Curr Genet (2016) 62:725–730
DOI 10.1007/s00294-016-0599-x

REVIEW

Timeless protection of telomeres

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Received: 24 March 2016 / Revised: 28 March 2016 / Accepted: 30 March 2016 / Published online: 11 April 2016

Abstract The DNA replication machinery encounters problems at numerous genomic regions that are inherently difficult to replicate. These genomic regions include telomeres, which contain repetitive DNA and telomere-binding proteins. If not properly regulated, replication of such genomic regions can result in DNA damage, leading to genomic instability. Studies implicated a role of Timeless-related proteins at difficult-to-replicate genomic regions, including telomeres. However, how these proteins maintain telomeres was elusive. In a recent report, we described the role of Swi1, a Timeless-related protein, in telomere maintenance in fission yeast. We demonstrated that Swi1 is required for proper replication of repeat DNA sequences at telomeres. We also showed that Swi1-deficient cells utilize recombination-based ALT (alternative lengthening of telomeres)-like mechanisms to maintain telomeres in the absence of telomerase. Here, we highlight these findings and present additional data to discuss the role of Swi1^{Timeless} in telomere protection and ALT prevention.

Communicated by M. Kupiec.

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Keywords Swi1 · Timeless · FPC · Fork protection complex · Telomeres · Myb/SANT · Tbf1 · ALT · Alternative lengthening of telomeres · Replication fork · Genomic integrity · Repeat DNA · Cancer

Role of Swi1^{Timeless} in replication of repeat DNA regions

Numerous chromosomal regions present obstacles for DNA replication. These include fork-blocking sites, DNA secondary structures caused by repeat sequences, highly transcribed regions, and DNA-binding proteins bound to the template DNA. These sites are considered difficult to replicate, because they are susceptible to replication fork arrest or breakage, resulting in replication stress during the normal course of DNA replication. To prevent these occurrences, eukaryotic cells have a dedicated sensor response mechanism, termed the DNA replication checkpoint, responsible for the coordination of DNA repair and cell cycle progression (Leman and Noguchi 2013; Mirkin and Mirkin 2007). Cells also need to protect or stabilize the replication fork when the replisome encounters difficult-to-replication regions. Central to this protection is the replication fork protection complex (FPC) that is comprised of two major components: Swi1 and Swi3 in fission yeast; Tof1 and Csm3 in budding yeast; and Timeless and Tipin in metazoans. The functions of the FPC are conserved among eukaryotes (Leman and Noguchi 2012).

In our recent report, we investigated the role of Swi1, a Timeless-related protein, in telomere DNA replication in fission yeast (Gadaleta et al. 2016). Because telomeres have various features that can hamper replisome progression (Ivessa et al. 2002; Makovets et al. 2004; Millet and Makovets 2016; Verdun and Karlseder 2006), we first sought to narrow down the list of

possible telomeric obstacles. This list includes repeat DNA sequences, telomere-binding proteins, heterochromatin, and other secondary structures such as the t-loop. Our genetic studies suggest that telomere-binding proteins and heterochromatin do not present major replication obstacles in the absence of Swi1. Telomerase activity is also intact in *swi1*Δ cells, indicating that Swi1 does not regulate telomerase. Instead, the repetitive nature of the telomeric DNA sequences was found to be the primary impediment during telomere replication and the main cause of telomere shortening in the absence of Swi1. These observations were further supported by the following findings: (1) An episomal plasmid carrying a 300-bp telomeric repeat tract undergoes extensive recombination when introduced into *swi1*Δ cells, while no recombination is observed when the same plasmid is introduced into wild-type cells; (2) a single tract of *E. coli LacO* repeats inserted at chromosome arm regions experience repeat instability in the absence of Swi1; and (3) telomeres, *LacO* repeats, and rDNA loci, all of which have repeat DNA sequences are enriched with Rad52, a recombinase known to bind ssDNAs at DNA lesions (Gadaleta et al. 2016). Consistently, *swi1* deletion also causes contraction of rDNA repeats (Rapp et al. 2010; Sommariva et al. 2005) and fork breakage at these loci (Noguchi et al. 2003). Therefore, Swi1's role in repeat DNA maintenance is independent of DNA sequence, repeat track length, and genomic location. We therefore propose that Swi1^{Timeless} is a novel regulator of repetitive DNA replication across the genome.

Swi1^{Timeless} as an anti-recombinase at telomeres

Both Rad52 ChIP-seq analysis and telomere-dysfunction induced foci (TIFs) quantification revealed significant enrichment of Rad52 at subtelomeric regions in *swi1*Δ cells. In addition, *swi1*Δ cells were also shown to recruit increased levels of Rad52 at *LacO* and rDNA repeats (Gadaleta et al. 2016). Altogether, these results suggest that Swi1 prevents recombination at multiple loci containing repeat DNA sequences throughout the genome. This function of Swi1 is conserved between fission yeast and mammalian cells. In HeLa cells, telomeres undergo extensive DNA damage and recombination, leading to telomere shortening in Timeless-depleted cells (Leman et al. 2012). Rad51 and Rad52 foci accumulate in mouse NIH3T3 cells and colocalize with PCNA, a marker for the replication fork (Urtishak et al. 2009). Therefore, Swi1^{Timeless} may function as an anti-recombinase at telomeres during DNA replication.

Swi1^{Timeless} may coordinate DNA polymerases at telomeres

How Swi1 loss causes repeat instability remains to be determined. Previous studies showed that the lagging-strand DNA polymerase (pol δ) arrives at telomeres much later than the leading-strand DNA polymerase (pol ε) even in wild-type cells (Moser et al. 2009a). Considering that Swi1 is involved in the coordination of leading- and lagging-strand synthesis (Noguchi et al. 2004; Sommariva et al. 2005), it is reasonable to suggest that *swi1*Δ cells experience severe uncoupling of the two DNA polymerases. Such uncoupling may result in extensive accumulation of ssDNA and replication fork collapse at telomeres, resulting in hyper-recombination. It is also possible that DNA secondary structures such as hairpins and G quadruplexes may promote replication slippage in the absence of Swi1, resulting in loss of repeats at telomeres and other loci with repeat DNA sequences. Further investigations are warranted to test this interesting possibility and address the role of Swi1 in preventing polymerase slippage at repeat DNA regions including telomeres, rDNA, and *LacO* repeats.

Role of Swi1–Myb/SANT protein interaction in DNA replication

Swi1 and its orthologues are required for replisome stability at natural barriers, including rDNA pausing sites, the fission yeast mating-type locus, highly transcribed loci, and now at telomeres (Cherng et al. 2011; Gadaleta et al. 2016; Leman et al. 2012; Leman and Noguchi 2012, 2013; Liu et al. 2012; Pryce et al. 2009; Razidlo and Lahue 2008; Rozenzhak et al. 2010; Sabouri et al. 2012; Shishkin et al. 2009; Voineagu et al. 2008). Thus, it is straightforward to suggest that Swi1-related proteins are required for the regulation of most difficult-to-replicate regions. However, the underlying mechanism by which Swi1 modulates DNA replication at these genomic regions is not well understood. Key to this mechanism appears to be the Myb/SANT family of DNA-binding proteins. These proteins bind specific sites along the genome, and a subset of them is required for replication fork pausing at natural replication barriers. For instance, Rtf1, a Myb/SANT protein, binds to the *RTS1* site at the fission yeast mating-type locus, in order to facilitate fork termination in a Swi1-dependent manner (Eydmann et al. 2008). Reb1, another Myb/SANT protein, is found at *Ter1-3* sites in the rDNA repeats and promotes fork pausing, which is also dependent on Swi1 (Dalgaard and Klar 2000, 2001; Krings and Bastia 2004). In addition, fission yeast telomeres also recruit Myb/SANT proteins including

TRF1 homologs, Taz1, and Tbf1 (Cooper et al. 1997; Pitt et al. 2008). Therefore, we hypothesized that Swi1 interacts with theses Myb/SANT family proteins at telomeres in order to stabilize replication forks passing along the telomeres. In fission yeast, roles of Taz1 at telomeres are well characterized, whereas the function of Tbf1 is elusive (Cockell et al. 2009; Moser and Nakamura 2009). In budding yeast, Tbf1 binds to telomeric repeats localized at subtelomeres, and it has been shown to play a role in telomere homeostasis by suppressing checkpoint activation at short telomeres (Fukunaga et al. 2012). Tbf1 is an essential protein in *S. pombe* due to its function for transcription;

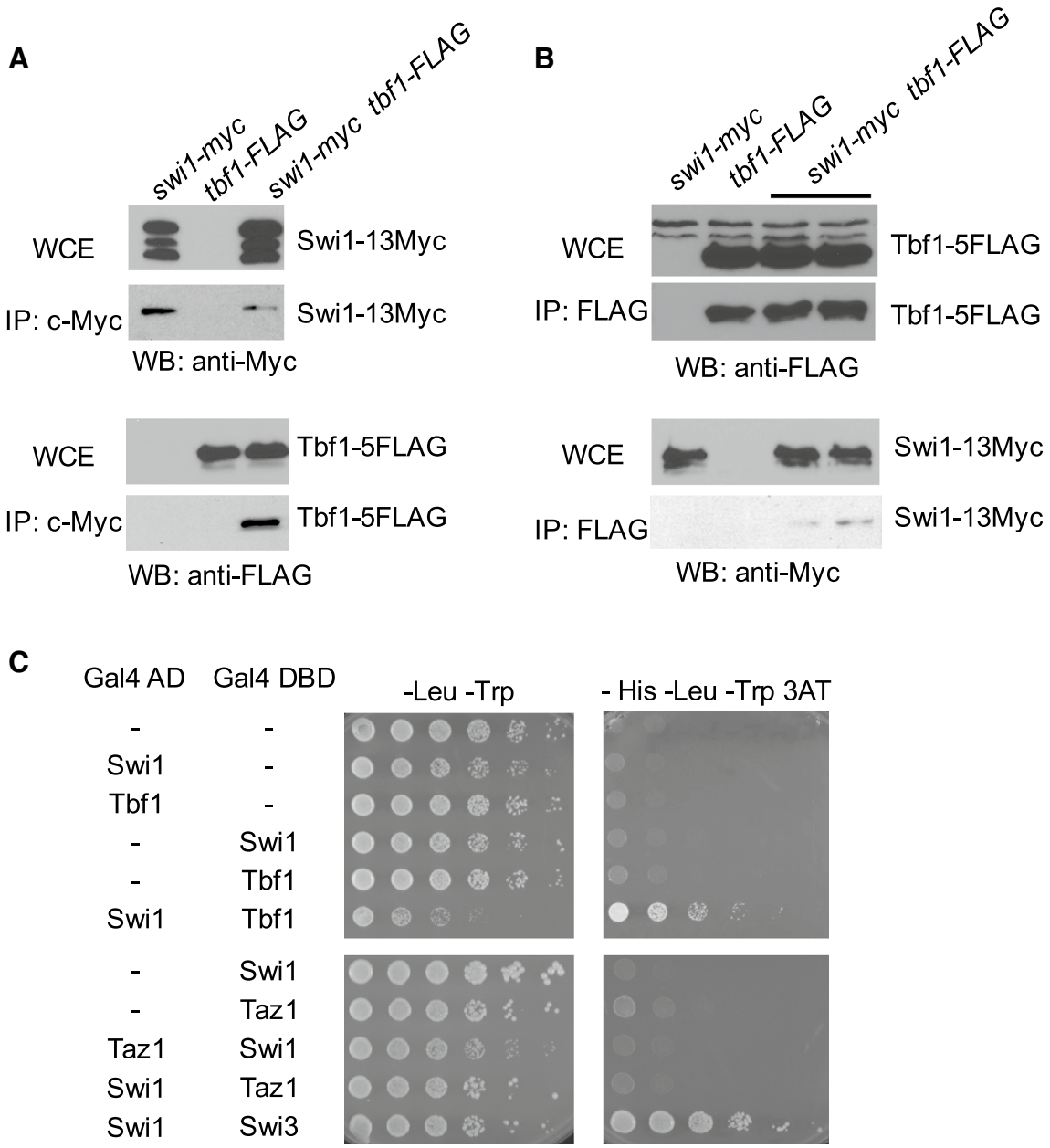

Fig. 1 Tbf1, a Myb-like protein, interacts with Swi1 in *S. pombe* cells. **a** *S. pombe* cells were engineered to express Swi1-13Myc and/or Tbf1-5FLAG. Cell extracts were prepared, and Swi1-13Myc was immunoprecipitated with anti-Myc polyclonal antibodies. Whole-cell extracts and precipitated fractions were probed with the anti-Myc (9E10) or anti-FLAG (M2) antibodies, in order to detect Swi1-13Myc and Tbf1-5FLAG by Western blotting (WB). **b** Tfb1-FLAG was immunoprecipitated from cell extracts expressing Swi1-13Myc and/or Tbf1-5FLAG. Swi1-13Myc and Tbf1-5FLAG in whole-cell extracts and precipitated fractions were detected by Western blotting (WB). **c** Two-hybrid interactions between Swi1 and Tbf1 or Taz1. Gal4-AD-Swi1 was tested in the Y190 strain for interaction with Gal4-DBD-Tbf1 or Gal4-DBD-Taz1. Growth on the -His-Leu-Trp 3-aminotriazole (3AT) plate indicates protein interaction. The interaction of Gal4-AD-Swi3 and Gal4-DBD-Swi1 was used as a positive control for protein interaction

however, its role in telomere maintenance in fission yeast is unknown (Cockell et al. 2009; Sarda and Hannenhalli 2015; Yan et al. 2015). We tested the idea that Swi1 modulates replication of the telomeric fork-block sites by interacting with the resident Myb/SANT proteins. Interestingly, we found that Swi1 physically interacts with Tbf1 (Fig. 1a–c), but not with Taz1 (Fig. 1c). Therefore, physical interaction between Swi1 and resident Myb/SANT proteins at telomeric repeats such as Tbf1 may ensure efficient replication of telomeric repeat DNA sequences. Such a mechanism also seems to be conserved between fission yeast and humans. We previously showed that Timeless physically interacts with TRF1 and TRF2 (Myb/SANT proteins) in human cells (Leman et al. 2012). Furthermore, TRF1 overexpression can induce replication fork stalling/pausing at telomeres (Ohki and Ishikawa 2004). Consistently, when TRF1 is overexpressed in HeLa cells, replication factors such as Cdc45 and RPA became enriched at telomeres. Strikingly, the enrichment of Cdc45 and RPA was abolished when Timeless was depleted via shRNA (Leman et al. 2012), suggesting that replication fork stalling at human telomeres is also dependent on Timeless-TRF1 interaction.

Role of Swi1 in ALT prevention

The study of the cellular mechanisms that control telomere length is central topic for the understanding of tumorigenesis and the development of age-related diseases. Approximately 10–15 % of cancer types, especially those of mesenchymal origin, survive without reactivation of telomerase. Instead, they maintain functional telomeres via the activation of ALT pathways; however, the mechanisms underlying ALT activation in these cancer cells are not clear (Dilley and Greenberg 2015). Recently, Zou and colleagues reported that cancer cells with ALT telomeres are hypersensitive to ATR inhibitors. Survival of ALT cells is highly dependent on ATR-ATRIP because ALT telomeres have increased levels of RPA-coated ssDNAs, which activate the ATR-ATRIP checkpoint kinase. Furthermore, ALT telomeres display high levels of telomeric repeat-containing RNA (TERRA) in S phase, which appear to correlate with RPA accumulation at telomeres and ATR-ATRIP activation (Flynn et al. 2015). Importantly, similar telomere defects were also seen in fission yeast *swi1*Δ cells. We observed that the simultaneous deletion of *swi1* and *rap1* disrupts the repressive telomeric chromatin state, potentially facilitating transcriptional activity at telomeric repeats (Fig. 2). As mentioned above, telomeres have increased levels of ssDNAs, represented by Rad52 recruitment in *swi1*Δ cells (Gadaleta et al. 2016). This is consistent with the decreased growth fitness of *swi1*Δ *rad26*Δ and *swi1*Δ *chk1*Δ cells (Noguchi et al. 2003). Rad26 is the fission yeast homolog of human ATRIP,

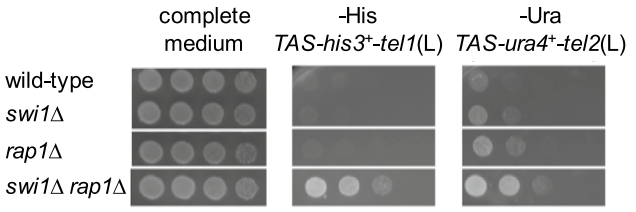

Fig. 2 Swi1 is involved in telomere silencing. *swi1*$^+$ and/or *rap1*$^+$ genes were deleted from an *S. pombe* strain bearing reporter genes. Rap1 has been shown to be involved in telomere silencing (Fujita et al. 2012; Moser et al. 2009b). The *his3*$^+$ and *ura4*$^+$ genes were inserted at silent subtelomeric regions of chromosome 1 [TAS-tel(L)] and 2 [TAS-tel2(L)], respectively. Fivefold-serial dilutions of the indicated strains were spotted onto YES agar medium or minimal medium lacking histidine or uracil. The plates were then incubated for 2–3 days at 32 °C. Representative images of repeat experiments are shown. Growth on medium lacking nutrients indicates defects in silencing at subtelomeres

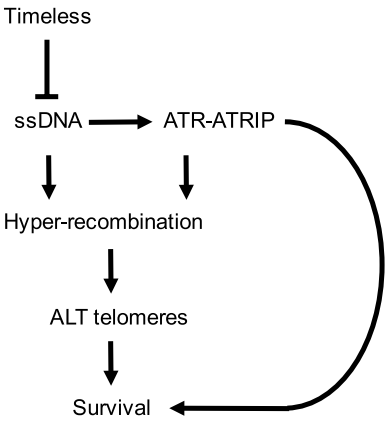

Fig. 3 Model of Timeless and ATR-ATRIP dependent ALT regulation. For details, see text

essential for ATR activation, and Chk1 is a downstream effector of ATR (Hustedt et al. 2013). *swi1*Δ *rad26*Δ and *swi1*Δ *chk1*Δ cells show increased levels of "cut" phenotype, indicative of mitotic catastrophe and death (Noguchi et al. 2003). These findings indicate that *swi1*Δ cells hyperactivate ATR^{Rad3}-ATRIP^{Rad26} due to increased levels of ssDNAs. Importantly, in telomerase-negative fission yeast cells, Swi1 loss leads to telomere hyper-recombination and an increase in the occurrence of ALT-type survivors (Fig. 3) (Gadaleta et al. 2016). Therefore, it would be interesting to test whether ALT activation in telomerase-negative *swi1*Δ cells is dependent on ATR^{Rad3}-ATRIP^{Rad26}. Similar experiments in human cells are also warranted to test the role of Timeless in preventing ALT phenotypes (Fig. 3).

In summary, our studies have demonstrated a novel and conserved role of the Timeless-related proteins in replication of repetitive DNA and telomere maintenance. Swi-1^{Timeless} prevents hyper-recombination and ALT activation

in the absence of telomerase activity in fission yeast. Considering the prevalence of human cancers with active ALT pathways as a means to maintain functional telomeres, our study provides a potential mechanism to explain ALT activation during cancer development in humans.

Acknowledgments This work was supported by the Aging Initiative at Drexel University College of Medicine. We thank Chiaki Noguchi and Grant Grothusen for technical assistance and National BioResource Project Japan for *S. pombe* strains.

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