)
	RIF1	Rif1	Rif1	Recruited by Rap1 (Sc) or Taz1 (Sp) to telomeres; HsRap1 may only be involved in recognizing dysfunctional telomeres
			Rif2	Rif2 has been found only in Sc
Checkpoint sensors	ATR	Rad3	Mec1	PIKK family kinases; recruited to RPA-coated single-stranded DNA
	ATRIP	Rad26	Ddc2	Binding partner of ATR/Rad3/Mec1
	ATM	Tel1	Tel1	PIKK family kinases; collaborate with MRN/MRX complex in telomere maintenance
	RAD1, RAD9, HUS1	Rad1, Rad9, Hus1	Rad17, Ddc1, Mec3	Rad1-Rad9-Hus1/Rad17-Ddc1-Mec3 forms PCNA-like clamp; loaded to telomeres and sites of DNA damages by the RFC-like clamp loader Rad17-Rfc$_{2-5}$ (Hs, Sp) or Rad24-Rfc$_{2-5}$ (Sc)
DNA repair proteins	MRE11, RAD50, NBS1	Rad32, Rad50, Nbs1	Mre11, Rad50, Xrs2	MRN/MRX complex; involved in HR and NHEJ; Nbs1/Xrs2 bind ATM/Tel1
	Ku86, Ku70	Ku80, Ku70	Ku80, Ku70	DSB binding; involved in NHEJ and telomere length regulation
	DNA-PK$_{cs}$			PIKK family kinase; involved in NHEJ; only found in higher eukaryotes

Note: DB, duplex-binding; DSB, double-stranded break; HR, homologous recombination; MRN, Mre11/Rad32-Rad50-Nbs1 complex; MRX, Mre11-Rad50-Xrs2 complex; NHEJ, nonhomologous end-joining; PIKK, phosphoinositide-3-kinase-related protein kinase; RFC, replication factor C; RPA, replication protein A; TERT, telomerase reverse transcriptase; TERRA, telomeric repeat-containing RNA.

lomeres must be able to prevent signaling between telomere-bound checkpoint sensors and their downstream regulators for cell-cycle arrest. Moreover, checkpoint sensor proteins must have telomere-specific targets required for telomere maintenance.

Indeed, telomere proteins, such as mammalian TRF1, TRF2, and POT1, are important for attenuating checkpoint signaling regulated by ATM and ATR kinases (Denchi and de Lange 2007; Guo et al. 2007; Karlseder et al. 2004). Recent studies have shown that budding yeast Stn1, a subunit of the Cdc13-Stn1-Ten1 complex, and fission yeast Ccq1, a subunit of the Pot1 complex (composed of Pot1-Tpz1-Poz1-Ccq1), are involved in attenuating checkpoint signaling at telomeres (Gasparyan et al. 2009; Tomita and Cooper 2008). Conversely, ATM and ATR checkpoint sensor kinases and their orthologs are involved in phosphorylating proteins localized at telomeres, such as the budding yeast telomere capping protein Cdc13 (Tseng et al. 2006) and the human telomere duplex-binding protein TRF1 (Wu et al. 2007), to regulate telomere accessibility.

The second important function telomeres must fulfill is to allow complete replication of chromosomal ends. Because of the semi-conservative synthesis of DNA, ends of DNA molecules cannot be completely replicated by conventional DNA polymerases (end-replication problem) (Fig. 1). To avoid continued DNA sequence loss at chromosome ends during successive cell divisions, most eukaryotic cells utilize a telomere-specific reverse transcriptase, known as telomerase, to synthesize repetitive GT-rich telomeric repeat DNA (Blackburn 2001). Telomerase can utilize its RNA subunit

as a template to synthesize a GT-rich strand (G-strand) beyond the end of the original genomic DNA.

Telomeric DNA consists of species-specific GT-rich repetitive telomere duplex DNA, as well as a 3′ single-stranded GT-rich strand overhang (G-tail). Both duplex and single-stranded portions of telomeres are important for telomere functions, as they recruit unique sets of telomere-specific proteins and telomerase. Components of known telomere-associated factors are summarized in Table 1 (see also Fig. 2). Since telomerase cannot extend blunt ends, the G-tail is essential for telomere length maintenance (Lingner and Cech 1996). The length of the G-tail increases during S-phase in both budding and fission yeast (Tomita et al. 2004; Wellinger et al. 1993b). In budding yeast, S-phase-specific long G-tails are generated independent of telomerase action, but depend partially on the MRX (Mre11-Rad50-Xrs2) complex (Larrivée et al. 2004; Wellinger et al. 1996). In fission yeast, the MRN complex and Dna2 helicase have been implicated in the generation of the G-tail (Tomita et al. 2003, 2004).

In several organisms, including humans, telomeric ends are folded into a "t-loop" structure in which the 3′ end of the G-tail invades the duplex tract and assumes a lariat-like structure (Griffith et al. 1999; Palm and de Lange 2008; Smogorzewska and de Lange 2004). Whether the t-loop exists in fission yeast telomeres remains to be established, but the telomere duplex-binding protein Taz1 has been found to promote t-loop formation of model fission yeast telomeres in vitro (Tomaska et al. 2004). Budding yeast telomeres appear to form an alternative higher order structure, unrelated to the t-loop (de Bruin et al. 2001). In humans, TRF2 promotes t-loop formation (Griffith et al. 1999). By sequestering telomeric DNA ends, the t-loop is thought to protect telomeres from DNA repair and checkpoint proteins. However, t-loop structures or telomeres bound by a capping protein complex, such as the budding yeast Cdc13-Stn1-Ten1 complex, are not accessible to telomerase and, thus, telomeres have to be partially unfolded or uncapped to allow access to telomerase to extend telomeric DNA (Blackburn 2001).

An important point to note from Table 1 and Fig. 2 is that budding yeast *Saccharomyces cerevisiae* has diverged significantly in telomere protein composition from humans, while telomere components in fission yeast *Schizosaccharomyces pombe* are closely related to human telomere proteins. While fission yeast Taz1 shows sequence and functional similarity to the human telomeric duplex DNA-binding proteins TRF1 and TRF2, the budding yeast genome does not encode orthologs of TRF1 and TRF2. Human and fission yeast Rap1 proteins are recruited to telomeres by protein–protein interaction with TRF2 and Taz1, respectively, while budding yeast Rap1 directly binds to telomeric duplex DNA (Kanoh and Ishikawa 2001; Li et al. 2000; Marcand et al. 1997) (Fig. 2). The fission yeast Pot1 complex (Pot1-Tpz1-Poz1-Ccq1) is important for telomere capping and the recruitment of telomerase to telomeres (Miyoshi et al. 2008; Tomita and Cooper 2008). This complex interacts with the telomere duplex-binding protein complex Taz1-Rap1 through Rap1 to form a higher order complex that closely resembles the mammalian "shelterin" complex (TRF1-TRF2-RAP1-TIN2-TPP1-POT1) (de Lange 2005; Miyoshi et al. 2008) (Fig. 2). However, budding yeast appears to en-

tirely lack the telomere capping Pot1 complex. Instead, budding yeast utilizes the G-tail-binding Cdc13-Stn1-Ten1 complex to protect telomeres (Gao et al. 2007). Interestingly, Stn1 and Ten1 subunits were recently identified and shown to be essential for telomere capping in fission yeast (Gao et al. 2007; Martín et al. 2007), and a plant Stn1 ortholog was found to be important for telomere protection (Song et al. 2008). Furthermore, plant and mammalian genomes appear to encode Cdc13-like proteins (F. Ishikawa, personal communication, 2009; C. Price and D. Shippen, personal communication, 2009). Thus, unlike budding yeast, most eukaryotic cells appear to utilize two independent G-tail-binding complexes to protect their telomeres. Therefore, while studies in budding yeast have provided the most detailed molecular description of telomere components to date, fission yeast should serve as an excellent model system that more closely resembles telomere maintenance mechanisms in higher eukaryotes.

Telomere length regulation and protection by fission yeast shelterin

A recent discovery of a fission yeast shelterin-like complex highlighted evolutionarily conserved elements of telomere length regulation between fission yeast and mammalian cells (Miyoshi et al. 2008) (Fig. 2). This shelterin-like complex consists of the Pot1 complex (Pot1-Tpz1-Poz1-Ccq1) and Taz1-Rap1 (Table 1). Tpz1 is an ortholog of mammalian TPP1, a known interaction partner of the telomere capping protein Pot1 (Palm and de Lange 2008). Much like *pot1Δ* cells, *tpz1Δ* cells experience severe immediate telomere dysfunction, and they can only survive as cells carrying circular chromosomes, suggesting that the evolutionarily conserved Pot1-Tpz1 complex is crucial for telomere protection (Baumann and Cech 2001; Miyoshi et al. 2008). The Ccq1 and Poz1 subunits are also redundantly required for telomere protection, since *ccq1Δ poz1Δ* cells experience severe immediate telomere dysfunction and can survive only after circularizing their chromosomes (Miyoshi et al. 2008).

Ccq1, a protein with homology to a structural maintenance of chromosomes (SMC) coiled-coil domain at its C-terminus, has previously been identified as a telomere protein involved in telomere length maintenance (Flory et al. 2004; Sugiyama et al. 2007). Ccq1 also promotes formation of telomeric heterochromatin by recruiting the Snf2/histone-deacetylase-containing repressor complex (SHREC) to telomeres (Sugiyama et al. 2007). However, the role of Ccq1 as a SHREC-associated factor seems to be distinct from its role as a Pot1 complex subunit, since stable interactions among other Pot1 complex subunits and SHREC subunits were not detected (Miyoshi et al. 2008). In fact, Ccq1 is essential for the association between telomerase and Tpz1 and the recruitment of telomerase to telomeres (Miyoshi et al. 2008; Tomita and Cooper 2008). In mammalian cells, POT1-TPP1 proteins have been shown to interact with telomerase and to increase its processivity (Wang et al. 2007; Xin et al. 2007). Given the similarities between the Pot1-containing complexes in recruitment and (or) activation of telomerase, mammalian cells might utilize an unidentified SMC-domain protein that plays a role analogous to fission yeast Ccq1 in

Fig. 1. End-replication problem and possible steps of action for telomerase. Because of the G-tail at telomeres and the limitation of semi-conservative replication by DNA polymerases, telomeres synthesized by leading-strand polymerases will be blunt-ended and shorter. After 5′-end resection to regenerate the G-tail, telomeres replicated by leading-strand synthesis may be further shortened. Telomeres replicated by lagging-strand polymerases can potentially retain their original length and end structure. #1, #2, and #3 indicate G-tail structures that can potentially be extended by telomerase. Heavy lines indicate newly synthesized strands. The strand synthesized by lagging-strand polymerases is CA-rich, whereas the strand synthesized by leading-strand polymerases is GT-rich.
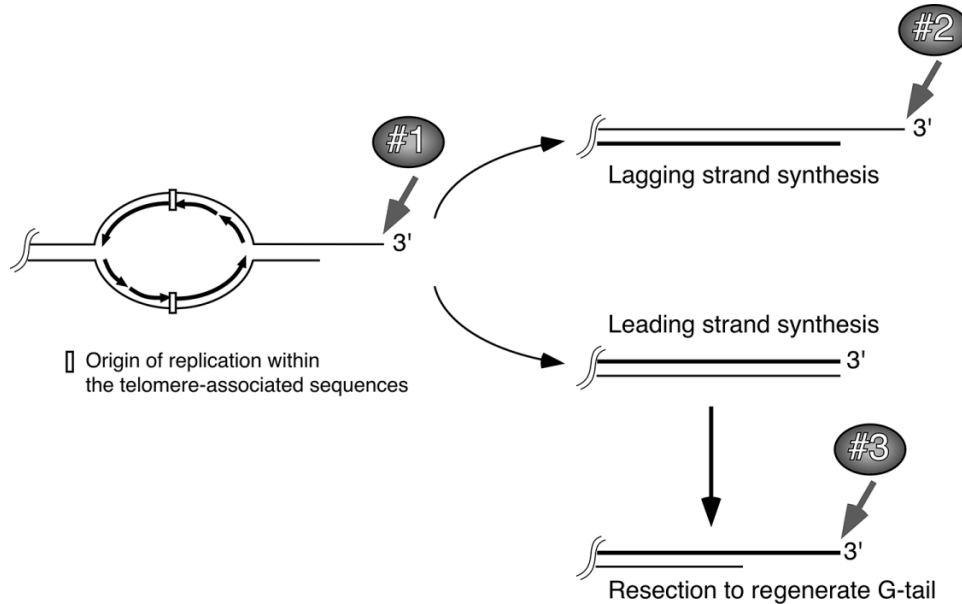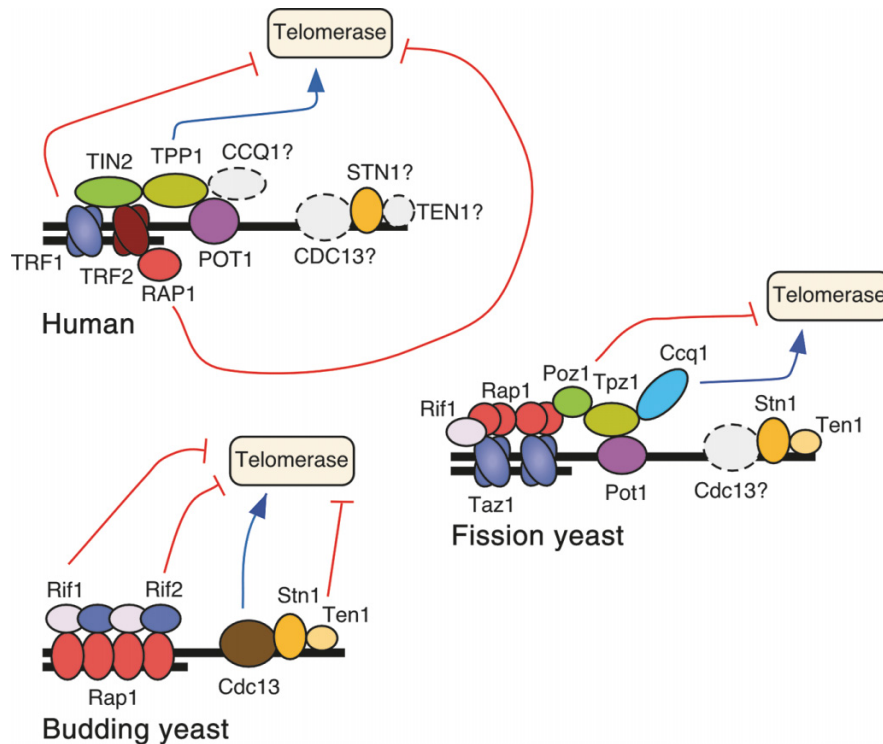

Fig. 2. Models of telomere proteins in humans, fission yeast, and budding yeast. Evolutionarily-related proteins are the same color. Blue arrows indicate proteins that promote telomere addition by telomerase, and red inhibitory signs indicate proteins that negatively regulate telomere addition. See Table 1 and main text for details on the proteins shown.

telomere maintenance (Fig. 2). On the other hand, in budding yeast, the G-tail-binding protein Cdc13 plays an important role in the recruitment of telomerase to telomeres by interacting with the Est1 subunit of the telomerase complex (Chan et al. 2008; Pennock et al. 2001). Interestingly, recent studies have shown that the budding yeast specific telomerase subunit Est3 may be structurally and functionally related to mammalian TPP1 (Lee et al. 2008; Yu et al. 2008). Thus,

the use of a Tpz1/TPP1-like protein appears to be universally required for the recruitment of telomerase to telomeres.

Fission yeast Poz1 was shown to connect the G-tail-binding Pot1 complex to the duplex telomere-binding protein complex Taz1-Rap1 via interaction with Rap1 (Miyoshi et al. 2008). Since *poz1Δ*, *rap1Δ*, and *taz1Δ* cells all show telomerase-dependent hyperelongation of telomeric GT-rich repeats, it was proposed that when Poz1 binds to Rap1, telomeres assume a highly protected state that inhibits telomerase recruitment. Since longer telomeres are bound by more Taz1-Rap1, Poz1 is more likely to interact with Rap1 and to inhibit further telomere elongation. However, when telomeres are short, Rap1-Poz1 interaction would not form efficiently; thus, the Pot1 complex can promote telomerase recruitment to elongate telomeres (Miyoshi et al. 2008; Tomita and Cooper 2008). This model for fission yeast telomere length regulation is similar to a model proposed for mammalian shelterin (Loayza and de Lange 2003; Palm and de Lange 2008), and given the similar role of Poz1 in connecting duplex-binding proteins to G-tail-binding protein, it has been speculated that fission yeast Poz1 might represent a functional homolog of mammalian TIN2 (Miyoshi et al. 2008). However, there is no obvious sequence homology between TIN2 and Poz1, and TIN2 connects POT1-TPP1 to duplex-binding proteins by directly interacting with TRF1 and TRF2, rather than through RAP1 (Chen et al. 2008; O'Connor et al. 2006; Ye et al. 2004). Thus, fission yeast Poz1 is likely to only represent a functional homolog of TIN2, not the true evolutionarily conserved ortholog of TIN2. It should be noted that although *poz1Δ*, *rap1Δ*, and *taz1Δ* cells all exhibit telomerase-dependent telomere elongation, only *taz1Δ trt1Δ* cells can efficiently maintain telomeres in the absence of telomerase by recombination (Subramanian et al. 2008; our unpublished results). Thus, it appears that Rap1-Poz1 interaction is not essential for the protection of telomeres against recombination.

Fission yeast Taz1 also interacts with Rif1, and loss of Rif1 causes telomere elongation, much like budding yeast *rif1* mutant cells (Kanoh and Ishikawa 2001; Marcand et al. 1997). However, it is currently unclear how fission yeast Rif1 inhibits telomere elongation. Unlike in budding yeast, fission yeast Rap1 and Rif1 do not interact directly, and they appear to regulate two distinct mechanisms to inhibit telomere elongation (Kanoh and Ishikawa 2001; Miller et al. 2005). A recent study in budding yeast has established that Rif1 and another Rap1-interacting protein, called Rif2, negatively regulate telomere elongation by inhibiting the recruitment of Tel1 kinase to long telomeres (Hirano et al. 2009). It will be interesting to see if fission yeast Rif1 regulates the localization of Tel1 and (or) Rad3 kinases at telomeres. Interestingly, mammalian Rif1 does not appear to interact with normal telomeres; it is only recruited to dysfunctional telomeres in an ATM-dependent manner (Silverman et al. 2004; Xu and Blackburn 2004). Since Rif1 recruitment appears to increase in *rap1Δ* cells (Kanoh and Ishikawa 2001), it is possible that Rif1 also preferentially binds to abnormal or elongated telomeres in fission yeast.

Fission yeast Stn1-Ten1 complex is essential for telomere capping

A demonstration that orthologs of budding yeast Stn1 and Ten1 are involved in telomere capping in fission yeast was surprising, since it was originally thought that the fission yeast Pot1 complex might represent the functional homolog of the budding yeast Cdc13-Stn1-Ten1 telomere capping complex (Baumann and Cech 2001; Martín et al. 2007). Because studies have failed to detect a stable interaction between Pot1 and Stn1-Ten1 (Martín et al. 2007; Moser et al. 2009) and because Stn1 was efficiently recruited to telomeres even when Pot1 recruitment to telomeres was inhibited (Moser et al. 2009), fission yeast cells likely contain two distinct telomere capping complexes.

Open-reading frames showing homology to fission yeast and budding yeast Stn1 have been found in mammalian cells and other higher eukaryotic species by database searches (Gao et al. 2007; Martín et al. 2007), and a recent study has demonstrated that *Arabidopsis* Stn1 plays a crucial role in telomere capping (Song et al. 2008). It has been proposed that budding yeast Cdc13-Stn1-Ten1 functions as a telomere-specific replication protein A (RPA)-like trimeric complex (Gao et al. 2007). Recent studies have found that Cdc13-like proteins (F. Ishikawa, personal communication, 2009; C. Price and D. Shippen, personal communication, 2009), as well as Ten1-like proteins (F. Ishikawa, personal communication, 2009), exist in higher eukaryotes. Thus, a Cdc13-like partner for Stn1-Ten1 most likely exists in fission yeast (Fig. 2), although sequence analyses have thus far failed to identify a Cdc13 ortholog in fission yeast. On the other hand, since budding yeast Stn1-Ten1 can provide telomere protection function independent of Cdc13 (Petreaca et al. 2006, 2007), it is possible that the Cdc13 ortholog may not be essential for telomere protection, and may have been lost in fission yeast. In any case, further characterization of the Stn1 and Pot1 complexes in fission yeast should provide new insight into telomere protection mechanisms in the future.

Coordination of semi-conservative DNA replication and telomere addition by telomerase

The replication of linear chromosomes by the semi-conservative DNA replication machinery will generate two structurally distinct termini at telomeres (Ohki et al. 2001) (Fig. 1). The strand replicated by lagging-strand synthesis will end up with a single-stranded overhang after removal of the last Okazaki fragment, whereas the strand replicated by leading-strand synthesis will have a blunt terminus. Therefore, leading-strand telomeres must be postreplicatively processed to regenerate the G-tail. The presence of processing events is supported by studies showing that both ends of chromosomes terminate in a 3′ overhang in yeasts, ciliates, and humans (Jacob et al. 2001; Makarov et al. 1997; Muñoz-Jordán et al. 2001; Wellinger et al. 1993b). In budding yeast and human cells, this processing event does not require telomerase, as the 3′ overhang was found to be still present at both ends of chromosomes in the absence of telomerase (Makarov et al. 1997; Wellinger et al. 1996). The

existence of two distinct types of end processing mechanisms at telomeres was also demonstrated by a study in human cells that observed chromosomal fusions only among leading-strand telomeres in cells carrying mutant versions of TRF2 or DNA-PK$_{cs}$ (Bailey et al. 2001). In addition, loss of the RecQ-like helicase WRN causes preferential loss of lagging-strand telomeres in human cells (Crabbe et al. 2004). Analysis of G-tail length in human cells lacking active telomerase revealed that lagging-strand telomeres carry much longer G-tails than leading-strand telomeres (Chai et al. 2006a). Interestingly, careful cell-cycle studies in telomerase-minus human cells found that de novo nucleotide incorporation at telomeres occurs in two phases, one throughout S-phase and another during G$_2$-phase (Verdun and Karlseder 2006). Since the G$_2$-phase incorporation of nucleotides occurs shortly after recruitment of the MRN complex and coincides with the recruitment of ATM kinase, it might represent some type of delayed replication or post-replicative processing at the extreme ends of telomeres.
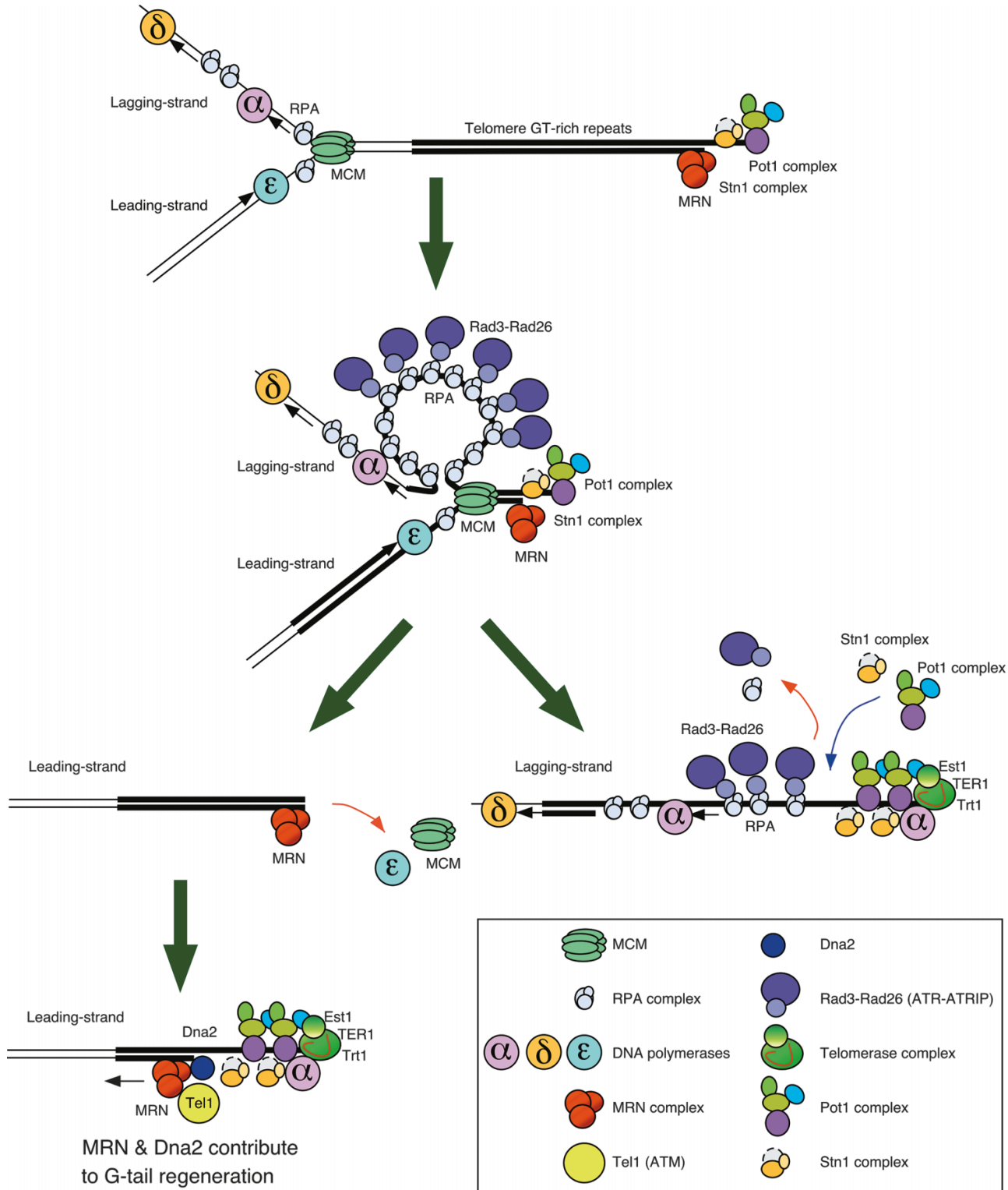
Telomerase may act on telomeres before conventional DNA replication machineries fully replicate telomeres (#1 in Fig. 1), on the lagging-strand replicated telomeres (#2 in Fig. 1) or on the leading-strand replicated telomeres, after nucleolytic processing occurrs to regenerate the G-tail (#3 in Fig. 1). In fission yeast, telomeres are replicated very late in S-phase (Kim and Huberman 2001), and quantitative chromatin immunoprecipitation (ChIP) analyses of synchronized fission yeast cell cultures have recently revealed that the arrival of the lagging-strand DNA polymerases (Polα and Polδ) at telomeres is significantly delayed, compared with the arrival of the leading-strand DNA polymerase (Polε) (Moser et al. 2009) (Fig. 3). Moreover, recruitment timing of the telomerase catalytic subunit Trt1 (TERT) matched very well with recruitment timing of DNA Polα (Moser et al. 2009). These data suggest that telomerase recruitment occurs after the arrival of the replication fork (at least after the arrival of the leading-strand DNA polymerase) at telomeres (i.e., #2 and (or) #3 in Fig. 1). In budding yeast, significant accumulation of the G-tail has been found to occur only after the replication fork arrives at telomeres (Wellinger et al. 1993a). However, when fission yeast cells were allowed to enter S-phase synchronously in the presence of the DNA replication inhibitor drug hydroxyurea (HU), which inhibits the replication of late replicating regions, including telomeres, a small but significant amount of telomerase was still transiently recruited to telomeres as cells entered S-phase (Moser et al. 2009). Thus, fission yeast appears to also posses an S-phase-specific, but replication-independent, telomerase recruitment mechanism prior to the actual arrival of the replication fork at telomeres (i.e., #1 in Fig. 1). Another interesting point to note for the recruitment pattern of Trt1 is that, unlike budding yeast Est2, Trt1 is recruited to telomeres only during late S-phase. In budding yeast, Est2 is loaded to telomeres in G$_1$ through specific interaction between the Ku70-Ku80 complex and telomerase RNA, but it also shows increased association with telomeres during late S-phase (Fisher et al. 2004). However, a recent study has shown that only the late S-phase association of Est2 to telomeres is essential for telomere maintenance in budding yeast (Chan et al. 2008).

The significant delay in the arrival of the lagging-strand DNA polymerases (Polα and Polδ), compared with the arrival of the leading-strand DNA polymerase (Polε) observed in fission yeast, is expected to cause a large accumulation of single-stranded (ss)DNA on the lagging-strand telomeres (Fig. 3). Consistently, large amounts of the ssDNA-binding protein complex RPA were recruited to telomeres just as Polε arrived at telomeres; this was followed by a decrease in RPA binding as Polα and Polδ arrived at telomeres (Moser et al. 2009). The checkpoint sensor protein Rad26 (ATRIP), which is recruited to RPA-coated ssDNA, was also recruited to telomeres, with very similar timing to Polε and RPA (Moser et al. 2009). Moreover, in the presence of HU (no telomere replication), S-phase-specific recruitment of DNA polymerases, RPA, and Rad26 to telomeres no longer occurred (Moser et al. 2009). Taken together, these data suggest that fission yeast cells accumulate ssDNA on lagging-strand telomeres as they replicate because of the differential arrival of leading- and lagging-strand DNA polymerases (Fig. 3). Recruitment of lagging-strand polymerases to the very ends of telomeres might be promoted by Trt1, Pot1, and (or) Stn1, since studies have shown that fission yeast Trt1 associates with the Polα complex in S-phase (Dahlén et al. 2003), and the budding yeast Cdc13-Stn1-Ten1 complex associates with the Polα complex (Grossi et al. 2004; Qi and Zakian 2000). By promoting the recruitment of the lagging-strand synthesis machineries to telomeric ends, Pot1 and Stn1 complexes may help to reduce ssDNA at telomeres and attenuate checkpoint responses. Alternatively, Pot1 and Stn1 may be able to displace RPA off G-tails and attenuate checkpoint responses, since these telomere-specific proteins might have higher affinity to the G-tail than RPA.

Does the accumulation of RPA and Rad3-Rad26 complexes have any functional significance for telomere maintenance in fission yeast? Since the elimination of the Rad3-Rad26 complex or the mutation of the largest RPA subunit (rad11-D223Y) in fission yeast leads to substantial telomere shortening (~100 bp in mutant cells, compared with ~300 bp in wild-type cells) (Nakamura et al. 2002; Ono et al. 2003), cell-cycle-regulated accumulation of Rad26 and RPA at telomeres is likely to be very important for telomere maintenance. But, their precise role is currently unclear. Premature collapse of a replication fork at telomeres would likely hinder recruitment of telomerase; thus, one possibility is that the Rad3-Rad26 (ATR-ATRIP) complex may contribute to telomere length maintenance by stabilizing a stalled replication fork at telomeres. In support of this possibility is the fact that elimination of the replication fork protection complex Swi1-Swi3 (Noguchi et al. 2004) also results in telomere shortening comparable to deletions of Rad3-Rad26 (Xhemalce et al. 2007; our unpublished results). On the other hand, Rad3-Rad26 might promote the recruitment of telomerase by phosphorylating components of telomerase, Stn1 complex, Pot1 complex, and (or) Taz1-Rap1. Indeed, there is experimental evidence in other systems that Rad3 (ATR) and the related kinase Tel1 (ATM) are involved in phosphorylating proteins bound to telomeres. For example, in budding yeast, Cdc13 is phosphorylated redundantly by Mec1 (ATR) and Tel1 (ATM) to facilitate the interaction between Cdc13 and the telomerase subunit Est1 (Tseng et al. 2006). In addition, phosphorylation of human TRF1 by

Fig. 3. A model of fission yeast telomere replication incorporating differential recruitment of leading- and lagging-strand DNA polymerases to telomeres. For simplicity, duplex telomere protein complexes Taz1-Rap1-Rif1 and Ku70-Ku80 are omitted from the figure. We propose that differential recruitment of polymerases would result in the accumulation of single-stranded DNA on lagging-strand telomeres and the recruitment of replication protein A (RPA) and Rad3-Rad26. These proteins might then play an important role in controlling the accessibility of lagging-strand telomeres. On the other hand, Tel1-Mre11/Rad32-Rad50-Nbs1 (MRN) and Dna2 may play a critical role in controlling the accessibility of leading-strand telomeres by promoting the generation of G-tails on leading-strand telomeres.

ATM weakens TRF1 binding to telomeres, thus alleviating the inhibitory effect of TRF1 on telomere addition (Wu et al. 2007).

Does Rad3-Rad26 play important roles in telomere maintenance on the lagging-strand only, the leading-strand only, or both the leading- and lagging-strand telomeres? If the differential arrival of leading-strand and lagging-strand DNA polymerases was the major cause for the accumulation of ssDNA at telomeres during replication, one would expect that Rad3-Rad26 would play a more important role in regulating lagging-strand telomere maintenance than leading-strand telomere maintenance. Since postreplicative resection can generate the G-tail on leading-strand telomeres (Figs. 1 and 3), one cannot completely rule out the possibility that Rad3-Rad26 plays an important role at leading-strand telomeres. However, mutations in the MRN complex and Dna2 nuclease, proteins likely to be involved in the regeneration of the G-tail at leading-strand telomeres, show only very small effects on the overall telomere length maintenance in fission yeast (Nakamura et al. 2002; Tomita et al. 2003, 2004). Thus, we favor the model in which Rad3-Rad26 exerts its positive role in telomere length regulation primarily at lagging-strand telomeres (Moser et al. 2009). (Fig. 3)

In budding yeast, ChIP analyses have shown increased association of Cdc13 and the telomerase subunit Est1 with telomeres in late S-phase, and recruitment of Cdc13 and Est1 coincided with recruitment of the leading-strand polymerase (Polε) (Bianchi and Shore, 2007a; Schramke et al. 2004; Taggart et al. 2002). Cdc13 and Est1 make direct protein–protein contact at telomeres (Pennock et al. 2001), Tel1, Mec1, and cyclin-dependent kinase Cdk1 promote Cdc13-Est1 interaction in late S/G_2 by phosphorylating Cdc13 (Li et al. 2009; Tseng et al. 2006). Currently, it is not known if the arrival of lagging-strand DNA polymerases (Polα and Polδ) at telomeres is delayed, compared with Polε, in budding yeast. Increased loading of RPA to telomeres also coincided with the peaks of Est1 and Cdc13 loading (Schramke et al. 2004). Thus, budding yeast might also accumulate ssDNA at lagging-strand telomeres because of the delayed synthesis of Okazaki fragments; however, it is also possible that MRX-dependent regeneration of the G-tail at leading-strand telomeres is primarily responsible for the observed RPA loading. In fact, increased binding of the G-tail-binding protein Cdc13 to telomeres in S-phase appears to require the generation of a long G-tail by the MRX complex (Larrivée et al. 2004; Takata et al. 2005).

Simultaneous loss of both ATR-ATRIP (Rad3-Rad26 in fission yeast and Mec1-Ddc2 in budding yeast) and ATM-MRN (Tel1-MRN in fission yeast and Tel1-MRX in budding yeast) pathways leads to the catastrophic loss of telomere stability in both fission and budding yeasts (Craven et al. 2002; Naito et al. 1998; Nakamura et al. 2002; Ritchie and Petes 2000). However, loss of only one of these two pathways shows contrasting telomere phenotypes between these two yeast species. Budding yeast cells mutated in the Tel1-MRX pathway show substantial telomere shortening, whereas mutations in the Mec1-Ddc2 pathway on its own have relatively minor defects in telomere maintenance (Craven et al. 2002). In contrast, fission yeast cells mutated in the Rad3-Rad26 pathway show substantial telomere shortening, whereas mutations in the Tel1-MRN pathway have

very little effect on telomere length (Nakamura et al. 2002). In addition, while the elimination of the S-phase checkpoint protein Mrc1 causes telomere shortening in budding yeast, it does not affect telomere length in fission yeast (Grandin et al. 2005; our unpublished results). In contrast, deletion of the replication fork protection complex (Swi1-Swi3 in fission yeast and Tof1-Csm3 in budding yeast) causes severe telomere shorting in fission yeast, but does not affect telomere length in budding yeast (Grandin et al. 2005; Xhemalce et al. 2007; our unpublished results). Therefore, it appears that there are some fundamental differences in the way these two yeast species achieve telomere length homeostasis with DNA damage response proteins.

It is possible that the ATM and ATR pathways are switched between budding yeast and fission yeast, because budding yeast might primarily regulate telomere length by controlling Tel1-MRX activity on the leading-strand, whereas fission yeast might primarily regulate telomere length by controlling Rad3-Rad26 activity on the lagging-strand (Fig. 3). Currently, there are no data available to support or refute this hypothesis in yeasts. However, studies in mammalian cells have suggested that telomerase and the MRN complex are involved in preferentially extending leading-strand telomeres (Chai et al. 2006a, 2006b). Moreover, it was recently shown in vitro that while ATM-MRN preferentially binds blunt DNA ends and cannot efficiently bind DNA ends with extended single-stranded regions, ATR-ATRIP preferentially binds DNA ends with single-stranded regions (Shiotani and Zou 2009). Therefore, long extended G-tails caused by the delayed arrival of lagging-strand polymerases might preclude the recruitment of Tel1/ATM and favor the recruitment of Rad3/ATR to lagging-strand telomeres. Conversely, initial blunt-ended leading-strand telomeres would be expected to be preferred substrates for Tel1/ATM until G-tails are regenerated by resection and become good substrates for Rad3/ATR.

DNA replication-independent changes in telomere protein association during cell cycle

While previous studies have suggested that changes in telomere accessibility are closely regulated by the replication of telomeric DNA (Chakhparonian and Wellinger 2003; Gilson and Geli 2007), some of the cell-cycle-regulated recruitment of proteins to telomeres in fission yeast were found to be independent of the replication of telomeric DNA (Moser et al. 2009). In particular, the addition of HU, which inhibits DNA replication at telomeres, did not inhibit the recruitment of MCM, Nbs1, or Stn1 to telomeres during S-phase (Moser et al. 2009). Thus, it appears that these factors undergo changes in telomere association during S-phase in a manner independent of the actual arrival of the DNA replication fork. The duplex telomere-binding protein Taz1 showed reduced binding prior to the replication of fission yeast telomeres, reminiscent of the reduced binding observed for mammalian TRF1 during S-phase (Moser et al. 2009; Verdun et al. 2005). Budding yeast Rap1 and Rif1, proteins important for the negative regulation of telomerase, also show reduced association during early S-phase, followed by a much more robust binding in late S/G_2-phase (Smith et al.

2003). Therefore, a universally conserved mechanism to remove inhibitory duplex telomere-binding proteins prior to the actual arrival of the replication fork at telomeres may exist. On the other hand, it is worth noting that Taz1 is also important in promoting replication fork progression through telomeric repeat sequences (Miller et al. 2006), and that the modest reduction in Taz1 binding detected by ChIP assays does not suggest the complete removal of Taz1 from telomeres during S-phase. In any case, increased reassociation of fission yeast Taz1 and budding yeast Rap1-Rif1 in late S-phase may play important roles in preventing the uncontrolled addition of telomeric DNA by telomerase (Smith et al. 2003).

The recruitment of fission yeast Stn1 to telomeres in the absence of DNA replication is quite intriguing. Since Stn1 recruitment to telomeres is increased in cells carrying longer G-tails (Martín et al. 2007) and since the MRN complex is involved in G-tail generation at telomeres (Tomita et al. 2003), it may be that a gradual increase in telomere association of MRN during S-phase in HU-treated cells could lead to increased G-tail formation without DNA replication, and could allow the recruitment of Stn1 to telomeres in late S-phase. If long G-tails are indeed generated on unreplicated telomeres, it might be that Stn1-Ten1 can bind much more strongly to the G-tail than RPA or Pot1 complexes would in the absence of an active DNA replication fork, since the binding of RPA and Pot1 to telomeres was greatly reduced in HU-treated cells (Moser et al. 2009). Alternatively, Stn1 may be recruited to telomeres by interacting with other factors (such as Nbs1 and MCM) that show increased and sustained recruitment to telomeres without an actual increase in G-tail length at telomeres.

Closing remarks

While studies in budding yeast have provided researchers with the most detailed mechanistic understanding of the telomere maintenance mechanism, limited conservation in telomere-associated proteins has made it difficult to apply the knowledge gained from budding yeast studies to studies in mammalian cells. With recent advances in the identification of a highly conserved shelterin-like complex and the Stn1-Ten1 complex, and with our understanding of the recruitment timing of various proteins involved in telomere metabolism during the cell cycle, fission yeast should serve as a very useful model system for mammalian telomere studies in coming years.

Acknowledgements

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