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# Using Circular Mini-chromosomes to Probe a Role for Stn1 in DNA Replication

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ABSTRACT OF THE THESIS

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Master of Science, Graduate Program in Cell, Molecular, and Developmental Biology
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Dr. Constance I. Nugent, Chairperson

CST complex, which is composed of Cdc13p, Stn1p, and Ten1p, is the telomere capping complex that can protect the chromosome end from degradation, inappropriate recombination, and chromosome end fusion. Our lab previously showed that if we overexpress *STN1* in wild-type yeast cells under HU treatment, the S phase checkpoint is interrupted, and the late origins are fired inappropriately. A direct test looking for extragenic suppressor of the induced HU sensitivity yields a surprising result as *STN1* overexpression suppresses the temperature sensitivity of *cdc7-1*, *dbf4-1*, and *mcm7-1*. To study how overexpressed *STN1* act to promote the viability of these replication deficient mutants, I use the classic plasmid stability assay with the CEN-ARS plasmid in these mutants, reasoning that perhaps overexpressed *STN1* could facilitate the general

replication. The outcomes illustrate that *STN1* overexpression enhances the CEN-ARS plasmid stability in *cdc7-1* and *mcm7-1*, suggesting overexpressed *STN1* can act away from telomere to improve replication directly or indirectly. Moreover, I test the TEL-ARS plasmid stability in the same mutants trying to identify in which part of the replication machinery is overexpressed *STN1* facilitating. Interestingly, *STN1* overexpression enhances the TEL-ARS plasmid stability in *cdc7-1* and *dbf4-1*, suggesting *STN1* overexpression is probably acting on the initiation step of the replication machinery. Plasmid stability assay with the TEL-ARS plasmid in the *sir4Δ* and *rap1-5* alleles is to examine if *STN1* overexpression can also facilitate the telomere-specific segregation mechanism, however the results suggest *STN1* overexpression probably has no affect on such segregation mechanism. The TEL-ARS plasmid stability has no significant change in both *sir4Δ* and *rap1-5* after STN1 overexpression. Finally, the CST mutants, *stn1-281t* and *ten1-105* demonstrate high TEL-ARS plasmid stability. The unexpected result could be due to the delaying in S phase caused by both mutants. In conclusion, my data suggest overexpressed *STN1* can act away from telomeres to facilitate general replication directly or indirectly perhaps at the initiation step of the replication.

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# Chapter 1

## Introduction

### Discovery of Telomeres and Telomerase

Telomeres, the ends of linear eukaryotic chromosome, were discovered in the late 30's and early 40's by Hermann Muller and Barbara McClintock. They both observed unique phenomena at the chromosome termini. Muller found that X-ray induced chromosome rearrangements never occurred at the termini in *Drosophila* (Muller 1938). McClintock observed that the terminal fragment was never involved in breakage-fusion-bridge cycles in *Maize* (McClintock, 1941). These observations led to the conclusion that chromosome termini were distinct from double strain breaks (DSB) and these termini were protected by other elements preventing cell death, chromosome end to end fusions, inappropriate degradation by nucleases, and illegitimate recombination (Blackburn 1999, de Lange 2001, Rudolph et al. 1999). (Figure 1.1)

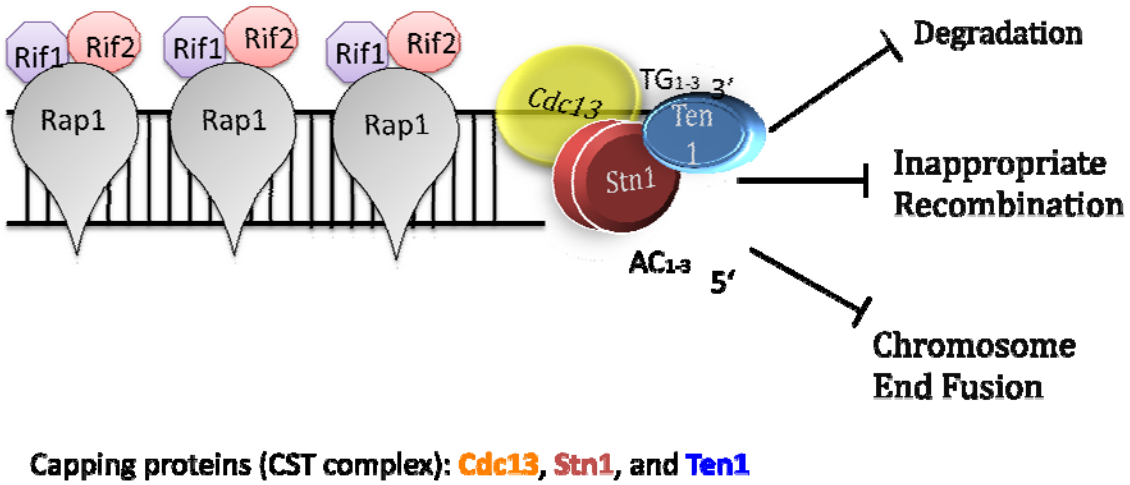


**Figure 1.1 End protection by the CST complex.**
The CST complex is composed of Cdc13p, Stn1p, and Ten1p. The Cdc13p of the CST

complex binds to 3' G-rich single-stranded DNA and serves as a critical component of the cap that protects the chromosome ends from degradation, inappropriate activation of the DNA damage checkpoint or recombination, and from chromosome end fusion.

In 1953, James Watson and Francis Crick solved the double helix structure of DNA. In addition, they proposed a model for replication involving the separation of the double stranded DNA helix to allow for synthesis of the new strands. As cells replicate chromosomes shorten from both ends, known as the end replication problem. (Figure 1.2) The terminal erosion of chromosomes occurs due to the unidirectional replication of DNA polymerases and because the termini of chromosomes contain a 3' overhang. Thus, leading strand replication results in a daughter strand that is shorter than the original strand by the size of the overhang, if the polymerase can replicate to the very terminus of the chromosome. Interestingly, recent evidence from human cells suggests that at least for the lagging strand the cell is unable to prime the very terminus of the chromosome and the terminal primer is actually placed 70-100 nucleotides from the end (Wright et al. 1997, Chow 2012). Thus, for the lagging strand, sequence is lost due to incomplete replication. Further sequence loss arises from nucleolytic processing of the leading strand product, which is required to generate the necessary 3' overhang (Faure 2010).

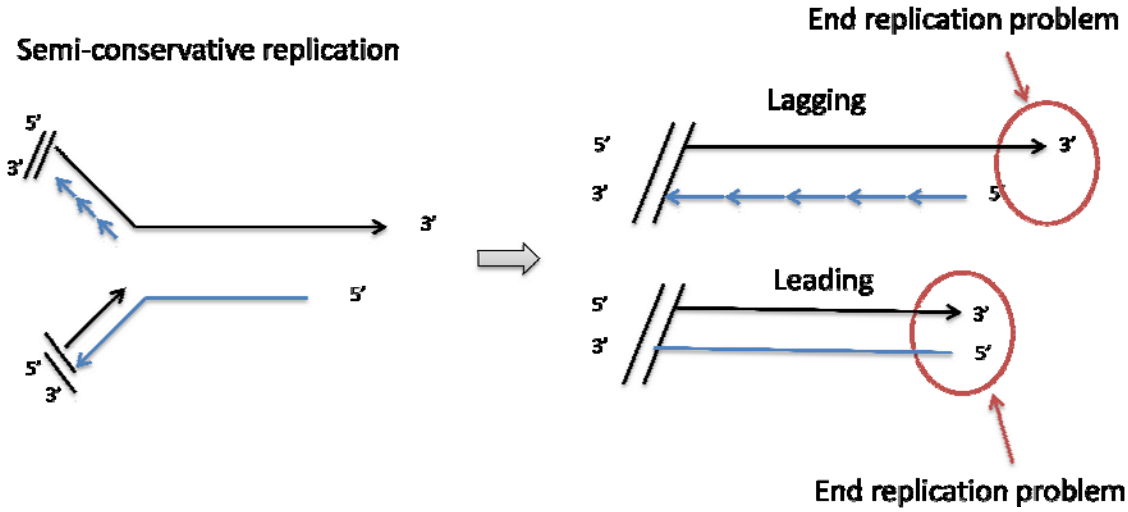


**Figure 1.2 End replication problem from semi-conservative replication.**
The semi-conservative replication will result in shortened 5' end in lagging strand and blunt ended chromosome ends. The next round of semi-conservative replication using the shortened 5' strand as the template in the lagging strand will result in even shortened daughter strands. On the other hand, the blunt ended leading strand cannot serve as the binding platform for Cdc13p in order to "cap" the telomeres, thus cells have to degrade the 5' strand to generate the 3' overhang. This process will also result in shortened 5' strand and even shortened chromosomes in the next round of semi-conservative replication. As chromosomes become shorter and shorter, essential genetic information will be lost and cell will eventually senescence.

The end replication problem was thought to be resolved by the palindromic hairpin structure in early 1970's (Karrer and Gall 1976, Engberg et al. 1976, Shore and Bianchi 2009), and this idea was soon extended when Blackburn's group revealed the guanine rich 3' DNA strand at the end of the chromosome and demonstrated that the hairpin structure is not enough for solving end replication problem (Blackburn and Gall 1978). More surprisingly, the G rich telomeric DNA sequence from *Tetrahymena* can "seed" the formation of functional telomere in *Saccharomyces cerevisiae* (Szostak and

Blackburn 1982). This finding suggested that the solution to the end replication is evolutionarily conversed.

In 1984, Carol Grieder along with Elizabeth Blackburn discovered telomerase and with it the mechanism by which cells could compensate for the gradual erosion of chromosome ends. Telomerase consists of RNA and proteins, and this enzyme can extend the G rich single strand telomere overhang after semi-conservative replication by using its RNA component (CCCCAA) as a template (Greider and Blackburn 1985, Greider and Blackburn 1989). Different groups further confirmed the function of telomerase by constructing telomerase defective mutants and revealing that those mutants contain shortened telomeres (Lundblad and Szostak 1989, Singer and Gottschling 1994, Blasco et al. 1997, Lendvay et al. 1996, Linger et al. 1997).

As Greider and Blackburn discovered the sequence of the telomerase RNA component in *Tetrahymena*, Lundblad and Szostak also found a gene that when mutated, will cause progressive telomere shortening over generations, a phenotype that is similar to telomerase deficient mutants. Thus, this budding yeast gene was named *EST1* for "ever short telomere 1" (Lundblad and Szostak 1989). The main function of *EST1* was characterized later by several groups of scientists. It was discovered that Est1 functions in the recruitment of telomerase to the single strand G-overhang of telomeres through interactions with the telomere associate protein, Cdc13p (Qi and Zakian 2000, Li et al. 2009, Chan et al. 2008, Dezwaan and Freeman 2009). A few years after the discovery of *EST1*, another telomerase component was revealed in a screen looking for telomeric silencing suppressors in *S. cerevisiae*. The new component was named *TLC1* for

"telomerase component 1" and was found to be the RNA component of telomerase in budding yeast, consisting of sequence CACCACACCCACACAC (Singer and Gottschling 1994). The *tlc1* mutant also displayed the shortened telomere phenotype. The rest of the telomerase components were identified in the same year by Lundblad's group and Cech's group through both genetic and biochemical approaches looking for genes that are essential for telomerase (Lendvay et al. 1997, Lingner et al. 1997). The results of the screen were three ever short telomere genes: *EST2*, *EST3*, and *EST4. EST2* was characterized and shown to be the catalytic subunit of the telomerase (Lingner et al. 1997, Counter et al. 1997). *EST3* can bind to *TLC1* and *EST1* to form a stable telomerase complex (Taggart and Zakian 2002).

**Telomere End Binding Proteins in *Saccharomyces cerevisiae***

While many research laboratories focused on the composition and function of telomerase in the 90's, other groups were focusing on proteins, besides telomerase, that can regulate the length of telomeres. These proteins can be separated into to two major groups: proteins that are associated with duplex telomeric DNA and proteins that are associated with the single stranded 3' overhang. Rap1p is the protein that associates with duplex telomeric DNA, and Rif1p and Rif2p bind to Rap1p. In the late 80's, Murray proposed that there is a feedback mechanism which can sense the telomere length and adjust it to a constant average length in yeast (Murray et al. 1988). This feedback mechanism was then shown to be conducted by Rif1p and Rif2p though Rap1p (Murray et al. 1988). The functions of Rap1p are transcriptional silencing, transcriptional activation, and telomere maintenance (Elledge and Davis 1989, Shore and Nasmyth 1987,

Buchman et al. 1988). In telomere maintenance, the Myb domain allows Rap1p to bind dsDNA, and the carboxyl termini of Rap1p can interact with the negative regulator of telomere length, Rif1p and Rif2p to form a Rap1-Rif1-Rif2 complex (Hardy et al 1992, Wotton and Shore 1997, Conrad et al. 1990). Deletion of either *RIF1* or *RIF2* results in long telomeres: about 600bp for *rif1Δ* and about 150bp for *rif2Δ*. Double deletion of RIF1 and RIF2 resulted in a synergistic elongation of telomeres suggesting that Rif1p and Rif2p function to regulate telomere length through different mechanisms (Shore and Bianchi 2009). In summary, the feedback mechanism for regulating telomere length is actually through the counting of Rif1p/Rif2p on telomeres instead of counting on Rap1p. (Levy and Blackburn 2004) In addition to the negative regulation of telomere length, Rap1p is shown to prevent NHEJ (non-homologous end joining) as *rap1* mutant shows increased chromosome end-to-end fusions (Greenwood and Cooper 2009). Moreover, Rap1p can protect telomeres from excessive end resection (Negrini and Shore 2007).

Another protein complex that regulates telomere length and is associated with double stranded telomeric DNA is Ku70/80. The Ku heterodimer is composed of Ku70p and Ku80p and is a multifunctional complex. It can recognize and repair DSB via NHEJ and prevent homologous directed recombination (HRD), regulate telomere length homeostasis, protect telomeres from degradation, aids in nuclear spatial organization, heterochromatin formation for transcriptional silencing, and regulation of late replication origin firing. (Boulton and Jackson 1996a, Boulton and Jackson 1996b, Martin et al. 1999, Milne et al. 1996, Mages et al. 1996, Boulton and Jackson 1998, Porter et al. 1996, Laroche et al. 1998, Bertuch and Lundblad 2003a, Bertuch and Lundblad 2003b, Daley et

al. 2005, Banerjee et al. 2006, Fisher et al. 2004, Stellwagen et al. 2003, Gravel et al. 1998, Nugent et al 1998, Polotnianka et al. 1998, Maringele and Lydall 2002, Cosgrove et al. 2002) Interestingly, the capping function of Ku complex in budding yeast is independent of the Rap1p demonstrating that at least three capping mechanisms (which will be discussed later) protect telomeres through out the cell cycle.

The other major group of proteins that associate with telomeric DNA in budding yeast are Cdc13p, Stn1p, and Ten1p. These proteins, especially Cdc13p, are thought to bind to ssDNA (single-stranded DNA). *CDC13* was first identified in a screen looking for cell cycle division mutants that could trigger *RAD9* dependent checkpoint arrest at G2/M (Carson and Hartwell 1985, Garvik et al 1995). This mutant, *cdc13-1*, accumulates excessive single stranded telomeric DNA at the non-permissive temperature, induces telomere recombination and causes mis-regulation of telomere length (Carson and Hartwell 1985, Garvik et al 1995, Diede and Gottschling 1999). By using *in vivo* telomere addition assay, Cdc13p was shown to protect chromosome ends from exonucleolytic activity (Garvik et al. 1995). One of the nucleases responsible for this degradation was shown to be ExoI and in the case of cdc13-1, resection can proceed 30~40 kb into the chromosome (Diede and Gottschling 1999, Tsubouchi and Ogawa 2000, Booth et al. 2001, Garvik et al. 1995). Cdc13 was shown to directly bind single stranded telomeric sequence through a gel mobility shift assay (Nugent et al. 1996, Lin and Zakian, 1996).

Besides the capping function of Cdc13p, research from Lundblad's group uncovered the second role of Cdc13p as they characterized the mutant, *cdc13-2*. *cdc13-2*

displays shortened telomeres similar to telomerase deficient mutants (Nugent et al. 1996). In addition, *cdc13-2 tlc1Δ* double mutants did not result in a synergistic reduction in telomere length (Nugent et al. 1996), implying that *CDC13* and telomerase work in the same pathway. Further evidence that placed *CDC13* and telomerase in the same pathway came from Qi and Zakian, who demonstrated a physical interaction between Cdc13p and Est1p through pulldown and yeast two-hybrid assays (Qi and Zakian 2000). The mechanistic details of how telomerase is recruited via *CDC13* were fleshed out resulting in a model in which *CDC13* directly interacts with *EST1* and in turn recruits the catalytic core of the telomerase holoenzyme (Wu and Zakian 2011). (Fig 1.3) The function of *EST1* can be bypassed if Cdc13p is fused directly to Est2p (Evans and Lundblad 1999). Along with Cdc13p's positive regulation of telomerase, *CDC13* has also been shown to negatively influence telomere length as *cdc13-5* mutants display long telomeres (Chandra et al 2001). In all, Cdc13p plays duel role in telomere protection and telomere length regulation.

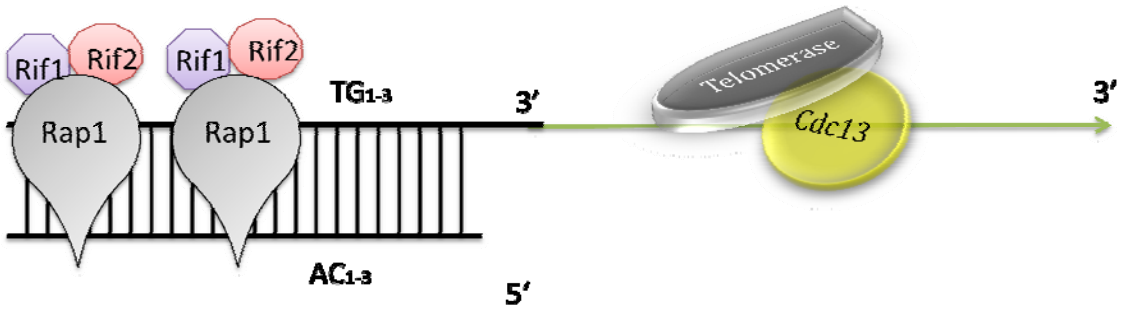


**Figure 1.3 Cdc13p recruits telomerase to extend the 3' strand.**
To solve the end replication problem, cells evolved with a mechanism that can extend the shortened telomeres. The Rap1-Rif1-Rif2 complex associates with the dsDNA (double-stranded DNA) at telomeres. As the telomeres become shortened after each replication, the number of Rap1-Rif1-Rif2 complexes will also reduced. Once the number of the Rap1-Rif1-Rif2 complexes is critically low, Cdc13p will recruit telomerase to extend the 3' overhang.

Another telomere associating protein, Stn1p (Suppressor of cdc ThirteeN), was discovered in a screen looking for dosage suppressors of *cdc13-1* (Grandin et al. 1997). Overexpression of Stn1p can both suppress the temperature sensitivity and excessively elongated telomeres of *cdc13-1* (Grandin et al. 1997). In addition, *stn1-13* displays a similar telomere defect phenotype as *cdc13-1* including accumulation of telomeric ssDNA and lengthened telomeres under restrictive temperature (Grandin et al. 1997). Finally, Cdc13p and Stn1p is shown to interact in the yeast two-hybrid assay, and *cdc13-1 stn1-13* double mutant is synthetic lethal even at permissive temperature (Grandin et al. 1997). All these findings lead to a hypothesis that Stn1p could interact with Cdc13p directly or indirectly in telomere protection and length regulation. This hypothesis was further elaborated when Chandra's group proposed a model showing Stn1p competes with Est1p in binding with Cdc13p. If Est1p is binding to Cdc13p, then telomere extension will be executed; whereas if Stn1p is binding to Cdc13p, then telomere length

will be negatively regulated and shortened because of the inhibition of telomerase recruitment (Chandra et al. 2001).

One attractive speculation derived from seeing the direct interaction between Stn1p and Pol12p was that Stn1p might play a role outside its niche, the very end of chromosomes. *CDC13* and *STN1* orthologs in human were first identified as the "alpha accessory factor" *AAF132/CTC1* and *AAF44/STN1* (Goulian et al. 1990). The follow up biochemical studies by the same group indicated that *AAF/CTC1-STN1* functions by increasing affinity of DNA Polα/primase in synthesis of the lagging strand of a replication fork (Goulian and Heard 1990).

Moreover, Cdc13p, Stn1p, and Ten1p were found to have similar structure and function as to replication protein A (RPA), a "global" replication factor (Gao et al. 2007, Gelinas et al. 2009, Sun et al. 2009). Cdc13p, Stn1p, and Ten1p are structurally similar to Rpa1p, Rpa2p, and Rpa3p in budding yeast, respectively. The OB-fold or winged helix domain between CST and RPA are well conserved (Price et al. 2010). Both CST and RPA are ssDNA binding protein and are shown to facilitate the movement of replication fork (Stweart and Price 2011, Giraud-Panis et al. 2010).

Finally, Stn1p was shown to mislocalize through out the genome in budding yeast after overexpression. More surprisingly, the mislocalized Stn1p perturbs the S phase DNA damage checkpoint possibly by misregulating the firing of late origins (Gasparyan et al. 2009). Together with the previous findings regarding Stn1p, it is strongly suggesting that Stn1p might not be a telomere specific binding protein after overexpression and may have a role in replication with a more global perspective.

The last telomere associating protein, Ten1p (TElomeric pathways with stN1), was also identified by the same group in a screen looking for dosage suppressor of the conditional mutants *stn1-13* and *stn1-154* (Grandin et al. 2001). Overexpression of *TEN1* can rescue the growth defect and restore telomere length in *stn1-13* and *stn1-154*. Also, physical interaction between Ten1p and Stn1p was shown through yeast two hybrid assay and co-immunoprecipitation (Grandin et al. 2001). Furthermore, *ten1* alleles share similar telomere defects as *stn1* mutants. Although overexpression of Ten1p alone does not suppress *cdc13-1* growth defects, it does help in suppressing the *cdc13-1* temperature sensitivity when Stn1p is also overexpressed (Grandin et al. 2001). In a more detailed examination performed by Xu et al., *ten1* mutants that display similar telomere defects as to *cdc13-1* could be partially suppressed after deletion of *EXOI* (Xu et al. 2009). Altogether, Ten1p is suggested to protect telomere from degradation as well as to regulate telomere length. A model was proposed where Cdc13p, Stn1p, and Ten1p, form a heterotrimer that caps the telomere end by binding to the G-rich single stranded telomeric DNA preventing inappropriate exonucleolytic activity from degrading telomeres (Grandin et al. 2001, Vodenicharov and Wellinger 2006). In addition to protect the telomeres, the binding of Stn1p-Ten1p to the elongated 3' overhang could inhibit the recruitment of the telomerase, which then regulates the telomere length (Grandin et al. 2001).

**Telomere End Binding Proteins in other Organisms**

Telomere protection in budding yeast is mainly conducted through the Rap1-Rif1-Rif2 complex and the Cdc13-Stn1-Ten1 (CST) complex. However, a Cdc13p homologue

has not yet been found in fission yeast (Martin et al. 2007). More surprisingly, fission yeast Rap1p does not bind to double stranded telomeric DNA (Price et al. 2010, Giraud-Panis et al. 2010). Instead, fission yeast Rap1p interacts directly with Taz1, and Taz1 binds to the telomeric duplex DNA. To complete the telomere protection in fission yeast, Rap1p also interact with Poz1, Poz1 binds to Tpz1, and Tpz1 interact with both Ccq1 and Pot1 where Pot1 binds the 3' single stranded overhang. Ccq1p is thought to recruit telomerase (Price et al. 2010). Disruption of Taz1p, Rap1p, and Pot1p results in a reduction of telomere length as well as activation of the DNA damage checkpoint (Martin et al. 2007, Price et al. 2010, Giraud-Panis et al. 2010). Surprisingly, *pot1*$^{-}$ survivors can form a circularized chromosome via end-to-end fusion (Baumann and Cech 2001).

The identification of budding yeast Stn1p and Ten1p orthologs in fission yeast came from structural profiling studies that detected the conserved oligonucltide/oligosaccharide-binding fold (OB fold) domains (Martin et al. 2007). Mutants with disruption of either fission yeast Stn1p or Ten1p display rapid telomere loss and end-to-end fusions, suggesting that both proteins function in telomere end protection. In addition, fission yeast Stn1p interacts with Ten1p and can localize to telomeric ssDNA, but no Pot1p interaction has been found (Martin et al. 2007). Although the protection function of these end binding proteins are similar between budding yeast and fission yeast, the mechanisms can be fairly different, suggesting rapid divergence might occur during telomere evolution (Price et al. 2010).

End binding proteins in plants include TRF-like proteins, Ctc1p, and Stn1p. TRF-like proteins bind to telomeric dsDNA in vitro and can negatively regulate telomere

length in vivo (Hong et al. 2007, Karamysheva et al. 2004). Arabidopsis Stn1p and Cdc13p paralogs were identified recently in the same lab via sequence alignment, BLAST, and examination of putative mutant phenotypes (Song et al. 2008, Surovtseva et al. 2009). Defects in either AtCtc1p or AtStn1p result in accumulation of single stranded G-rich telomeric DNA, high frequency of end-to-end fusions, subtelomeric homologous recombination, telomere length shortening, and activation of the DNA damage checkpoint (Song et al. 2008, Surovtseva et al. 2009). These phenotypes suggest AtCtc1p and AtStn1p act in a similar manner as their yeast paralogs. Unlike CST in budding yeast, loss of AtCtc1 or AtStn1 does not result in immediate cell death but plants still die gradually (Song et al. 2008, Surovtseva et al. 2009). Oddly, no Ten1p homolog has yet been found in plants, and AtPot1 does not bind to telomeric ssDNA in vitro nor does it function in telomere capping (Price et al. 2010, Shakirov et al. 2009, Surovtseva et al. 2007). However, Pot1p from moss *Physcomitrella patens* can bind to G-rich single stranded telomeric DNA in vitro and is important for telomere end protection in vivo (Shakirov et al. 2010). Even though fission yeast Pot1 ortholog in *Arabidopsis* (AtPot1 and AtPot1b) does not act in telomere capping, altogether, the end protection mechanism in plant is suggested as follows: TRF-like proteins bind to duplex telomeric DNA region for telomere length homeostasis, and Ctc1p and Stn1p form a complex that cap the telomere end via binding to the G-rich telomeric ssDNA (Price et al. 2010, Giraud-Panis et al. 2010). Finally, an interesting speculation was proposed where plants might serve as an evolutionary bridge, in telomeres and its associating proteins, that connects budding yeast and vertebrates (Price et al. 2010).

The telomere capping complex in mammals is called shelterin, as it "shelters" mammalian telomeres from inappropriate nucleolytic attack and other harmful events that can alter or destroy the telomere structure (de Lange 2005, de Lange 2009). Shelterin is composed of Trf1p, Trf2p, Tin2p, Tpp1p, Pot1p, and Rap1p (Palm and de Lange 2008, Xin et al. 2008, Stewart et al. 2011). Briefly, Trf1p and Trf2p bind to duplex region of telomeres via their SANT/Myb domain at C-terminus (Linger and Price 2009). Tin2p (N-terminal) then binds to both Trf1p and Trf2p and the C-terminal of Tin2p binds to Tpp1p. Tin2p and Tpp1p form a bridge that links Trf1/Trf2 to Pot1p, which binds to G-rich telomeric ssDNA. Rap1p independently binds to Trf2p instead of interacting with telomeric DNA directly (de Lange 2009, Price et al. 2010, Giraud-Panis et al. 2010, Stewart et al. 2011). The shelterin complex binds to the telomere sequence TTAGGG and helps mammalian telomeres form a T (telomere)-loop structure by folding back the telomere. In addition to the T-loop structure, a D (displacement)-loop structure is created as the G-rich ssDNA from the end of T-loop invades to the duplex region of the telomeric DNA (Stansel et al. 2001, de Lange 2004). This T-loop serves to protect the mammalian telomere as well as preventing the recruitment of telomerase, indicating that other proteins are involved in resolving the T-loop for telomerase extension (de Lange 2004, de Lange 2009).

Shelterin was thought to be the only capping complex in mammals until the discovery of budding yeast CST ortholog, Ctc1p, Stn1p, and Ten1p (Miyake et al. 2009). Interestingly, mammalian Ctc1p and Stn1p were identified as polymerase alpha accessory factors (AAF) and aid polymerase alpha during DNA replication (Casteel et al 2009,

Goulian et al. 1990). Human Ctc1p was shown to interact with Stn1p and Ten1p as well as to bind to G-rich telomeric ssDNA without sequence specificity independent of Pot1p (Miyake et al. 2009). Knocking out any of the CST component in humans results in telomere shortening and increased DNA damage foci, suggesting that mammalian CST-like complex also functions in telomere protection (de Lange 2009, Miyake et al 2009). Furthermore, the discovery of mammalian CST-like complex also suggests that mammals have evolved multiple complexes to coordinate different aspects of telomere biology including telomere capping and telomere replication (Miyake et al 2009, Price et al. 2010).

**Dynamics of Telomere Maintenance**

Maintaining proper telomere length in higher eukaryotes is crucial because too long or too short of telomeres will result in cancer cell development or cell senescence and apoptosis. In addition, telomere length homeostasis is crucial to prevent loss of essential genetic information, prevent gross chromosomal rearrangements, and probably serves a function in higher order nuclear organization. How cells maintain telomere homeostasis is still not fully understood, yet recent studies have shed some light on the subject and identified many factors essential to telomere homeostasis: such as cell cycle regulation proteins, telomere binding proteins, DNA damage checkpoint proteins, and replication proteins. These discoveries explain how complex telomere regulation is in keeping a healthy cell.

The first problem cells face regarding telomere maintenance is the gradual erosion of DNA sequence after semi-conservative replication. Telomerase was identified to

overcome this problem by extending the telomere length or more specifically, elongating the 3' G-rich telomeric ssDNA. Such simple task is actually a combination of different processes by varies types of proteins. In budding yeast, telomere extension begins by sensing critically short telomeres with the counting mechanism by Rap1/Rif1/Rif2 complex (Levy and Blackburn 2004). This idea was supported with the finding that by using a single telomere addition assay, not all but a few telomeres were extended with a preference to elongate short telomeres (Teixeira et al. 2004). Moreover, the elongation process was identified to be coordinated with cell cycle progression and occurred during late S/G2 phase in budding yeast (Marcand et al. 2000). Interestingly, the "sensing" of short telomeres involves the checkpoint proteins, mainly Tel1p and Mec1p (Stewart et al. 2011).

Many crucial pieces of data led to the development of a working model describing how these proteins interact to promote telomere extension. First, the extension of telomeres requires the association of telomerase to the end of the chromosome. Part of this association requires the binding of Cdc13p to the telomeric G-rich ssDNA (Lingner and Cech 1996). However, after the conventional DNA replication, the leading strand product is blunt ended while the lagging strand will have a short single stranded G-rich overhang. Nevertheless, both strands cannot serve as the substrate for Cdc13p binding. Since different outcomes were observed at telomere ends, Chakparonian proposed that there are two different end processing mechanisms for leading and lagging strand for generating 3' G-overhang (Chakparonian and Wellinger 2003). Interestingly, both leading and lagging strands often terminate with a precise 3' end sequence, suggesting at least

one common feature is shared among the two (Shore and Bianchi 2009).

The Mre11p-Rad50p-Xrs2p (MRX) complex was shown to be necessary to generate the 3' overhang required for Cdc13p binding. In addition, the resection via MRX complex requires the cell cycle regulator, cyclin-depend kinase 1 (Cdk1) (Ira et al. 2004, Frank et al. 2006). After resection, newly generated 3' G-rich telomeric ssDNA can serve as a substrate for Cdc13p binding allowing for the recruitment of telomerase via Tel1p and Mec1p (Larrivee et al. 2004, Tseng et al. 2006). To recruit telomerase, Cdc13p is phosphorylated by Tel1p and Mec1p with their association of Xrs2. The association of Xrs2 triggers the phosphorylation of Tel1p and Mec1p and subsequently phosphorylates Cdc13p. The phosphorylated Cdc13p then recruits telomerase via interaction with Est1p to chromosome ends (Nugent et al. 1996, Sabourin et al. 2007, Bianchi et al. 2004, Marcand et al. 1999, Pennock et al. 2001, Taggart et al. 2002, Bonetti et al. 2009). After telomerase recruitment, Est2p serves as a catalytic subunit and Tlc1 serves as the RNA template, together with the other telomerase components, telomerase can now add the $TG_{1\text{-}3}$ repeats to the processed short telomeres (Lewis and Wuttke 2012, Stewart and Price 2011).

A current problem regarding telomere replication is how the complementary strand is synthesized following extension of the 3' end by telomerase. This "fill-in" synthesis was shown to require Polα (Fan and Price 1997). Moreover, they suggested that telomerase extension mechanism and DNA polymerase fill-in mechanism might depend on each other as inhibition of DNA polymerases resulted in longer 3' G-rich overhangs compare to wild type. The fill-in hypothesis was further confirmed when

Diede and Gottschling used a *de novo* telomere addition assay to show DNA polymerase mutants, specifically defects in Polα and Polδ, fail to add 5' C-rich telomeric ssDNA with long 3' G-rich ssDNA. Their findings suggested that 5' C-rich strand synthesis requires Polα and Polδ, and 5' C-rich strand fill-in is coordinated with telomerase extension of the 3' G-rich overhang (Diede and Gottschling 1999).

How 3' extension and 5' fill-in are coordinated is still poorly understood. Recently, a research group discovered the interaction between Cdc13p and Pol1p (the catalytic subunit of Polα) via yeast two hybrid and Co-immunoprecipitation (Qi and Zakian 2000). They further hypothesized that Cdc13p might recruit Polα to telomeres for fill-in synthesis of the 5' C-rich strand. This idea together with the findings from Chandra's group led to an intriguing model where recruitment of Polα through Cdc13p triggers the 5' fill-in and negatively regulates telomerase extension of the 3' strand (Qi and Zakian 2001, Chandra et al. 2001). This model was further expanded after the detection of direct interactions between Stn1p and Pol12p (the regulatory subunit of Polα) and the interaction between Ten1p and Pol12p. Thus, the recruitment of Polα not only involves Cdc13p but also Stn1p and maybe Ten1p (Grossi et al. 2004, Petreaca et al. 2006, Xu et al. 2009).

In sum, telomeres are gradually shortened as cells continue to divide. The erosion of telomeres reduces the binding sites for Rap1p-Rif1p-Rif2p and triggers the Mre11p-Rad50p-Xrs2p resection of the shortened telomeres and generates 5' G rich-over hang. Tel1p and Mec1p sense the processing event of MRX complex and further phosphorylate Cdc13p for recruiting the telomerase in extending the G-overhang. After the extension,

Stn1p and Ten1p together bring in Polα to prime the complementary C-rich strand and together with the help from Polδ to complete the C-rich strand fill-in. The fill-in mechanism in addition inhibits the telomerase extension of the G-overhang in regulating the telomere homeostasis. (Figure 1.4)

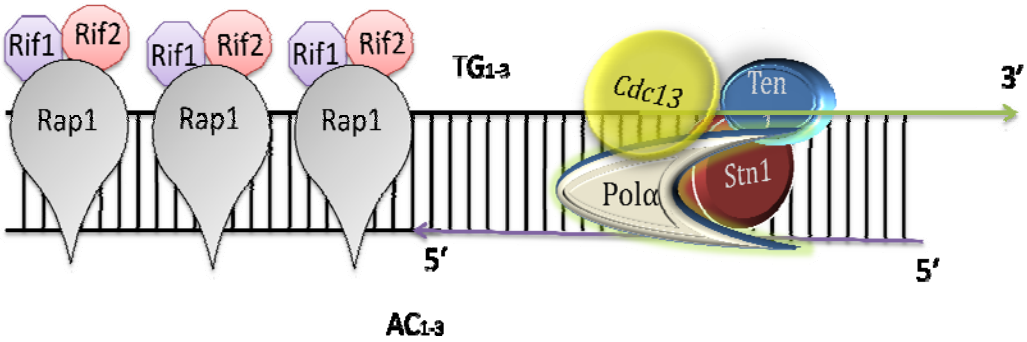


**Figure 1.4 Fill-in synthesis by Polα via the help from the CST complex.**
A hypothesized mechanism for synthesizing the complementary 5' strand after the telomerase elongates the 3' overhang is the fill-in synthesis. It is proposed that Stn1p and Ten1p together with Cdc13p will recruit Polα to the telomere ends, and synthesize the complementary 5' end.

## Chapter 2

## Effects of *STN1* Overexpression on the CEN-ARS Plasmid Stability

### Introduction

Stn1p is thought to be a telomere binding protein that helps to protect telomeres from nucleolytic degradation, inappropriate recombination, and end-to-end fusions (Dewar and Lydall 2011; Moser and Nakamura 2009). In addition to Stn1p's telomere capping function, it is also hypothesized that Stn1p regulates telomere length through fill-in synthesis via interaction with Pol12p, the regulatory subunit of Polα (Grossi et al. 2004; Petreaca et al. 2006; Xu et al. 2009; Stewart and Price 2011). Interestingly, the discovery of yeast Stn1p orthologs in mammals as polymerase accessory factors and the structural similarities between CST and RPA complex lead to an idea suggesting that Stn1p might have a role in conventional replication (Miyake et al. 2009; Grossi et al. 2004; Petreaca et al. 2006; Gao et al. 2007; Gelinas et al. 2009; Sun et al. 2009; Giraud-Panis et al. 2010). This idea is further supported by evidence showing hCST aiding fork restart under replication stress because hSTN1 knockdown decreases in new origin firing under HU treatment (Stewart et al. 2012). Also, data from *Xenopus* extracts demonstrates that xCST can increase the efficiency of replication by promoting priming of a single stranded DNA template (Nakaoka et al. 2011), as chromosome DNA replication is functional after xStn1p immunodepletion, but ssDNA template cannot be synthesized unless it is primed (Nakaoka et al. 2011).

Despite the normal function of Stn1p at telomeres, previous work from our lab showed that overproduction of Stn1 increases the HU sensitivity of cells and this appears

to occur through a disruption of the S phase checkpoint. In addition, both observations are dependent on Pol12p as chromosome binding and S phase defects were suppressed in *pol12* mutants (Gasparyan et al. 2009). Together, these findings suggest overproduced Stn1p could be recruited to non-telomeric sites via direct or indirect interactions with Polα and interrupt the normal function of the S phase checkpoint (Gasparyan et al. 2009).

In an effort to elucidate the mechanism by which overproduced Stn1p exacerbates replication stress, a screen was attempted to identify extragenic suppressors of the *STN1* induced sensitivity to HU. Unfortunately, no extragenic suppressors were found other than the Pol12 alleles. While looking for suppressors of the HU sensitivity of overproduced Stn1p, a surprising observation was made that *STN1* can act as a dosage suppressor of the temperature sensitivity of *cdc7-1*, *dbf4-1*, and *mcm7-1*. These mutants are replication deficient mutants as *CDC7* and *DBF4* are thought to be function at initiation of DNA replication and *MCM7* is thought to be function at both initiation and elongation of DNA replication (Heller et al. 2011; Pospiech et al. 2010; Tye 1999a; Bochman and Schwacha 2009). However, the mechanism by which overproduction of Stn1p is able to suppress these replication mutants' temperature sensitivity remain elusive. Such suppression could simply be the consequence of the disruption to the normal cellular response to the replication stress. Another speculation could be that *STN1* has a role in global replication when overexpressed.

Before examining the role of overexpressed *STN1* in global replication, to understand the properties of these replication deficient mutants is crucial. *CDC7* was first identified in the cell-division cycle screen by tracking mutated yeast cell morphology

after radiation exposure (Culotti and Hartwell 1971). One of the mutants, *cdc7-1,* is a conditional allele that will arrest as large, budded cells (with a dumbbell shape). This mutant contains Gly to Ala change at amino acid position 384 (Patterson et al. 1986). The early genetic studies determined that *CDC7* encodes an initiation factor that controls S phase entry during the cell cycle (Culotti and Hartwell 1971; Hereford and Hartwell 1974). First, a reciprocal shift method was used where the mutant cells were blocked in G1 with alpha factor, and released from the block at non-permissive temperature. Examination of the *cdc7-1* cell morphology revealed that *CDC7* function is required for cells to enter S phase. The double-mutant method further identified a specific order of genes that act on cell cycle progression by comparing the morphology of each single mutant and the double mutants. From these studies, they propose that *CDC7* is involved in initiation of DNA synthesis (Hereford and Hartwell 1974).

The function of *CDC7* was further elaborated by different groups that characterized Cdc7p as a serine/threonine protein kinase that not only acts before S phase to fire the origin but also throughout S phase for re-initiation of replication fork after stalling (Hollingsworth and Sclafani 1990; Bousset and Diffley 1998). Sclafani's group overproduced Cdc7p and tested whether immunoprecipitated Cdc7p could phosphorylate mammalian histone H1 on serine/threonine residues. The results indicated that Cdc7p is a kinase and the kinase activity is thermolabile (Hollingsworth and Sclafani 1990). In addition, Donaldson proposed that Cdc7p acts in firing both early and late origins. This group used alpha factor to block cells in G1 and observed origin firing by 2D gel after release into different conditions (Donaldson et al. 1998). Furthermore, Cdc7p was shown

to interact with Orc2p, which is one subunit of the origin recognition complex, by yeast two hybrid. This finding further support the idea that Cdc7p is involved in origin firing (Hardy 1996). Cdc7p also interact with Dbf4p and MCM complex, which are involved in initiation of the DNA replication. The interaction and function of the association of Cdc7p and these proteins will be further discussed in the later paragraphs.

*CDC7* could act to re-initiate replication origins after HU treatment as well as *cdc7-1* has enhanced HU sensitivity, suggesting *CDC7* also plays a role in S phase checkpoint (Bousset and Diffley 1998; Jares et al. 2000). A screen was done to search for mutations that are lethal in combination with *cdc7-1*, and *rad53-1* (a checkpoint mutant) was one of the hit. This genetic interaction between *CDC7* and *RAD53* in budding yeast further links the function of *CDC7* to the checkpoint (Dohrmann et al. 1999). When under replication stress, S phase checkpoint is activated and one of the target is *CDC7*. The function of the phosphorylated Cdc7p is then inhibited, thus preventing late origin from firing (Jares et al. 2000). Last but not the least, *S. pombe* Cdc7p/Hsk1p undergoes Csd1-dependent phosphorylated under HU treatment (Snaith et al. 2000) and such phosphorylation alleviates the Cdc7p/Hsk1p kinase activity (Weinreich and Stillman 1999).

Besides the role in initiation at replication origin and S phase checkpoint, other studies revealed some minor functions of Cdc7p such as regulation of chromatin silencing at telomeres, prevention of inappropriate exit from mitosis by targeting Cdc5p, and involvement in DSB repair through break-induced replication (BIR) (Miller et al. 2009, Lydeard et al. 2010).

It was also found that *dbf4-1* temperature sensitivity could be suppressed by overexpressing *STN1*. *DBF4* was first identified and characterized as an initiation factor similar to Cdc7p in Thomas' laboratory by using temperature sensitive mutant, *dbf4*-1 (Johnston and Thomas 1982a; Johnston and Thomas 1982b). They first use ethyl methane sulphonate mutagenesis to make mutants and observe their terminal cellular morphology after incubation at 37°C. They observe certain mutants arrest in large bud that looks like a dumbbell thus named them DBF (dumbbell forming) (Johnston and Thomas 1982a). At the same time, they found that *DBF4* is required for initiation of S phase. They observe no DNA synthesis after arresting *dbf4-1* with alpha factor and releasing the cells at the restrictive temperature before the start of the S phase. When they arrest the mutant cells and release them into restrictive temperature after the start of S phase, they observe DNA synthesis. Thus they conclude that *DBF4* functions similar to *CDC7* in initiating DNA synthesis. (Johnston and Thomas 1982b).

Later studies indicate that Dbf4p can regulate and interact with Cdc7p where Cdc7p acts as the catalytic subunit and Dbf4p acts as the regulatory subunit, and Dbf4 associates with Cdc7p at the G1/S transition (Kitada et al. 1992; Jackson et al. 1993). Sugino's group first found the association between *CDC7* and *DBF4* as they observed *cdc7-1 dbf4-1* double mutant is synthetic lethal and overexpression of *DFB4* can suppress *cdc7-1* temperature sensitivity (Kitada et al. 1992). Sclafani's group later proposes that *DBF4* could regulate *CDC7* kinase activity. They showed that Dbf4p can interact with Cdc7p both in vivo by yeast two-hybrid assay and in vitro by reconstitution assay. In addition, Dbf4p is shown to be required for Cdc7p kinase activity as *dbf4-1* mutant

reduces the Cdc7p kinase activity at non-permissive temperature (Jackson et al. 1993). The discovery of an interaction between Dbf4p and the origin of replication (Dowell et al. 1994) supports the idea of Dbf4p as a DNA replication initiation factor required for origin firing. They use one-hybrid screen with origin DNA replication as bait to isolate *DBF4* (Dowell et al. 1994).

*DBF4* is involved in initiation of DNA replication is suggested again when Owens demonstrates that both *CDC7* and *DBF4* are required to trigger DNA synthesis with *CDC45* (Owens et al. 1997). *CDC45* is part of the pre-replication complex at replication origin. This group uses reciprocal shift experiments with *cdc7-4 cdc45-1* and *dbf4-1 cdc45-1* double mutants to examine if the double mutants can begin and complete DNA synthesis or not (Owens et al. 1997). The results identify the dependency of *CDC7/DBF4* with *CDC45* for replication initiation. The role of *DBF4* in origin firing was further solidified when its downstream targets, the *MCM* helicase complex, was discovered (Lei et al. 1997). *DBF4* was identified as a suppressor of *mcm2-1* and removal of *DBF4/CDC7* kinase activity will also block Mcm2p phosphorylation, suggesting phosphorylation of Mcm2p by Cdc7p/Dbf4p at the G1/S phase transition is a critical step in the initiation of DNA synthesis at replication origins (Lei et al. 1997).

*DBF4* is also suggested to be associated with S phase checkpoint pathway as Dbf4p is phosphorylated under HU treatment, and this phosphorylation is Rad53/Cds1-dependent (Brown and Kelly 1999; Takeda et al. 1999; Weinreich and Stillman 1999). A more precise role of *DBF4* in the S phase checkpoint is demonstrated by a group of scientist showing that Dbf4p is a direct substrate for ATM and ATR mediated S phase

checkpoint. The phosphorylation of Dbf4p is critical for inhibiting initiation of late origin from firing, thus to maintain genome integrity (Lee et al. 2011).

The MCM complex is composed of Mcm2-7 and is suggested to melt the dsDNA during DNA replication (Davey et al. 2003, Bochman and Schwacha 2009). *mcm7* was first isolated and identified as a cell-division cycle mutant and a suppressor of the other two cold-sensitive cell-division cycle mutants, *cdc45* and *cdc54/mcm4* (Moir et al. 1982, Hennessy et al. 1991). Further characterization suggested Mcm7p acts as a licensing factor that can bind to chromatin for replication initiation (Tanaka et al. 1997, Donovan et al. 1997, Hennessy et al 1991, Homesley et al. 2000, Lei and Tye 2001). In addition, Mcm7p's association with origins depends on Cdc6p, a protein that is essential for the assembly of pre-replicative complexes (pre-RCs) at origins of DNA replication (Tanaka et al. 1997; Donovan et al. 1997) More precisely, Mcm7p interacts with other subunits of the MCM complex to form a heterohexamer complex that is loaded onto DNA replication origins by Cdc6 in late G1 with other pre-replicative complex (Aparicio et al. 1997, Davey et al. 2003, Biswas-Fiss et al. 2005, Wilmes and Bell. 2002, Kawasaki et al. 2006). After loading, subunits of the MCM complex (Mcm4p, Mcm6p, and Mcm7p) can be phosphorylated by the DDK complex (Weinreich and Stillman 1999).

MCM complex is loaded during G1 phase and is activated during S phase via the phosphorylation from the DDK complex (Bochman and Schwacha 2009; Remus and Diffley 2009; Araki 2010). When the complex is loaded onto the origin, they form the double hexamer where the N-terminal domains face each other (Sclafani et al. 2004). Furthermore, phosphorylation of the MCM complex is suggested to perform

conformational change to allow the helicase activity during the S phase. This idea comes from *bob1* mutant that can bypass the requirement of *CDC7/DBF4*, but evidence is needed for confirmation. In addition to the conformational change, the phosphorylation of MCM complex also reduces its binding affinity to the dsDNA and allows the MCM complex to unwind it, which is an ATP dependent process, that allows elongation step to occur (Davey et al. 2003, Homesley et al 2000, Labib et al. 2000, Kaplan et al. 2003, Biswas-Fiss et al. 2005, Bochman and Schwacha 2009).

As mention before, one speculation for *STN1* behavior after overexpression could simply be that disrupting the S phase checkpoint by *STN1* overexpression is sufficient to allow the strains to grow at higher temperature. However, our lab observed that the temperature sensitivity of *dbf4-1 mrc1Δ* double mutant is not suppressed (Gasparyan, data not published). Another speculation could be that overexpressed *STN1* plays a role in facilitating replication under abnormal condition and could possibly act away from telomeres. Often times, replication deficient mutants enhance the loss rate of circular mini-chromosomes, plasmids that contain a centromere and a yeast replication origin. (Maine et al. 1984; Hartwell and Smith 1985; Loo et al. 1995). This phenotype was referred to as the MCM (mini-chromosome maintenance) phenotype, and the replication deficient mutants *cdc7-1*, *dbf4-1*, and *mcm7-1* all exhibit such property (Maine et al. 1984; Donaldson et al. 1998; Sclafani 2000; Kitada et al. 1992; Tye 1999b). As previously mentioned, *cdc7-1* and *dbf4-1* cannot fire origins properly and *mcm7-1* is thought to handicap the elongation step perhaps by impairing helicase activity. Also, *mcm7-1* displays defects in replication initiation. Thus, the MCM phenotype arises in these

mutants as they have a harder time initiating and/or finishing DNA replication appropriately, and perhaps *STN1* overepxression could help to restore the crippled replication machinery.

The MCM phenotype in these mutants is a handy property for testing my hypothesis that overexpressed *STN1* facilitates replication directly or indirectly at non-telomeric loci. If overexpressed *STN1* can suppress this MCM phenotype with a circular plasmid that contains no telomere sequence then it would demonstrate a role for Stn1p in promoting replication away from telomeres.

## Material and Methods

*Plasmids and Strains*

Plasmids and yeast strains used are shown in Appendix A. Wild-type (hC160), *cdc7-1* (hC2403), *dbf4-1* (JBY999), and *mcm7-1* (DBY2029) were co-transformed with the desired plasmids. The test mini-chromosome plasmid was the CEN-ARS plasmid (pJBN218), the vector control plasmid was pCN416 and pAS2, and the high-copy number plasmid constitutively over-expressing *STN1* was pCN421 and pCN188.

*Transformation*

Wild type, *cdc7-1*, *dbf4-1*, and *mcm7-1* were grown on yeast extract peptone dextrose (YPD) plates at 23°C for 3 to 5 days. Colonies from the YPD plates were inoculated in 50 ml of liquid YPD and left in the shaker at 23°C overnight. Optical density (OD) of the overnight cell cultures was measured to make sure the cultures contained 1-2 x $10^7$ cells/ml ($OD_{600}$ = 0.3-0.5). Cells were harvested by centrifugation at 3000 rpm for 5 min at room temperature. The pellets were resuspended in 10 ml of sterile 1X TE buffer (10 mM of Tris and 1 mM of EDTA at pH 8.0). Resuspended cells were again pelleted by centrifugation at 3000 rpm for 5 min. The pellets were washed with 10 ml of sterile 1X TE/Lithium acetate (LiTE) solution (1X TE at pH 8.0 with 0.1mM lithium acetate) and then were gently resuspended in 0.5 ml of sterile 1X LiTE solution and incubated at room temperature for 30 min. 5 μL (1μg/μL) of carrier DNA and 2 μL (1μg/μL) of desired plasmids were added into 150 μL of the cell mixtures containing LiTE for another 30 min at room temperature. 0.7 mL of 50% (w/v) polyethylene glycol (PEG) was added into each cell mixture and allowed to incubate for an additional 30 min.

The final cell mixtures were heat shocked at 42°C for 15 min and incubated on ice for 10 min. The cooled cell mixtures were centrifuged at room temperature for 7-10 secs and the supernatant was removed sterilely. Pellets were resuspended in 150 μL of 1X TE buffer and plated onto appropriate selective medium according to the nutritional markers on the plasmids. All plates were incubated at 23°C until colonies appeared, usually 5 days.

*Assay for temperature sensitivity*

Single colonies from wild type [pAS2], wild type [pCN188], *mcm7-1* [pAS2], and *mcm7-1* [pCN188] were inoculated in 2 mL of SC –trp liquid medium. Cell cultures were grown for 4 to 6 days until saturation at 23°C. Ten-fold serial dilutions of the saturated cultures were stamped onto SC –trp plates and incubated at 23°C, 28°C, 30°C, 32°C, 34°C, and 36°C for 3 to 5 days.

*Plasmid stability assay- determining plasmid loss rate under non-selective conditions*

The percentage of cells that lose the plasmid per generation (X) was determined as follows: $X= 1-e^{r}$, where $r = \ln (R_f - R_i)/N$. N = number of generation in selective media and was calculated as [(log(final cell number)-log(initial cell number))/log2]. $R_f$ = percent plasmid retained at $N^{th}$ generation. $R_i$ = percent plasmid retained at start (Dani and Zakian 1983). $R_i$ and $R_f$ were calculated by first taking the difference between the number of colonies on YPD plates and number of colonies on SC –Ura plates and then divided by 100. A brief flow chart demonstrating the plasmid stability assay process is shown in figure 2.3. pJBN218 was cotransformed with either pCN416 or pCN421 into wild type, *cdc7-1*, *dbf4-1*, and *mcm7-1*, and the plasmids were selected by plating on SC –Leu, -Ura plates. A single colony from the transformation plates was struck onto

SC –Leu, -Ura plates to reduce the possibility of selecting cells that lacked the desired plasmids. A single colony from the streak out was inoculated into 5 ml of SC –Leu, -Ura liquid media overnight at 23°C. Next, using a hemocytometer to determine cell density, approximately $10^5$ cells from the overnight culture (initial cell number for calculating N) from the selective liquid media were inoculated into 10 ml of SC –Trp media. Thus, nutritional selection was maintained for pCN421, the *STN1* overexpression plasmid, but not for the experimental pJBN218 plasmid. This step was the starting point for monitoring the plasmid loss rate. To determine the fraction of cells containing the plasmid at this initial point, $R_i$, approximately 100 to 300 cells were plated onto YPD plates, and then incubated at 23°C. After 5 days of growth, these colonies were replica-plated onto SC –Ura or SC –Ade plates and to YPD plates. Cells that had already lost the CEN-ARS plasmid, and colonies that lost the plasmid within the first few divisions following plating, would score as Ura- or Ade-. Colonies that lost the CEN-ARS plasmid after 5-$7^{th}$ division could still have plenty of cells with the plasmid and would score as Ura+ or Ade+. Cell cultures in 10 ml SC-Leu media were grown 12 to 24 hours at their semi-permissive temperatures, 30°C for wild type, *dbf4-1*, and *mcm7-1* strains and 28°C for wild type and *cdc7-1* strains. After 12 to 24 hours, the experiment reached the end point for monitoring the plasmid loss rate, $R_f$.

The final density of the culture was determined using a hemocytometer and 100 to 300 cells were plated onto YPD plates. These YPD plates were incubated at 23°C for 5 days and were replica-plated onto SC –Ura plates and YPD plates to test for the presence of the experimental CEN-ARS plasmid. The number of cells that grew on the YPD plates

and SC –Ura plates would be used in calculating $R_f$.

*Statistical analysis*

Fluctuation analysis is designed to maximize the precision for estimating the mutation rate from the distribution of mutants (Pope et al. 2008). Luria and Delbrück first described the fluctuation analysis model for determining spontaneous mutation rate in bacteria (Luria and Delbrück, 1943). Lea and Coulson further extended the model for accommodating larger number of mutation events (Lea and Coulson, 1949). The general assumptions for the fluctuation analysis are 1) the cells are growing exponentially 2) mutation prior to the experiment is neglected 3) the probability of mutation per cell is constant 4) the growth rate for both non-mutants and mutants are the same 5) number of mutants is small than number of non-mutants 6) revertants are negligible 7) cell death is negligible 8) all mutants are detected 9) mutation happens after the experiment is neglected (Foster 2006). In addition to calculate mutation rate, fluctuation analysis has been used to calculate chromosome (or plasmid) loss rate (Runge 1991), and the "mutation" in the original fluctuation analysis is referred to as chromosome (or plasmid) loss. In this study, the percent plasmid-bearing cells and the percent of plasmids that are lost per generation were calculated by a modified fluctuation analysis with the method of median. The equation was $X= 1-e^r$, where $r = \ln (R_f - R_i)/N$ as described in the *plasmid stability assay* section. Since the result carried out by different colonies could vary greatly within one strain, I decided to use the method of median to minimize disproportional inflation caused by jackpot colonies.

Whiskers box plot including median and data range from minimum to maximum

was used for result presentation. The line within the box indicates the median value whereas the colored area below the median represents the first quartile showing the data range from 25 percentile to 50 percentile. The colored area above the median represents the third quartile, which shows the data range from 50 percentile to 75 percentile. The very top and very bottom lines outside the box represent the maximum value and minimum value of the data collected from the strain. Whiskers box plot from minimum to maximum and two-tailed unpaired student t test was performed using GraphPad Prism version 6.00 (Trail) for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

## Results

*Suppression of mcm7-1 Temperature Sensitivity by Overproducing Stn1p*

In the process of screening mutants to try to identify extragenic suppressors of the *STN1* induced sensitivity to HU, our lab found that *STN1* can act as a dosage suppressor of *cdc7-1*, *dbf4-1*, and *mcm7-1*. Over-expressed *STN1* increases the semi-permissive temperature of *cdc7-1* to 28°C and *dbf4-1* to 30°C (C.N. and H.G., data not shown). Here, we confirm that *mcm7-1* temperature sensitivity can be suppressed at 36°C by overexpressing *STN1*. (Figure 2.1) One speculation of the outcome could be that because overproduced Stn1p disrupts the S phase checkpoint and increases late origin firing events (Gasparyan et al. 2009), the promotion of origin firing helps to compensate for the defects in *cdc7-1, dbf4-1*, and *mcm7-1*. Another interpretation could be that overproduced Stn1p could improve replication machinery at genomic regions that were more susceptible in these mutants.

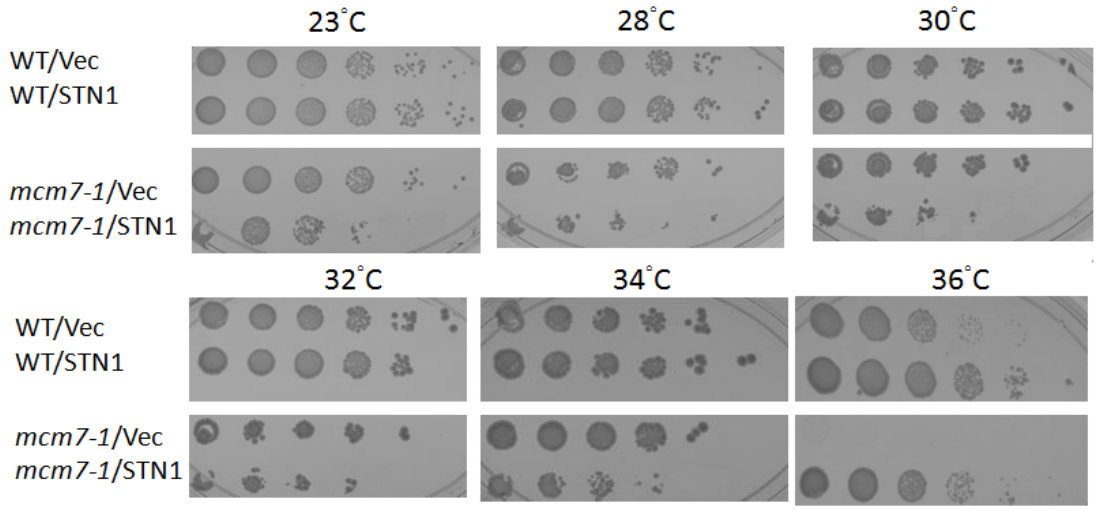


**Figure 2.1 *STN1* overexpression suppresses *mcm7-1* temperature sensitivity at 36°C**
Wild type (hC160) and *mcm7-1* (DBY2029) with vector plasmid (pAS2) or *STN1* overexpressing plasmid (pCN188) were grown to saturation in 2mls of YPD and 10-fold serial dilutions were performed. Strains were then plated onto YPD and placed at indicated temperatures for 4 days.

*Mitotic Stability Assay Was Used to Test if Overproduced Stn1p can Suppress the Mcm phenotype in cdc7-1 and mcm7-1*

Since we observed that overproduced Stn1p could increase the viability of the replication deficient mutants, *cdc7-1*, *dbf4-1*, and *mcm7-1*, it is possible that overproduced Stn1p could also suppress the Mcm phenotype carried by these mutants as we rationalized that overproduced Stn1p could improve replication directly or indirectly. In addition, our previous findings showed overproduced Stn1p could mislocalize to non-telomeric sites, implying that overproduced Stn1p could possibly improve replication directly or indirectly at regions away from telomeric sequences. To test this idea, I used a circular CEN-ARS plasmid that contains one centromere, one autonomous replication sequence, but lacking any telomeric sequence. (Figure 2.2) If excess Stn1p can enhance

the stability of the mini-chromosome (the CEN-ARS plasmid) in *cdc7-1*, *dbf4-1*, and/or *mcm7-1*, then overproduced Stn1p can be said to improve replication of non-telomeric DNA.

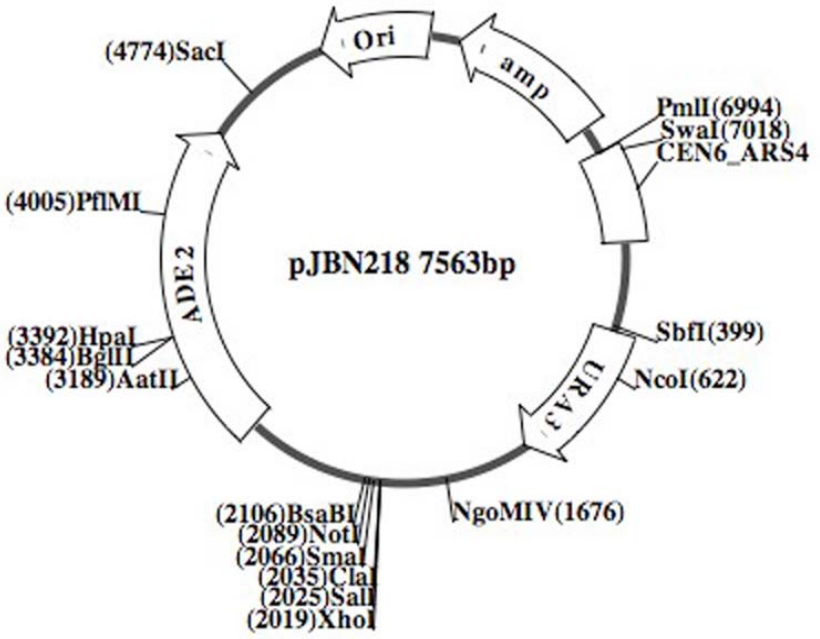


**Figure 2.2 Schematic drawing of pJBN218 (CEN-ARS plasmid)**
pJBN218 is a circular plasmid that consists of one centromere (CEN6), one autonomous replication sequence (ARSH4), one URA3 marker, and one ADE2 marker. This plasmid contains no telomere sequences, thus is named CEN-ARS plasmid.

A classic experiment for testing mini-chromosome stability inside the cells is the plasmid stability assay (PSA). PSA monitors the percentage of plasmid-bearing cells in a population under "nonselective conditions" (Ansari and Gartenberg 1997, Gehlen et al. 2011). Key parameters that will affect the results of this assay are plasmid replication efficiency and plasmid segregation efficiency. In PSA, there are two ways to examine which cell contains the tested plasmid or not. One way is to use selective medium with

selectable markers on the tested plasmid and another way is to introduce a colorimetric reporter into the cells.

The colorimetric technique was invented by Hieter and co-workers in 1985. The principle is to use an *ade2-101* mutant (a mutation that introduces an ochre stop codon prematurely in the *ADE2* ORF) that is defective in phosphoribosylaminoimidazole carboxylase (or 5AIR carboxylase) that catalyzes the sixth step in the *de novo* biosynthesis of purine nucleotides (Hieter et al. 1985). Such mutation will lead to the accumulation of purine precursors in the vacuole and result in red pigmentation of the colony. One way to revert the red colony back to white is to introduce *SUP11* (a $tRNA_{TYR}$ gene) that is an ochre mutation suppressor into the mutant cells where it can suppress the accumulation of red pigment from *ade2-101* strain. Another way for reversing the color is simply introducing the wild type *ADE2* gene back to the mutant cells.

In my experiments, I used both colorimetric and selectable markers to examine the number of plasmid containing cells. However the colorimetric assay became hard to interpret due to colony overlapping when the colony number exceed 250, thus I mainly use the selectable marker to examine the number of plasmid-bearing cells. In addition, the colorimetric reporter was confounded by the observation that white sectors seemed to develop more frequently than expected in red colonies. (This could be a result of cells losing mitochondrial function and becoming petites.) Nevertheless, the results calculated from the colorimetric assay were pooled together with the results calculated by using selectable marker.

A flow chart shows the detailed steps of the plasmid stability assay (Figure 2.3).

After transformation of the pJBN218 (CEN-ARS plasmid) together with either pCN416 (2μ plasmid encoding the ADH1 promoter and *LEU2* marker) or pCN421 (2μ plasmid expressing *STN1* from the ADH1 promoter, marked by *LEU2*), Ura+Leu+ colonies were struck onto new SC –ura, -leu plates to exclude dormant cells not bearing the desired plasmids (Figure 2.3 step 2).

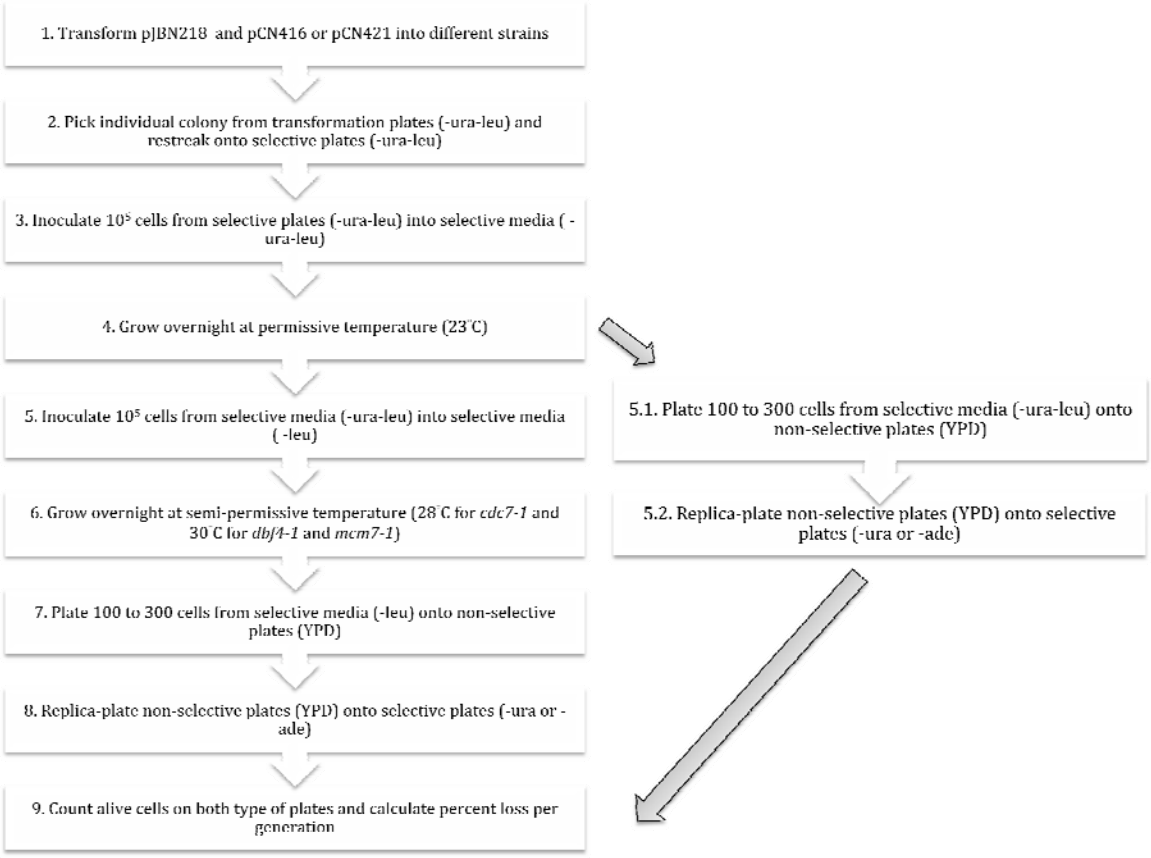


**Figure 2.3 Steps of plasmid stability assay**
pJBN218 (CEN-ARS-*URA3*) with pCN416 (2μ-*LEU2* vector) or pCN421 (2μ-*LEU2-STN1*) were first introduced into the yeast strain. Transformants were restreaked onto selective plates (SC –Ura, -Leu) to prevent choosing dormant cells without bearing the desired plasmids. Cells were then grown overnight at 23°C in selective medium (SC –Ura, -Leu) and inoculated into non-selective medium for the CEN-ARS plasmid (SC –Leu) at 28°C (for *cdc7-1*) or 30°C (for *dbf4-1* and *mcm7-1*) overnight. 100 to 300 cells from the selective medium (SC –Ura, -Leu) were plated onto YPD plates and replica platted onto selective plates (SC –Ura or SC-Ade). 100 to 300 cells from the non-selective medium

for the CEN-ARS plasmid (SC -Leu) were plated onto YPD plates and replica platted onto selective plates (SC –Ura or SC-Ade). Colonies bearing the CEN-ARS plasmid were calculated according to the formula presented by Dani and Zakian. (Dani and Zakian 1983)

Determination of the percent of cells maintaining the plasmid at the start of the experiment ($R_i$) was done under "selective condition" in SC –Leu, -Ura media to insure that cells retain the CEN-ARS plasmid and either pCN416, pCN421, pAS2, or pCN188. This step was monitored under permissive temperature for growth at 23°C. The cells were then allowed to grow in liquid media for a period without nutritional selection for the CEN-ARS minichromosome. The cultures were incubated at semi-permissive temperature where mutant defects should be triggered or enhanced, and nutritional selection was maintained for the vector or *STN1* overexpression plasmid. The percent of cells maintaining the CEN-ARS plasmid at the end of the experiment ($R_f$) and approximate number of generations the cells underwent in nonselective culture were determined; the loss rate of the CEN-ARS minichromosme was then calculated for each culture.

*Preliminary Results*

Before taking a large sample set, four independent colonies from each strain were tested in the preliminary experiment. Each colony was tested twice. Demonstration of PSA with colorimetric assay was shown in Figure 1.4. As mentioned before, the timing of testing the CEN-ARS plasmid stability was within the growth period in SC –Leu liquid medium under non-selective condition for the CEN-ARS plasmid. Thus, cells that lose the CEN-ARS plasmid during that period would grow into red colonies on YPD plates, as

*ADE2* on the CEN-ARS plasmid was lost. On the other hand, if the cells retained the CEN-ARS plasmid, they would grow into white colonies. (Figure 2.4) The color sectoring of the colonies was due to the loss of the CEN-ARS plasmid after plating onto YPD plates. (Figure 2.4 C) For example, cell that loss the CEN-ARS plasmid only after the first mitosis event would result in ½ white and ½ red colony. Thus, both white and sectoring colonies were considered as bearing the CEN-ARS plasmid whereas red colonies were considered to have lost the CEN-ARS plasmid.

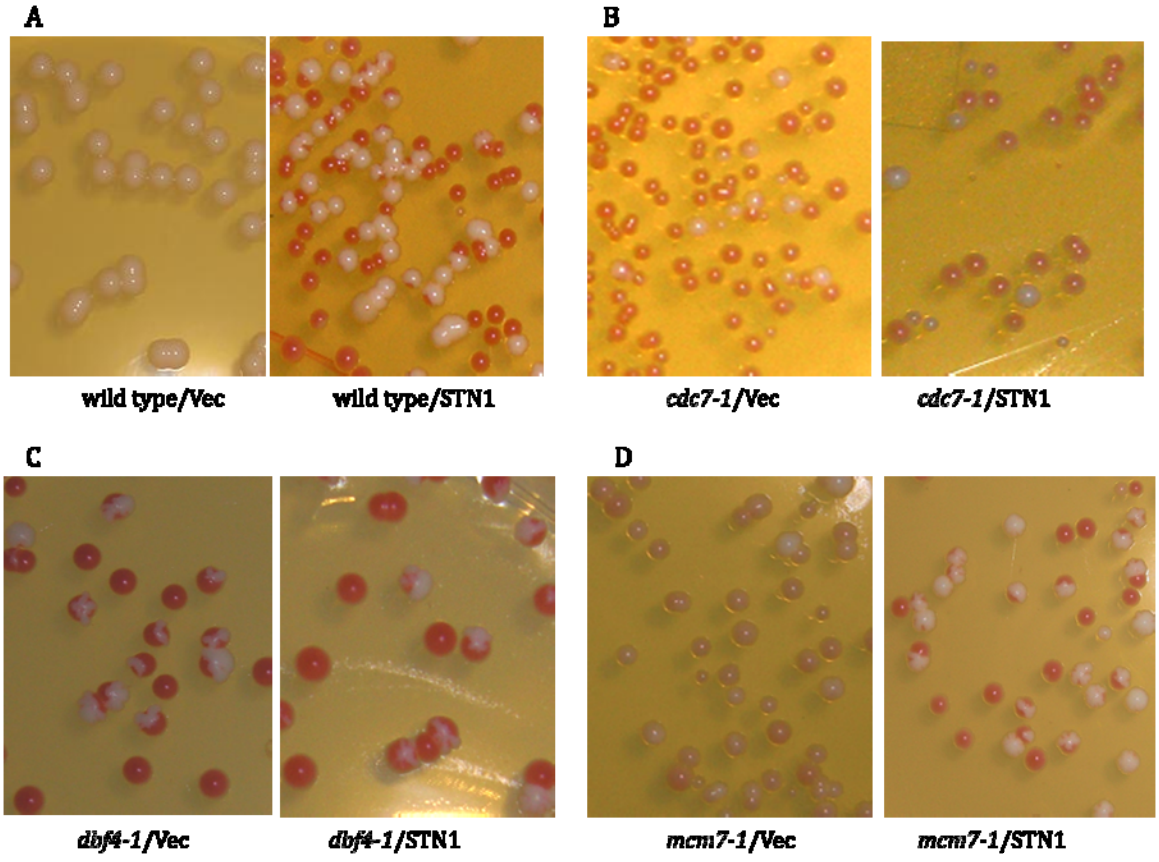


**Figure 2.4 Pictures of plasmid stability assay with color metric.**
Pictures were taken from the YPD plates that were before replica-platted onto SC –Leu plates after growing under non-selective conditions. All YPD plates were incubated at 23°C for 5 days. Vec= pCN416 and STN1= pCN421. (A) Left: wild type cells bearing vector. Right: wild type cells bearing STN1 overexpressing plasmid. (B) Left: *cdc7-1* bearing vector. Colonies in pink would turn into solid red colonies as pigmentation

accumulates overtime. White= cells bearing the CEN-ARS plasmid. Red= Cells without the CEN-ARS plasmid. Right: *cdc7-1* bearing STN1 overexpressing plasmid. (C) Left: *db4-1* bearing vector. Right: *db4-1* bearing STN1 overexpressing plasmid. (D) Left: *mcm7-1* bearing vector. Colonies in pink would turn into solid red colonies as pigmentation accumulated overtime. Right: *mcm7-1* bearing STN1 overexpressing plasmid.

The loss rate calculated here was higher than reported value for *cdc7-1* (5.2% average plasmid loss rate) bearing a CEN-ARS plasmid (with ARS306) at 26.5°C (Donaldson et al. 1998). However, unlike our experiment, the reported value was determined by quantifying the signal on a Southern blot and was tested at 26.5°C instead of 28°C. Besides the *cdc7-1* results, previously reported plasmid loss rate with CEN-ARS plasmid in *dbf4-1* (7.5%) and *mcm7-1* (5%) were slightly different from my calculations, 10% plasmid loss rate for *dbf4-1* and 10% for *mcm7-1* (Donato et al. 2006 and Homesley et al. 2000). The reported value for *dbf4-1* was using a CEN-ARS plasmid (with ARS1010) and was tested at 30°C. The CEN-ARS plasmid used in the report for *mcm7-1* contained ARS121 and the experiments were carried out at 30°C as well. Nevertheless, the reported values could not be used to directly compare with my results as the ARS elements and experimental conditions were different.

Although the sample size was small, the result revealed that overexpressed *STN1* could possibly act as a dosage suppressor of the plasmid instability in *cdc7-1* and *mcm7-1*. The student paired T test (two tailed) showed the significance of this result as the P value for *cdc7-1* with vector (median plasmid loss rate, 23%) or with pCN421 (median plasmid loss rate, 15%) was 0.0049 and for *mcm7-1* with vector (median plasmid loss rate, 10%) or with pCN421 (median plasmid loss rate, 1%) was 0.0009. (Figure 2.5) No significant

suppression was detected in *dbf4-1* [pCN421]. After seeing the enhancement of the CEN-ARS plasmid stability in *cdc7-1* and *mcm7-1* by overproducing Stn1p and the fluctuation of the data, I therefore performed the same experiment with a larger set of samples.

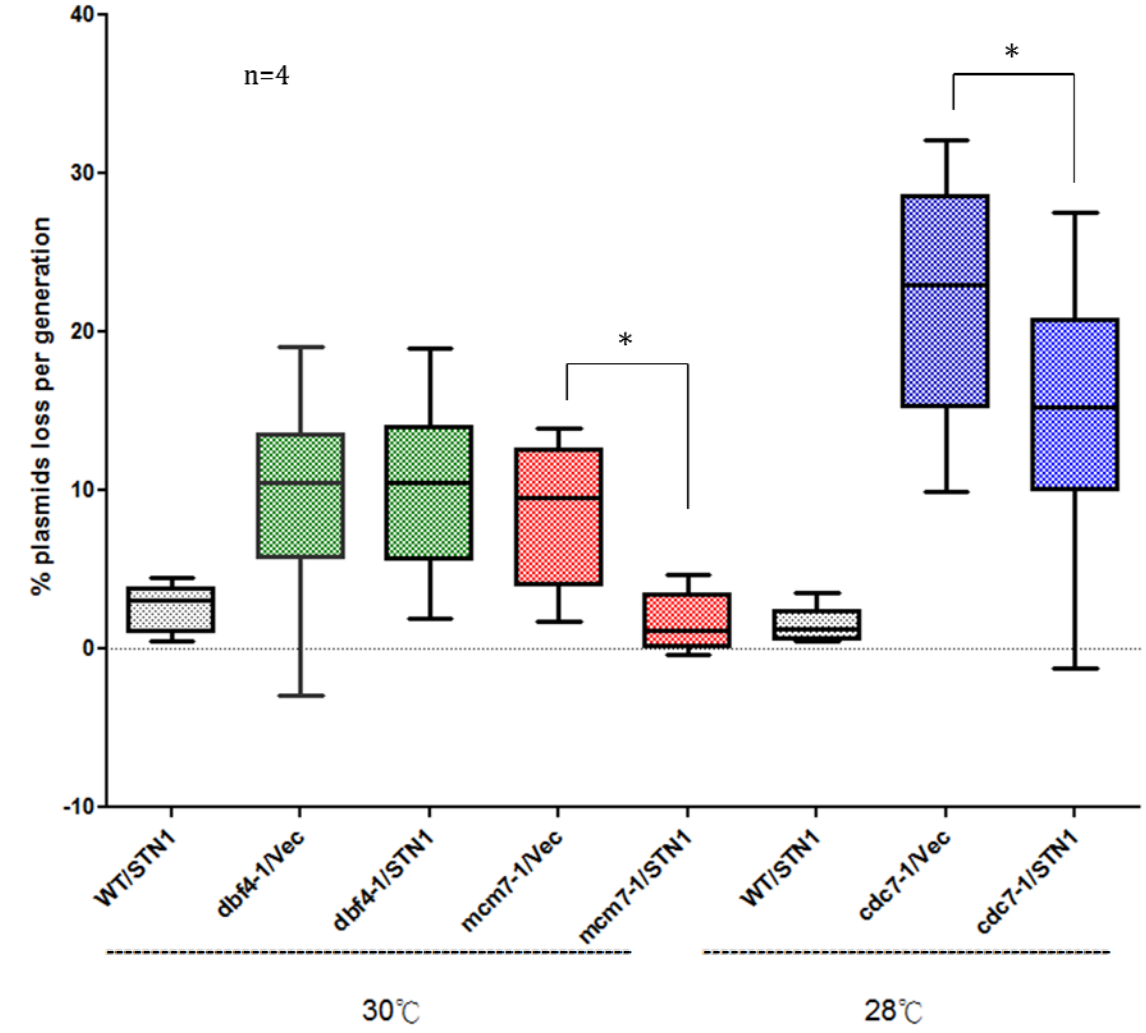


**Figure 2.5 Preliminary test of minichromosome stability in replication mutants.** The percent loss rate of the CEN-ARS plasmid (pJBN218) was tested following overnight growth of cells under selective conditions (5 mls of liquid SC-Leu –Ura) at 23°C in wild-type (hC160), *cdc7-1* (hC2403), *dbf4-1* (JBY999), and *mcm7-1* (DBY2029) strains. Cultures were then grown under nonselective conditions for the CEN-ARS plasmid (10 mls of SC -Leu) at 30°C for 1-2 days. (The CEN-ARS plasmid is marked by *URA3* and *ADE2*.) The percent of cells in the culture that were Ura+ or Ade+ following the growth in non-selective media was determined. Colonies tested in the wild-type, *cdc7-1, dbf4-1,* and *mcm7-1* alleles were each from one transformation plate. No pooled

data were used in the wild-type. (WT= wild-type)
n= number of independent colony tested
* = Indicating the result was significant.
P value for *mcm7-1*/Vec and *mcm7-1*/STN1 is 0.0009
P value for *cdc7-1*/Vec and *cdc7-1*/STN1 is 0.0049

*Plasmid Stability Assay Testing Dosage Suppression of the MCM phenotype by STN1 overexpression.*

Colonies from four different transformations were tested for each strain. Number of colonies tested for each strain was indicated in Figure 2.6. Fluctuation analysis looking at median value was used since the results vary for each strain due to jackpot colonies (such as revertants). The differences would mislead the interpretation if the average of the results were practiced. The number of total independent colonies tested is summarized in Table 2.1. The results were calculated from pooled data with strains containing either pCN416 or pCN421 and pAS2 or pCN188. Results obtained from these different *STN1* overexpressing plasmids were not significantly different. (Figure 2.7)

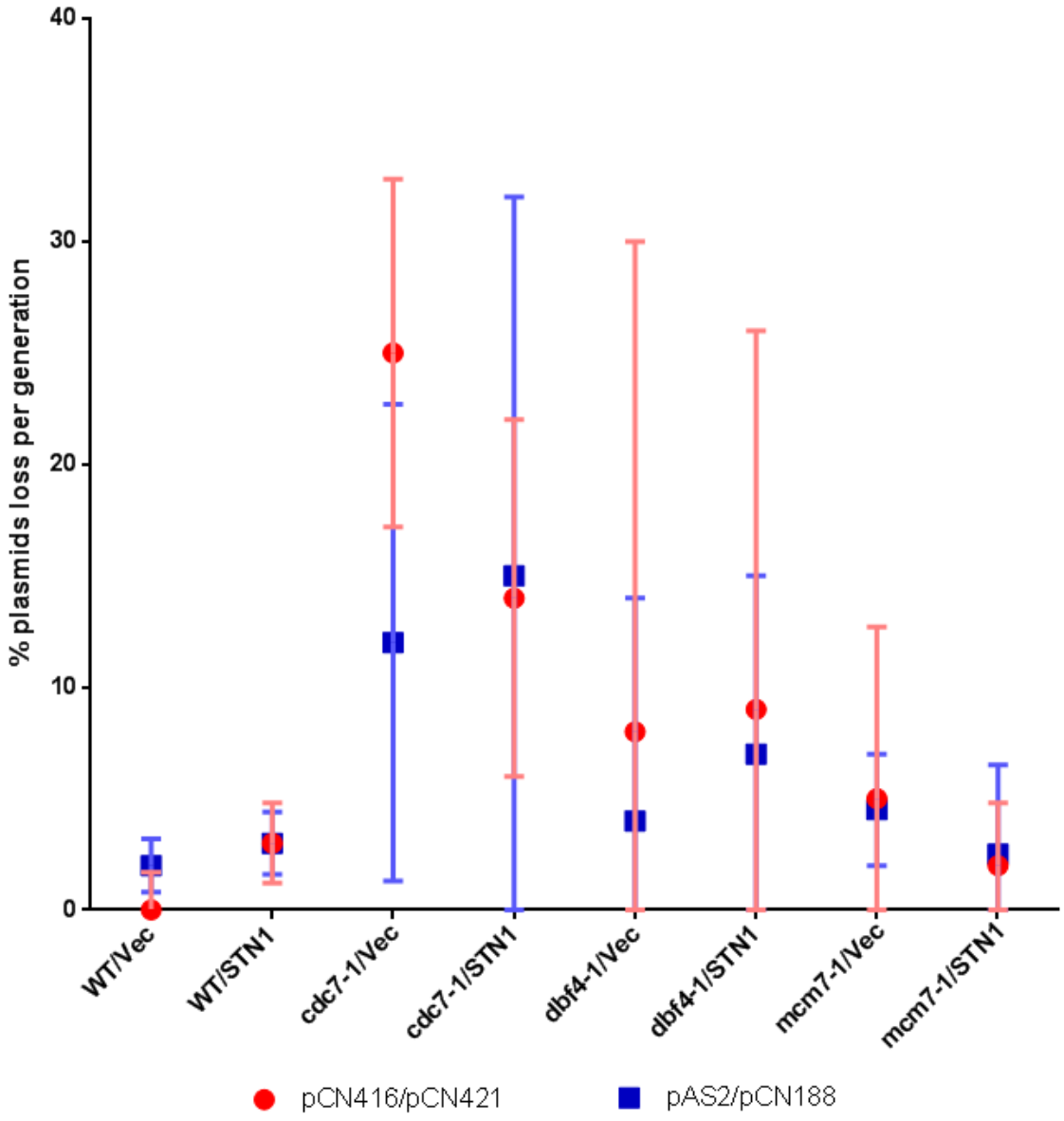


**Figure 2.7 Comparing percent plasmids loss rate between cells bearing pCN416 or pCN421 and pAS2 or pCN188**

The percent plasmids loss rate of the CEN-ARS plasmid (pJBN218) was tested following overnight growth of cells under selective conditions (5 mls of liquid SC-Leu –Ura) at 23°C in wild-type (hC160), *cdc7-1* (hC2403), *dbf4-1* (JBY999), and *mcm7-1* (DBY2029) strains. Cultures were then grown under nonselective conditions for the CEN-ARS plasmid (10 mls of SC –Leu for cells bearing pCN416 or pCN421 and 10 mls or SC –Trp for cells bearing pAS2 or pCN188) at 30°C for 1-2 days. The percent of cells in the culture that were Ura+ or Ade+ was determined. Colonies tested in the wild-type, *cdc7-1, dbf4-1,* and *mcm7-1* alleles were each from four transformation plates. (WT= wild-type)

The PSA results again indicated that overproduced Stn1p could significantly enhance the stability of the CEN-ARS plasmid in *mcm7-1* and *cdc7-1* (Figure 2.5). The P value from two-tailed student T test was 0.0091 for *mcm7-1* [Vector] (median plasmid loss rate, 5%) and *mcm7-1* [*STN1*] (median plasmid loss rate, 2%). The p value from two-tailed student T test was <0.0001 for *cdc7-1* [Vector] (median plasmid loss rate, 25%) and *cdc7-1* [*STN1*] (median plasmid loss rate, 14%). Not too surprising, overproducing Stn1p decreased the stability of CEN-ARS plasmid in wild-type cells. No significant difference was observed at 28°C, wild type [Vector] had 0% plasmid loss rate and wild type [*STN1*] had 1% plasmid loss rate. However, the difference was significant at 30°C, wild type [Vector] had 1% loss rate and wild type [*STN1*] had 3% loss rate.

As expected, the standard deviations were high for all strains because of the fluctuation of the data collected (Table 2.1). As the sample size increased, the results obtained were more similar to the published values; however, no direct comparison could be made because the experimental conditions were somewhat different.

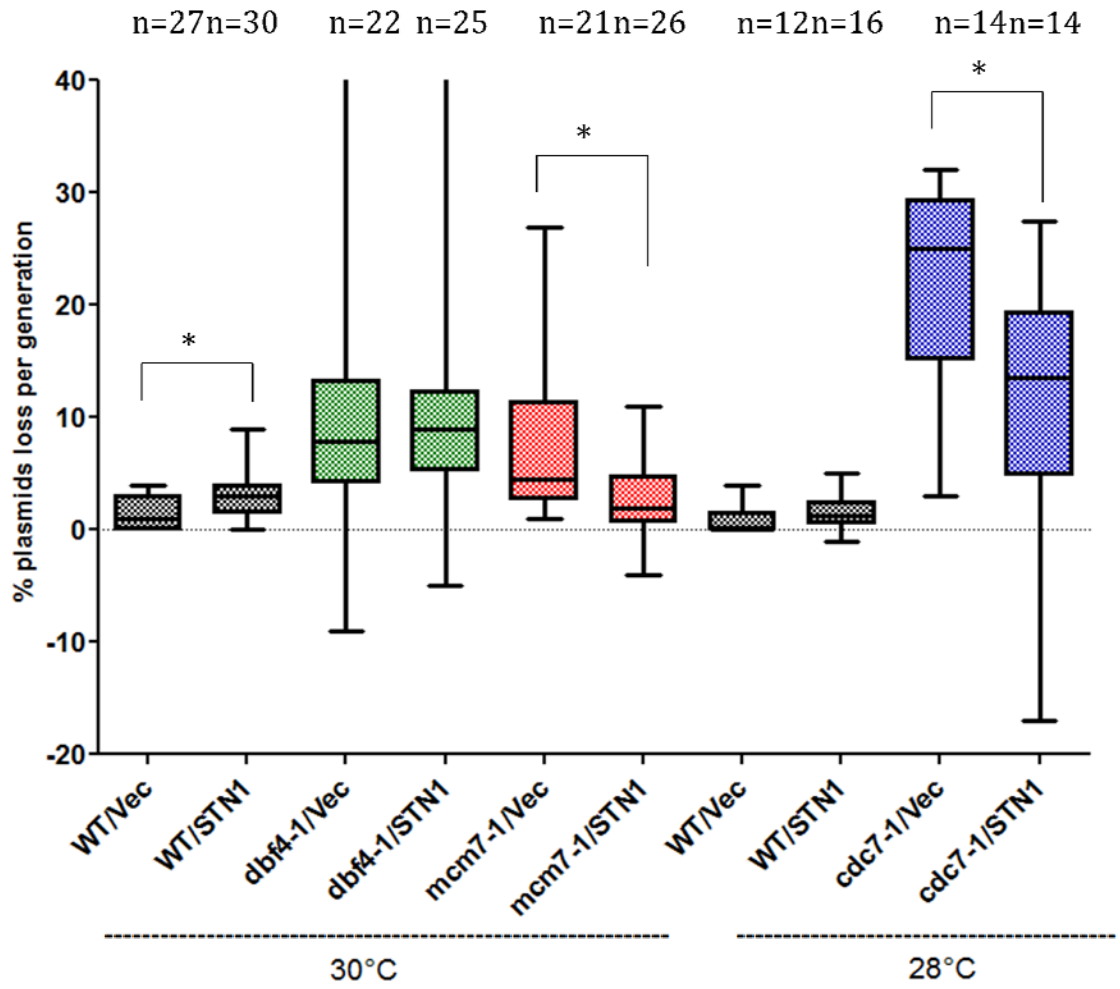


**Figure 2.6 Overexpression of *STN1* suppresses the MCM phenotype in *cdc7-1* and *mcm7-1*.**

The loss rate of the CEN-ARS plasmid (pJBN218) was tested following overnight growth of cells under selective conditions (5 mls of liquid SC-Leu –Ura) at 23°C in wild-type (hC160), *cdc7-1* (hC2403), *dbf4-1* (JBY999), and *mcm7-1* (DBY2029) strains. Cultures were then grown under nonselective conditions for the CEN-ARS plasmid (10 mls of SC –Leu for cells bearing pCN416 or pCN421 and 10 mls or SC –Trp for cells bearing pAS2 or pCN188) at 30°C for 1-2 days. The percent of cells in the culture that were Ura+ or Ade+ and the number of cell generations in the culture over this time were determined. Colonies tested in the wild-type, *cdc7-1, dbf4-1,* and *mcm7-1* alleles were each from four transformation plates. (WT= wild-type)
n= number of independent colony tested
* = Indicating the result was significant.
P value for WT/Vec and WT/ STN1 is 0.0007

P value for *mcm7-1*/Vec and *mcm7-1*/STN1 is 0.0091
P value for *cdc7-1*/Vec and *cdc7-1*/STN1 is <0.0001

**Table 2.1 Summary of the *STN1* overexpression results**

| | | Number of generation (Average) | | Number of independent colony tested | | Number of colonies counted/plate (Average) | | CEN-ARS plasmid loss rate (Median) | | Standard deviation | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | Temperature | Vector | OP STN1 | Vector | OP STN1 | Vector | OP STN1 | Vector | OP STN1 | Vector | OP STN1 |
| WT | 30°C | 10 | 10 | 27 | 30 | 301 | 228 | 1% | 3% | 2% | 2% |
| *dbf4-1* | 30°C | 6 | 7 | 22 | 25 | 250 | 166 | 8% | 9% | 22% | 16% |
| *mcm7-1* | 30°C | 9 | 9 | 21 | 26 | 146 | 180 | 5% | 2% | 7% | 3% |
| WT | 28°C | 9 | 9 | 12 | 16 | 340 | 418 | 0% | 1% | 1% | 1% |
| *cdc7-1* | 28°C | 5 | 6 | 14 | 14 | 339 | 291 | 25% | 14% | 8% | 10% |

Average number of generation, number of independent colony tested, average number of colonies counted/plate, median percent plasmids loss rate, and standard deviation were shown. Wild type (hC160), *cdc7-1* (hC2403), *dbf4-1* (JBY999), and *mcm7-1* (DBY2029) were used here. Vector = pCN416 or pAS2. OP STN1 = pCN421 or pCN188.

**Discussion**

The role of Stn1p is suggested to maintain telomere integrity by capping the telomeres and by promoting complementary C-strand synthesis. In addition, our lab previously showed overproduced Stn1p could associate with non-telomeric chromosome regions (Gasparyan et al. 2009). Furthermore, overproduced Stn1p increased the viability of *cdc7-1, dbf4-1,* and *mcm7-1* at their semi-permissive temperatures. My results here reinforce the observation of non-telomeric association of increased Stn1p levels under replication stress by testing the CEN-ARS plasmid stability in *cdc7-1, dbf4-1,* and *mcm7-1*. Together, my results suggest that overproduced Stn1p can act away from telomeres and perhaps facilitate replication machinery directly or indirectly, and such facilitation might require certain stress levels within the cells.

The dosage suppression of temperature sensitive mutants by overexpressing *STN1* could lead to at least two interpretations. One, overproduced Stn1p could simply improve replication machinery at genomic regions that are more susceptible to the replication stress in these mutants. Thus, increased viability of these mutants at their semi-permissive temperatures. However, how overproduced Stn1p improves replication remains unknown. Another interpretation is that loss of the S phase checkpoint allows viability of these replication deficient mutants. This interpretation is in agreement with the data showing *mrc1Δ*, *mrc1-3a*, and *cds1Δ* strains can bypass *HSK1* requirement (Matsumoto et al. 2011). *MRC1* and *CDS1* are both involved in S phase checkpoint as Mrc1p is the checkpoint mediator and Cds1p is the checkpoint kinase. *HSK1* is the fission yeast homologue of *S. cerevisiae CDC7*. The bypass of *hsk1Δ* might be due to a reduced

number of replication fork arrests and increased firing of dormant origins in these S phase checkpoint deficient mutants (Matsumoto et al. 2011). In addition, published data from our lab demonstrated that overexpressed *STN1* under HU or MMS treatment can interfere with different aspects of the S phase checkpoint such as causing spindle extension, inappropriately firing late origins, and destabilizing replication fork progression (Gasparyan et al. 2009). Therefore, the increased viability of *cdc7-1, dbf4-1,* and *mcm7-1* could be because of increased origin firing efficiency due to overproduced Stn1p interfered with the normal S phase checkpoint function and caused more firing of the dormant origins.

To show that high levels of Stn1p could act away from telomeric regions under stress conditions, and to have more understanding of the mechanism by which overproduced Stn1p could improve replication, I tested CEN-ARS plasmid stability in *cdc7-1, dbf4-1,* and *mcm7-1* with overexpression of *STN1*. The results confirmed our hypothesis that overproduced Stn1p could improve replication directly or indirectly at non-telomeric region as the CEN-ARS plasmid stability was significantly increased in *cdc7-1* and *mcm7-1* after overproducing Stn1p. Since the CEN-ARS plasmid contained no telomeric sequences, the enhanced CEN-ARS plasmid stability in *cdc7-1* and *mcm7-1* strongly suggested that high level of Stn1p could act away from telomeres. How does overproduced Stn1p improve the stability of the CEN-ARS plasmid? One idea was that, since Stn1p could interact with Pol12p (Grossi et al. 2004), overproduced Stn1p could improve the association of Polα with the replication fork, thus increased fork progression efficiency or priming efficiency. Another interpretation was that, Stn1p is structurally

similar to Rpa2p and the CST complex is also structurally and functionally similar to RPA complex (Miyake et al. 2009), thus overproduced Stn1p could interact with RPA components and further improve replication machinery by increasing and/or stabilizing the association of RPA complex with the replication fork. Lastly, *cdc7-1* and *mcm7-1* could cause replication stress, thus activating the S phase checkpoint. High levels of Stn1p disrupt the S phase checkpoint by increasing the efficiency of origin firing, and eventually enhanced the CEN-ARS plasmid stability.

On the other hand, overproduced Stn1p decreased the CEN-ARS plasmid stability in the wild type. This could be due to the S phase checkpoint interruption by overproducing Stn1p. Since wild type cells do not undergo much replication stress, the disruption of natural occurring S phase checkpoint in the wild type cells then became a problem upon maintaining the CEN-ARS plasmid. Although origin firing might be more efficient, high levels of Stn1p also resulted in a long spindle extension phenotype and decreased the fork stability. Increasing origin firing efficiency could not compensate for these other S phase checkpoint defects, thus decreased the CEN-ARS plasmid stability in the wild type cells. Another speculation could be that, overexpression of *STN1* is causing other difficulties independent of its checkpoint disruption.

Unexpectedly, overproducing Stn1p did not increase or decrease the CEN-ARS plasmid stability in *dbf4-1*. Since Dbf4p and Cdc7p work in a complex to fire the origin by phosphorylating the MCM complex, the CEN-ARS plasmid stability should also be increased in the *dbf4-1* with high levels of Stn1p. Since the results in *dbf4-1* fluctuated in a much broader range than other alleles, we could not rule out the possibility that

overproduced Stn1p could still enhance the CEN-ARS plasmid stability. Nevertheless, since overproducing Stn1p had no effect on *dbf4-1*, one speculation could be that, the defects caused by both *dbf4-1* itself and the S phase checkpoint interruption was balanced by increased origin firing efficiency.

It would be interesting to test the speculations regarding enhancing the CEN-ARS plasmid stability in *cdc7-1* and *mcm7-1*. To test if an increased Stn1p level improves replication via Polα, we could test the CEN-ARS plasmid stability in *pol12-40 cdc7-1* and *pol12-40 mcm7-1* double mutants with overexpression of *STN1*. To test the RPA complex association with Stn1p, we could try to co-immunoprecipitate Stn1p with Rpa1p or Rpa3p. If we could pull down both Stn1p and Rpa1p or Stn1p and Rpa3p, it would suggest overproducing Stn1p could improve replication via acting as one of the Rpa complex. To test the idea of increasing Stn1p level under replication stress will increase origin firing, we could use BrdU-IP-chip to examine the origin firing efficiency in *cdc7-1* and *mcm7-1* with or without overproducing Stn1p.

In sum, my results strongly suggested that Stn1p, when overproduced, could act away from telomeric sequences in stabilizing the CEN-ARS plasmid in *cdc7-1* and *mcm7-1* but by an unknown mechanism(s). A likely explanation is that OP Stn1 facilitates origin firing. However, such facilitation is delicate and sensitive to small perturbation due to the observation that overproducing Stn1p could increase viability of the replication deficient mutants but could also become harmful to cells when there is no replication stress.

## Chapter 3

## Testing the stability of a telomere-containing plasmid

### Introduction

From the results showing *STN1* overexpression can suppress the MCM phenotype in the replication deficient mutants with the CEN-ARS plasmid, several hypotheses could be made. 1) Over-expressed *STN1* could help to promote replication at non-telomeric sequences on the plasmid. 2) Increased *STN1* dosage improves plasmid segregation. 3) *STN1* over-expression interferes with the S phase checkpoint in a way that allows these replication deficient mutants to be more likely to complete replication of the CEN-ARS plasmid. In this chapter, I will be examining hypotheses 1 and 2 and will try to elaborate on hypotheses 1 to understand how *STN1* over-expression could improve general replication.

If over-expressed *STN1* could help to improve replication of the CEN-ARS plasmid, then it should be more likely to improve replication of a TEL-ARS plasmid since *STN1* is important for telomere maintenance. Thus, if the *STN1* function is compromised, we should expect to see low TEL-ARS plasmid stability. In addition to *STN1*, *TEN1* is also hypothesized to participate in the fill-in mechanism at the telomere. Within the same idea, *ten1* mutant might also reduce the TEL-ARS plasmid stability. Hence, I will be testing the TEL-ARS plasmid stability in CST mutants, *stn1-281t* and *ten1-105*.

The observation from chapter 1 demonstrates that *STN1* overexpression does not improve the CEN-ARS plasmid in the wild-type. However, it is possible that

overexpressed *STN1* could help to maintain the TEL-ARS plasmid stability because the normal role for *STN1* is to help maintain and protect telomeres. If *STN1* overexpression could help to maintain the TEL-ARS plasmid in the wild-type cells, it could suggest that either telomere replication or segregation is improved.

If overexpressed *STN1* does help to improve general replication, it would be interesting to identify at which step of the replication machinery does overexpressed *STN1* act on. To solve this problem, I will be comparing the results of the CEN-ARS and the TEL-ARS plasmid stability in the replication deficient mutants, *cdc7-1*, *dbf4-1*, and *mcm7-1*. *cdc7-1*and *dbf4-1*are thought to have defects in initiation of DNA replication and *mcm7-1* is thought to have defects in both initiation and elongation step of the replication machinery (Heller et al. 2011; Pospiech et al. 2010; Tye 1999a; Bochman and Schwacha 2009). Therefore, if *STN1* overexpression can enhance the plasmid stability of both the CEN-ARS and the TEL-ARS plasmids in the *cdc7-1*and *dbf4-1*alleles, it would suggest that *STN1* overexpression is helping to initiate DNA replication. If the plasmid stability for both plasmids is enhanced in the *mcm7-1* allele, it would suggest that *STN1* overexpression is more likely to help the elongation step of the DNA replication. If the plasmid stability for both plasmids is enhanced in all three alleles, then it would suggest that *STN1* overexpression is perhaps act to promote the initiation step of the DNA replication because it is the common defect in all three mutants. In addition, we can also separate out the possibility of *STN1* overexpression in helping segregating the CEN-ARS plasmid if both plasmids' stability could be improved in the same mutant because the segregation mechanism is different in between the CEN-ARS plasmid and the TEL-ARS

plasmid.

Finally, if we observe that overexpressed *STN1* is improving the TEL-ARS plasmid stability, it is still possible that the segregation of the TEL-ARS plasmid is enhanced by *STN1* overexpression. Thus, I will be testing the TEL-ARS plasmid stability in the telomere segregation deficient mutants, *sir4Δ* and *rap1-5*. If *STN1* overexpression helps to maintain the TEL-ARS plasmid in these mutants, then it would suggest overexpressed *STN1* plays a role in facilitating the telomere segregation. If no enhancement is observed, then the result might be inconclusive but still hint a possibility that *STN1* overexpression does not participate in telomere segregation.

## Material and Methods

*Cell culture and transformations*

Wild-type, *cdc7-1*, *dbf4-1*, *mcm7-1*, *stn1-281t*, *ten1-105*, *sir4Δ,* and *rap1-5* strains were grown on yeast extract peptone dextrose (YPD) plates at 23°C for 5 days. Colonies from the YPD plates were inoculated in 50 ml of liquid YPD and left in the shaker at 23°C overnight. Optical density (OD) of the overnight cell cultures was measured to make sure the cultures contained 1-2 x $10^7$ cells/ml ($OD_{600}$ = 0.3-0.5). Cells were harvested by centrifugation at 3000 rpm for 5 min at room temperature. The pellets were resuspended in 10 ml of sterile 1X TE buffer (10 mM of Tris and 1 mM of EDTA at pH 8.0). Resuspended cells were again pelleted by centrifugation at 3000 rpm for 5 min. The pellets were washed with 10ml of sterile 1X TE/Lithium acetate (LiTE) solution (1X TE at pH 8.0 with 0.1mM lithium acetate) and then were gently resuspended in 0.5ml of sterile 1X LiTE solution and incubated at room temperature for 30 min. 5 μL (1μg/μL) of carrier DNA and 2 μL (1μg/μL) of desired plasmids were added into 150μL of the cell mixtures containing LiTE for another 30min at room temperature. 0.7 mL of 50% (w/v) polyethylene glycol (PEG) was added into each cell mixture and allowed to incubate for an additional 30min. The final cell mixtures were heat shocked at 42°C for 15min and incubated on ice for 10min. The cooled cell mixtures were centrifuged at room temperature for 7-10secs and the supernatant was removed sterilely. Pellets were resuspended in 150μL of 1X TE buffer and plated onto appropriate selective medium according to the nutritional markers on the plasmids. All plates were incubated at 23°C until colonies appeared, usually 5 days.

*Plasmids and Strains*

Plasmids and yeast strains used are shown in Apendix III, respectively. Wild-type (hC160), *cdc7-1* (hC2403), *dbf4-1* (JBY999), *mcm7-1* (DBY2029), *stn1-281t* (hC671), *ten1-105* (hC2241), *sir4Δ* (hC1654), and *rap1-5* (YJB208) were co-transformed with the desired plasmids. The test mini-chromosome plasmid was the TEL-ARS plasmid (YRpRW41), the vector control plasmid was pAS2, and the high-copy number plasmid constitutively over-expressing *STN1* was pCN188.

*Plasmid stability assay- determining plasmid loss rate under non-selective conditions*

The percentage of cells that lose the plasmid per generation (X) was determined as follows: $X= 1-e^r$, where $r = \ln (R_f - R_i)/N$. N = number of generation in selective media and was calculated as [(log(final cell number)-log(initial cell number))/log2]. $R_f$ = percent plasmid retained at $N^{th}$ generation. $R_i$ = percent plasmid retained at start (Dani and Zakian 1983). $R_i$ and $R_f$ were calculated by dividing number of colonies on SC –Leu plates with number of colonies on YPD plates followed by multiplying with 100%. Single colonies from transformation plates were each inoculated into 5 ml of SC –Leu, -Trp liquid media overnight at 23°C. Next, using a hemocytometer to determine cell density, approximately $10^5$ cells from the overnight culture (initial cell number for calculating N) from the selective liquid media were inoculated into 10 ml of SC -Trp media. Thus, nutritional selection was maintained for pCN188, the *STN1* over-expression plasmid that encodes *TRP1*, but not for the experimental YRpRW41 plasmid, which encodes *LEU2*. This step was the starting point for monitoring the plasmid loss rate. To determine the fraction of cells containing the plasmid at this initial point, $R_i$,

approximately 100 to 300 cells were plated onto YPD plates, and then incubated at 23°C. After 5 days of growth, these colonies were replica-plated onto SC –Leu plates and to YPD plates. Cells that had already lost the YRpRW41 plasmid, and colonies that lost the plasmid within the first few divisions following plating, would score as Leu-. Colonies that lost the YRpRW41 plasmid after 5-7$^{th}$ division could still have plenty of cells with the plasmid and would score as Leu+.

To test the YRpRW41 stability in *stn1-t281* and *ten1-105* strains, transformed cells were inoculated in 5 ml SC -Leu media and grown overnight at 23˚C. Overnight cultures were then grow under nonselective conditions (10 mls of liquid YPD) at 30°C for 1-2 days. After 1-2 days, the experiment reached the end point for monitoring the plasmid loss rate, $R_f$. The final density of the culture was determined using a hemocytometer and 100 to 300 cells were plated onto YPD plates. These YPD plates were incubated at 23°C for 5 days and were replica-plated onto SC –Leu plates and YPD plates to test for the presence of the experimental TEL-ARS plasmid. The number of cells that grew on the YPD plates and SC –Leu plates would be used in calculating $R_f$.

*Plasmid stability assay- retention of YRpRW41 in cells under selective conditions*

Single colonies from strains transformed with YRpRW41 were streaked on SC-Leu plates, and then single colonies that grew on these plates were each inoculated into 1 ml of sterilized $dH_2O$. Next, using a hemocytometer to determine cell density, approximately 100-300 cells were plated onto YPD plates, and then incubated at 23°C. After colonies grew up (usually 5 days), these colonies were replica-plated onto SC –Leu plates and to YPD plates. Cells that had already lost the YRpRW41 plasmid, and

colonies that lost the plasmid within the first few divisions following plating, would score as Leu-. Colonies that lost the YRpRW41 plasmid after 5-7$^{th}$ division could still have plenty of cells with the plasmid and would score as Leu+. The number of cells grew on the YPD plates and SC –Leu plates would be used in calculating percent of plasmid-bearing cells, which is number of colonies on SC –Leu plates divided by number of colonies on YPD plates times 100%.

*Statistical analysis*

Fluctuation analysis is designed to maximize the precision for estimating the mutation rate from the distribution of mutants (Pope et al. 2008). Luria and Delbruck first described the fluctuation analysis model for determining spontaneous mutation rate in bacteria (Luria and Delbruck, 1943). Lea and Coulson further extended the model for accommodating larger number of mutation events (Lea and Coulson, 1949). The general assumptions for the fluctuation analysis are 1) the cells are growing exponentially 2) mutation prior to the experiment is neglected 3) the probability of mutation per cell is constant 4) the growth rate for both non-mutants and mutants are the same 5) number of mutants is small than number of non-mutants 6) revertants are negligible 7) cell death is negligible 8) all mutants are detected 9) mutation happens after the experiment is neglected (Foster 2006). In addition to calculate mutation rate, fluctuation analysis has been used to calculate chromosome (or plasmid) loss rate (Runge 1991), and the "mutation" in the original fluctuation analysis is refereed to as chromosome (or plasmid) loss. In this study, the percent plasmid-bearing cells and the percent of plasmids that are lost per generation were calculated by a modified fluctuation analysis with the method of

median.The equation was $X= 1-e^{r}$, where $r = \ln (R_f - R_i)/N$ as described in the *plasmid stability assay* section. Since the result carried out by different colonies could vary greatly within one strain, I decided to use the method of median to minimize disproportional inflation caused by jackpot colonies.

Whiskers box plot including median and data range from minimum to maximum was used for result presentation. The line within the box indicates the median value whereas the colored area below the median represents the first quartile showing the data range from 25 percentile to 50 percentile. The colored area above the median represents the third quartile, which shows the data range from 50 percentile to 75 percentile. The very top and very bottom lines outside the box represent the maximum value and minimum value of the data collected from the strain. Whiskers box plot from minimum to maximum and two-tailed unpaired student t test was performed using GraphPad Prism version 6.00 (Trail) for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

## Results

*Testing TEL-ARS plasmid stability in telomere maintenance deficient mutants.*

In comparison to the CEN-ARS plasmid, the TEL-ARS plasmid used here lacked the centromere but contained three tracts of telomeric sequences (280 bp each) that are oriented in direct and inverted directions. (Figure 3.1) Repetitive sequences are hard to replicate because it increases the probability of replication fork slippage during elongation step (Byzmek and Lovett 2001). Therefore, the repetitive telomere repeat tracks on this TEL-ARS plasmid may be hard to replicate. Moreover, the fact that the telomere tracts are present as in direct and inverted orientations may decrease the plasmid stability within the cells because these sequences could result in secondary structures that make fork progression more difficult, or could simply increase the probability of sequence deletion within the inverted repeats (Peeters et al. 1988, Henderson and Pete 1993). Finally, plasmids that lack centromeres will also result in low plasmid stability because of improper segregation during mitosis (Dani and Zakian 1983). The telomere-dependent plasmid segregation mechanisms are not likely to be as efficient as centromere-based segregation. Thus, for several reasons, this particular TEL-ARS plasmid containing the direct and inverted telomeric sequences may have low plasmid stability. Thus, it was reasonable to first test the stability of this TEL-ARS plasmid under selective conditions in wild-type cells to insure the feasibility of using this plasmid for our experiments. (Figure 2.2) If the wild-type cells could not even maintain the TEL-ARS plasmid at an acceptable range (such as retaining around 50% of the TEL-ARS plasmid in the whole population at the beginning of the experiment), testing TEL-ARS

plasmid stability in the CST mutants would not be ideal since they were thought to have more difficult time maintaining the TEL-ARS plasmid as reasoned above.

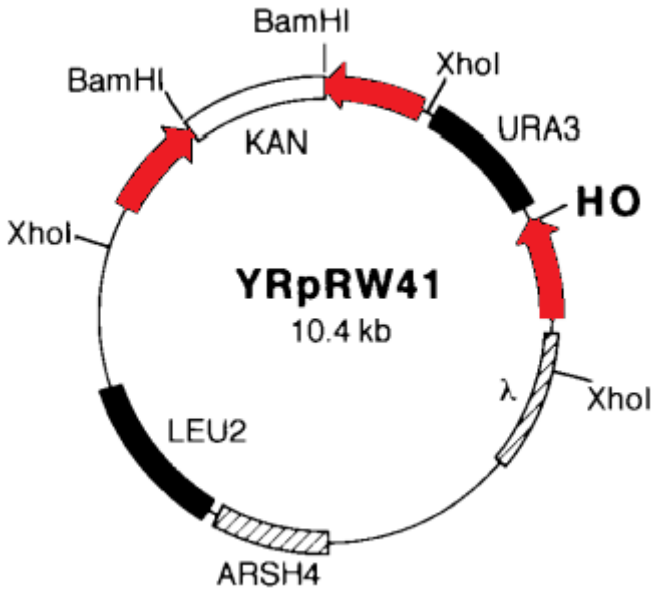


**Figure 3.1 Schematic drawing of the TEL-ARS plasmid.**
YRpRW41 (TEL-ARS plasmid) consists of three telomere repeats (red arrow), one autonomous replicating sequence (ARSH4), one *URA3* marker, and one *LEU2* marker. This TEL-ARS plasmid contains no centromere (Dionne and Wellinger 1998).

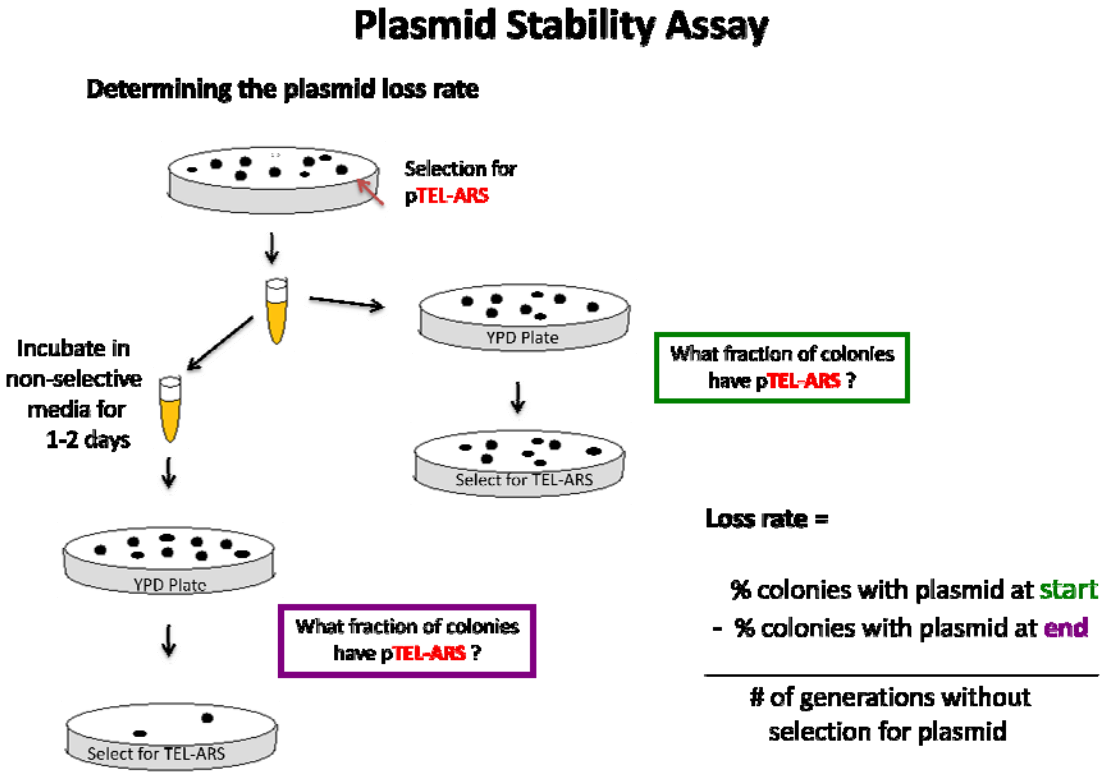


**Figure 3.2 Plasmid stability assay for determining the plasmid loss rate under non-selective conditions.**
YRpRW41 (TEL-ARS-*LEU2*-*URA3*) with pAS2 (2µ-*TRP1* vector) or pCN188 (2µ-*TRP1*-*STN1*) were first introduced into the yeast strain. Transformants were restreaked onto selective plates (SC –Trp, -Leu) to prevent choosing dormant cells without bearing the desired plasmids. Cells were then grown overnight at 23°C in selective medium (SC –Trp, -Leu) and inoculated into non-selective medium for the TEL-ARS plasmid (SC –Trp) at 30°C overnight. 100 to 300 cells from the selective medium (SC –Trp, -Leu) were plated onto YPD plates and replica platted onto selective plates (SC –Leu). 100 to 300 cells from the non-selective medium for the TEL-ARS plasmid (SC -Leu) were plated onto YPD plates and replica platted onto selective plates (SC –Leu). Colonies bearing the TEL-ARS plasmid were calculated according to the formula presented by Dani and Zakian. (Dani and Zakian 1983)

The observed data show that the stability, measured by percent plasmid-bearing cells, of the TEL-ARS plasmid (78%) was similar to the CEN-ARS plasmid (79%). (Figure 3.3 A) Therefore, since more than half of the cells do maintain the plasmid under selective growth conditions, it should be feasible to use the TEL-ARS plasmid to test

whether either compromised *stn1* function or overexpressed *STN1* affect the plasmid stability.

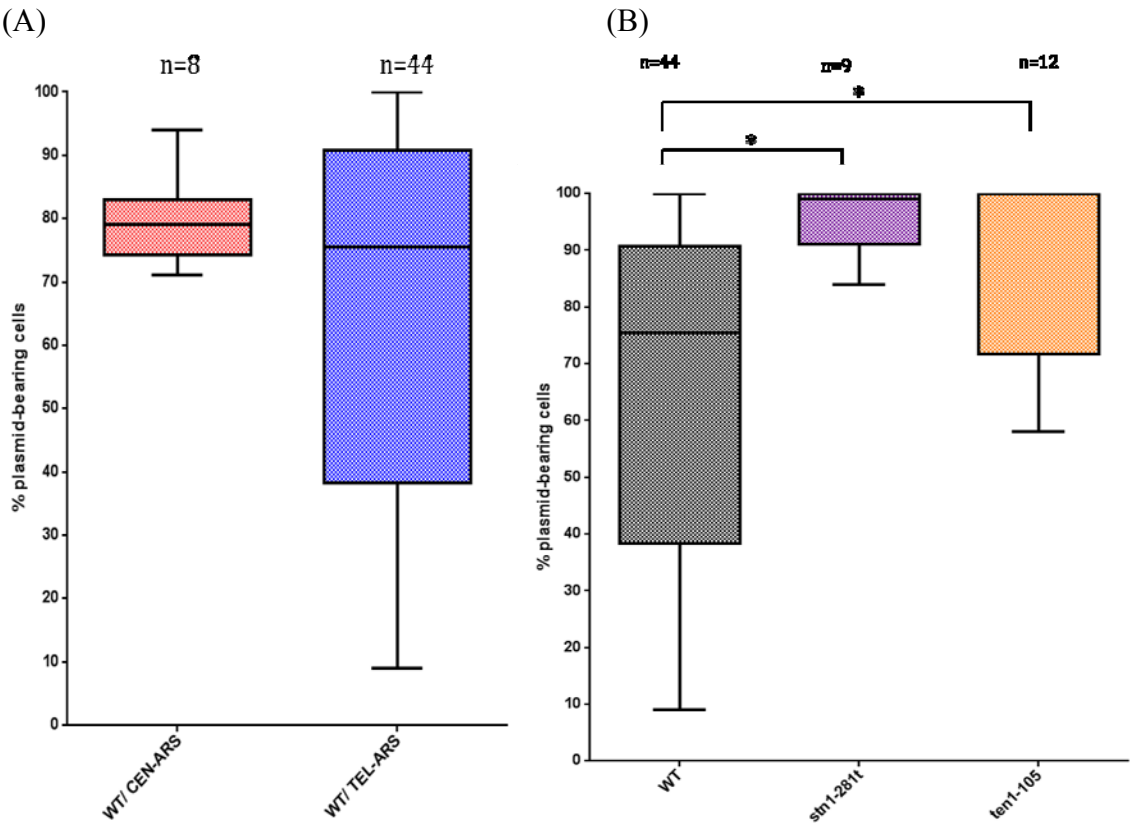


**Figure 3.3Testing the TEL-ARS plasmid stability by measuring percent plasmid-bearing cells in telomere maintenance deficient mutants.**
A) The percent of cells bearing only the CEN-ARS plasmid (pJBN218) or the TEL-ARS plasmid (YRpRW41) was tested following overnight growth of cells under selective conditions (SC –Ura for pJBN218 and SC –Leu for YRpRW41) at 23°C. The percent of cells in the culture that were Ura+ (for pJBN218) or Leu+ (for YRpRW41) was determined. Colonies tested in WT/CEN-ARS were from one transformation plate and in WT/TEL-ARS was from five different transformation plates. The result for WT/TEL-ARS was from pooled data from different experiments under the same condition. (WT= wild-type) B) The percent of cells bearing only the TEL-ARS plasmid YRpRW41 was tested following overnight growth of cells under selective conditions (5 mls of liquid SC - Leu ) in wild-type (hC160), *stn1-281t* (hC671), and *ten1-105* (hC2241) strains. The percent of cells in the culture that were Leu+ was determined. The experiment was performed at 23°C. Colonies tested in the wild-type were from five different transformation plates and the result was from the pool data from different experiments

under the same condition. Colonies tested in the *stn1-281t* and *ten1-105* were each from two different transformation plates. (WT= wild-type)
n= number of independent colony tested
* = Indicate the result was significant as the P value was less than 0.05.
P value for wild-type vs. *stn1-281t* was 0.004
P value for wild-type vs. *ten1-105* was 0.011

*Do CDC13, STN1 and TEN1 promote TEL-ARS stability?*

There is some evidence to suggest that Cdc13p, Stn1p and Ten1p promote replication through telomere repeats (Grossi et al. 2004, Petreaca et al. 2006, Petreaca et al. 2007, Xu et al. 2009, Price et al. 2010). If this is true, then one would predict that the TEL-ARS plasmid would be less stable in strains deficient for CST function than in wild-type cells. To see if compromised *STN1* function will affect the TEL-ARS plasmid stability, *stn1-281t* and *ten1-105* strains were used. These alleles were chosen because they both are deficient in telomere maintenance.

*stn1-281t* was created by integration of a PCR product that introduced a stop codon in *STN1* resulting in a truncated protein lacking amino acids 282 to 494 (Petreaca et al. 2007). This allele was characterized in the same paper and was shown to deficient for interaction with Cdc13p. In addition, the *stn1-t281* truncation mutant displayed long telomeres with increased internal single stranded TG repeats. Double mutants with *rad9-Δ* or *rad52*-Δ resulted in synthetic lethality, respectively. *stn1-281t* appears to be slow growing at permissive temperature with a prolonged G2/M phase. (Petreaca et al. 2007) As this allele is defective for interaction with Cdc13p, and Cdc13p was shown to interact with Pol1p, *stn1-281t* should have reduced interaction with Polα if both interactions are required for recruitment. Thus, *stn1-281t* may cause deficiency in DNA replication at

telomeres. Moreover, one explanation for *stn1-281t*'s increase in internal ssTG repeats could be from defects in end replication via Polα.

*ten1-105* was isolated as a temperature sensitive mutation from random PCR mutagenesis. The resulting plasmid was then integrated into the yeast genome. (Xu et al. 2009) This allele appears to have extremely elongated telomeres at permissive temperature and will accumulate extensive telomeric ssDNA at non-permissive temperature. Ten1-105p was also shown to lose interaction with Cdc13p but retains the ability to interact with Stn1p. At high temperatures, *ten1-105* induces a significant amount of Rad52-YFP foci even though Cdc13p is still shown to associate with telomeres. Finally, double mutant combinations with defects in Polα, *ten1-105 cdc17-1* are synthetic lethal, suggesting Ten1p plays a role in DNA replication. (Xu et al. 2009) Similar to *stn1-281t* mutant, *ten1-105* has defects in interaction with Cdc13p, which could result in reduced interaction with Polα. Subsequently, *ten1-105* may also cause deficiency in DNA replication at telomeres.

The TEL-ARS plasmid was transformed into the mutant *stn1* and *ten1* strains, and the stability of the plasmid under nutritional selection was tested in two different transformants from each strain. Unexpectedly, the percent of plasmid-bearing cells in *stn1-281t* (99%) and *ten1-105* (100%) was much higher than in wild-type cells (76%). (Figure 3.3 B) The P value from an unpaired student t test for comparing wild-type vs. *stn1-281t* and wild-type vs. *ten1-105* was 0.004 and 0.0107, respectively; suggesting the difference of both comparisons were significant. (Figure 3.3 B)

Since both of these mutant strains take a lot longer to complete one cell cycle than

wild-type cells, it was possible that the increased stability of the plasmids in the mutants was a bit deceptive. To rule out the possibility that the observed results reflected fewer cell cycles in the mutant strains, the loss rate of the TEL-ARS plasmid per generation was determined in the *stn1* and *ten1* deficient strains. (Figure 3.4) The results showed that the percent of cells losing the plasmid per generation was low in both *stn1-281t* (0%) and *ten1-105* (0.3%) and was similarly low in the wild-type (2%). No statistical significance was detected in comparing the loss rate between the wild-type and *stn1-281t* strains and between wild-type and *ten1-105* strains. The P value from unpaired student t test for both comparisons were greater than 0.05. (Figure 3.4) These loss rate data suggested the high stability in the mutants was probably not a result of fewer cell cycles.

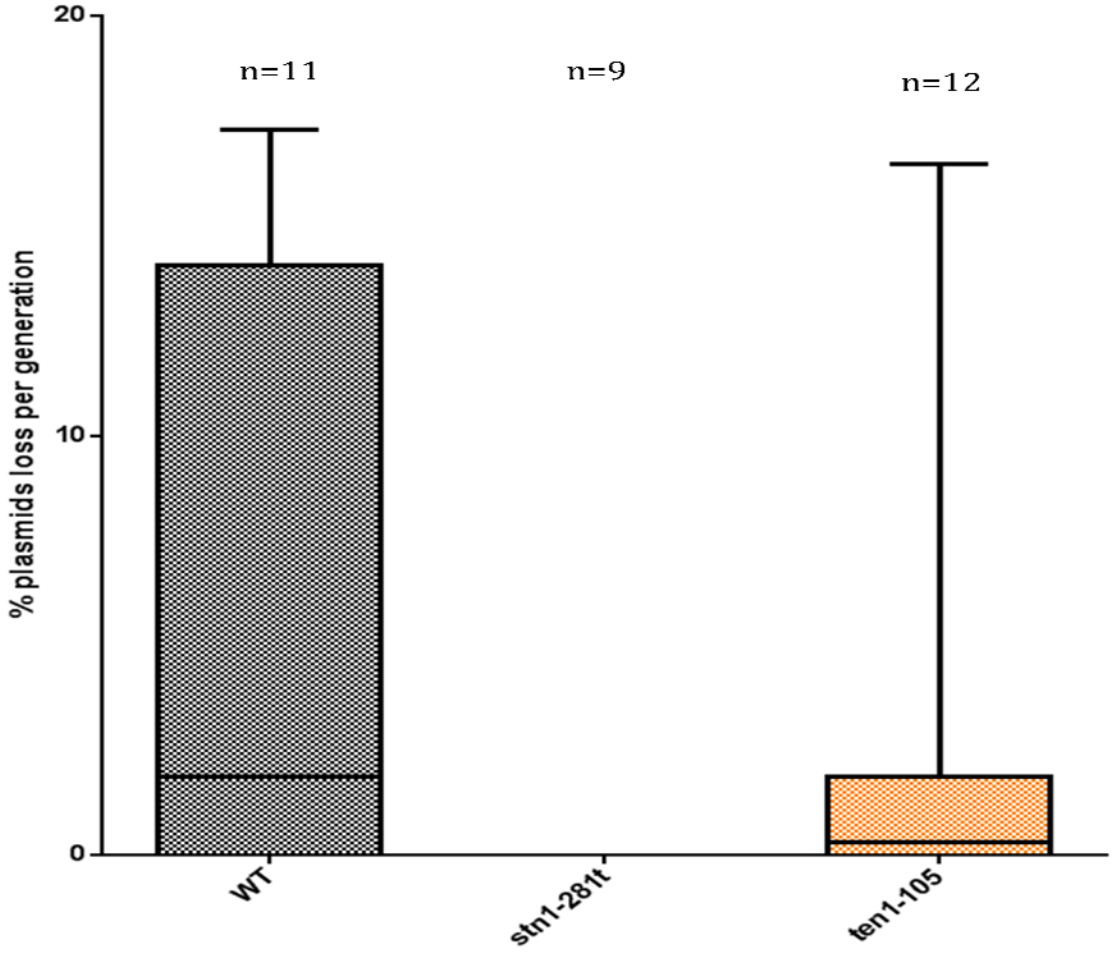


**Figure 3.4 Testing the TEL-ARS plasmid stability by measuring percent plasmid loss per generation in telomere maintenance deficient mutants.**
The percent plasmids loss rate of the TEL-ARS plasmid (YRpRW41) was tested following overnight growth of cells under selective conditions (5 mls of liquid SC -Leu) at 23°C in wild-type (hC160), *stn1-281t* (hC671), and *ten1-105* (hC2241) strains. Cultures were then grow under nonselective conditions (10 mls of liquid YPD) at 30°C for 1-2 days. The percent of cells in the culture that were Leu+ was determined. Colonies tested in the wild-type, *stn1-281t* and *ten1-105* were each from two different transformation plates. No pooled data were used in the wild-type. (WT= wild-type)
n= number of independent colony tested
P value for wild-type vs. *stn1-281t* was 0.0518
P value for wild-type vs. *ten1-105*was 0.1502

Since both *stn1-281t* and *ten1-105* lead to substantial Rad52 foci formation (Xu et al. 2009, Petreaca et al. 2007), a possibility was that the extremely high plasmid stability

was a result of the plasmid integrating into the genome. If the TEL-ARS plasmid is being integrated into the genome, then when counter-selection is imposed on the cells, the cells should not grow well because the integration should not be reversed at a high frequency. To test how easily the TEL-ARS plasmid could be lost from the mutant strains, I first streaked the mutant Leu+ colonies onto 5-FOA plates, which will kill cells expressing Ura3p. The *stn1-281t* [TEL-ARS] and *ten1-105* [TEL-ARS] strains all grew on the 5-FOA media, suggesting that either the plasmid is lost, or it lost its *URA3* in the process of either becoming linear or integrated. (Appendix III) Next, to test whether additional plasmid markers were present in the Ura- colonies from the 5-FOA plates, I re-streaked the survivors onto SC –Leu plates. For *stn1-281t* strain, five out of the eight colonies tested were Leu+, suggesting either the revertant occurred at high frequency, plasmid rearrangement looping out *URA3* marker at higher rate, or plasmid containing only the *LEU2* marker was integrated into the yeast genome at high occurrence. On the other hand, *ten1-105* demonstrated no Leu+ colonies, consistent with a loss of the plasmid rather than an integration event. (Appendix II)

*Testing TEL-ARS plasmid stability in cdc7-1, dbf4-1, and mcm7-1.*

After seeing that *STN1* over-expression can reduce the CEN-ARS plasmid loss rate in *cdc7-1* and *mcm7-1*, I decided to test the TEL-ARS plasmid loss rate in the same replication deficient mutants: *cdc7-1*, *dbf4-1*, and *mcm7-1*. Although the CEN-ARS and TEL-ARS plasmids use the same origin of replication (ARSH4), the telomere tracts might pose more of a replication challenge. If *STN1* does promote replication through telomere sequences, then its increased dosage might have more of an impact on

improving the plasmid stability.

Unexpectedly, I observed very low transformation efficiencies for the TEL-ARS plasmid in these mutant strains, especially in *cdc7-1* and *dbf4-1*. Because of the severity of the problem, I could not examine the percent of cells losing the plasmid per generation as the mutants could not maintain the TEL-ARS plasmid in the non-selective condition overnight (Data not shown). Thus, instead of looking at the plasmid loss rate, I only examined the percent of plasmid-bearing cells in the whole population. Since I observed the low transformation efficiency for the TEL-ARS plasmid in these mutants, I decided to compare the stability of the TEL-ARS plasmid to the CEN-ARS plasmid in the same strains before examining the effect of *STN1* over-expression on the TEL-ARS plasmid stability. The thinking was that the comparison might give me a better understanding toward the behavior of the TEL-ARS plasmid or to help dissect the deficiency in these mutants in more details.

The results showed that the TEL-ARS plasmid stability was much lower compared to the CEN-ARS plasmid in all strains. The CEN-ARS plasmid stability as measured by the percent plasmid-bearing cells was 100% (wild-type), 81% (*cdc7-1*), 74% (*dbf4-1*), and 80% (*mcm7-1*). (Figure 3.5) On the other hand, the TEL-ARS plasmid stability measured by the percent plasmid-bearing cells was 45% (wild-type), 20% (*cdc7-1*), 29% (*dbf4-1*), and 25% (*mcm7-1*). (Figure 3.5) Such low plasmid stability might explain the low transformation efficiency observed previously. One thing to note here is that all strains tested also contain the 2μ vector in addition to the TEL-ARS plasmid. We observed that the addition of the 2μ vector plasmid lowered the TEL-ARS plasmid

stability at least in the wild-type. This will be discussed in the later paragraph.

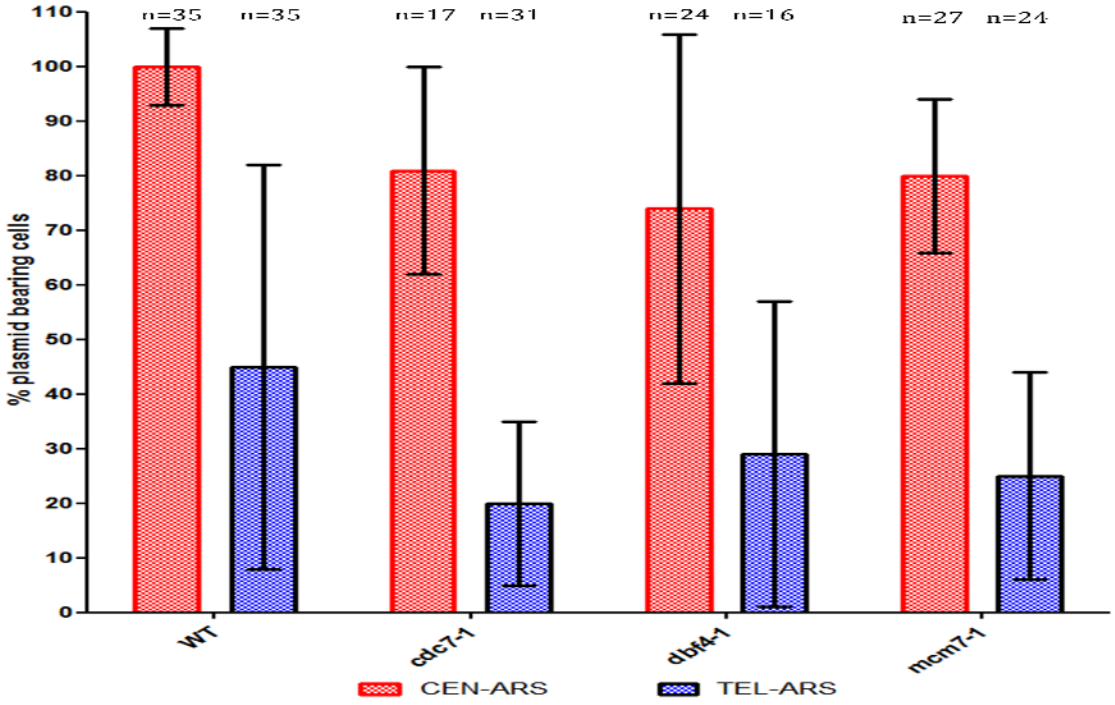


**Figure 3.5 Comparison of the stability of CEN-ARS plasmid and TEL-ARS plasmid in replication deficient mutants by measuring percent plasmid-bearing cells**
The percent of cells bearing TEL-ARS plasmid (YRpRW41) or CEN-ARS (pJBN218) plasmid was tested under selective conditions in wild-type (hC160), *cdc7-1* (hC2403), *dbf4-1* (JBY999), and *mcm7-1* (DBY2029). The CEN-ARS plasmid bearing cells were grown overnight in 5 mls of liquid SC -Leu -Ura). The percent of cells in the culture that were Ura+ was determined. The percent plasmid bearing cells for TEL-ARS plasmid was tested under selective conditions (SC –Leu –Trp, transformation plates) without overnight growth. The percent of cells in the culture that were Leu+ was determined. All strain tested contain an additional vector plasmid, pCN416 or pAS2 for cells bearing the CEN-ARS plasmid and pAS2 for cells bearing the TEL-ARS plasmid. All experiments were performed at 23°C. Colonies tested in the wild-type, *cdc7-1*, *dbf4-1*, and *mcm7-1* were each from four different transformation plates while testing the CEN-ARS plasmid and were each from five transformation plates while testing the TEL-ARS plasmid. (WT= wild-type) Median with standard deviation was shown.
n= number of independent colony tested for both CEN-ARS and TEL-ARS plasmids
* = Indicate the result was significant as the P value was less than 0.05
P value for WT/CEN-ARS vs. WT/TEL-ARS was <0.0001
P value for *cdc7-1*/CEN-ARS vs. *cdc7-1*/TEL-ARS was <0.0001
P value for *dbf4-1*/CEN-ARS vs. *dbf4-1*/TEL-ARS was 0.0078
P value for *mcm7-1*/CEN-ARS vs. *mcm7-1*/TEL-ARS was <0.0001

*Enhancement of TEL-ARS stability by STN1 over-expression*

As shown in Figure 3.5, the TEL-ARS plasmid is not maintained well in these replication mutant strains. I went on to test if *STN1* over-expression could still enhance the TEL-ARS plasmid stability in these strains. The results were somewhat surprising as *STN1* over-expression significantly enhanced the TEL-ARS plasmid stability in both *cdc7-1* and *dbf4-1*, but not in *mcm7-1*. (Figure 3.6) The percent plasmid-bearing cells were 20% (*cdc7-1*/Vec) vs. 37% (*cdc7-1*/STN1), 29% (*dbf4-1*/Vec) vs. 52% (*dbf4-1*/STN1), and 25% (*mcm7-1*/Vec) vs. 31% (*mcm7-1*/STN1). (Figure 3.6) The unpaired student t test suggested that the TEL-ARS plasmid stability enhancement in *mcm7-1* was not significant as the P value was greater than 0.05.

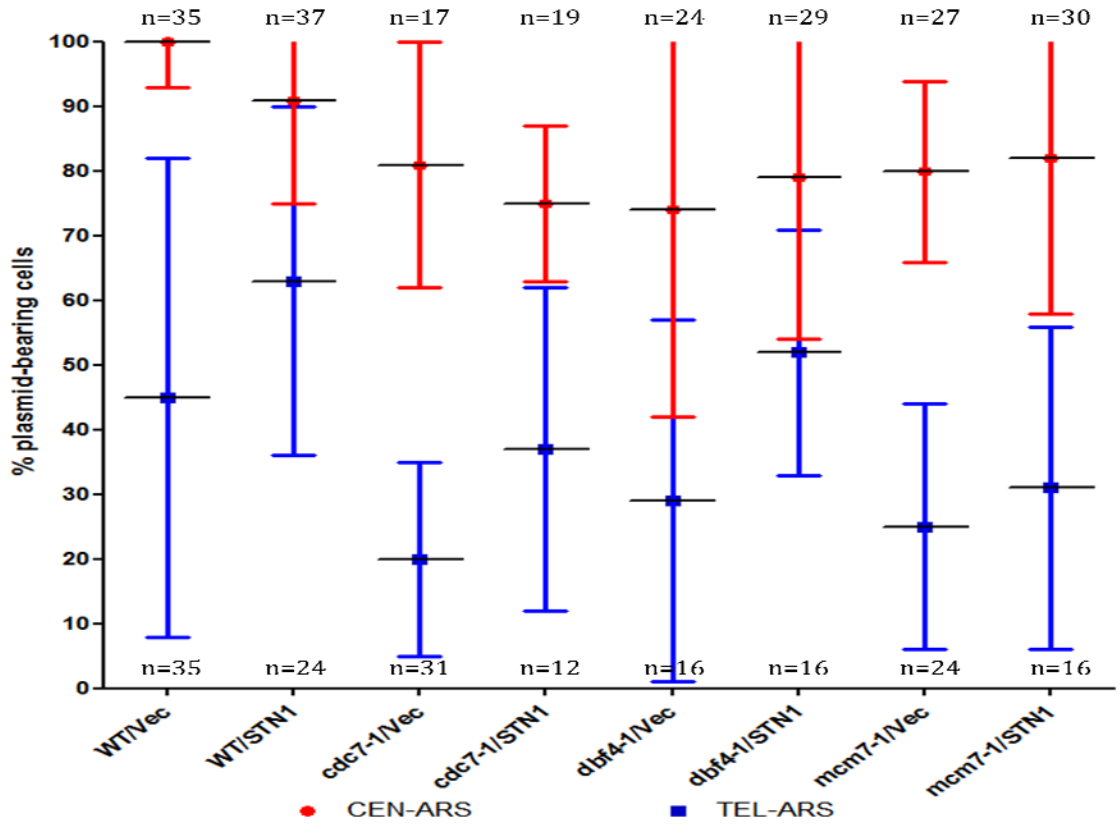


**Figure 3.6 Comparing the stability of CEN-ARS and TEL-ARS plasmids with *STN1* over-expression in replication deficient mutants.**
The percent of cells bearing the TEL-ARS plasmid (YRpRW41) or CEN-ARS (pJBN218) plasmid was determined from tests under selective conditions in wild-type (hC160), *cdc7-1* (hC2403), *dbf4-1* (JBY999), and *mcm7-1* (DBY2029). The CEN-ARS plasmid bearing cells were grown overnight in 5 mls of liquid SC -Leu -Ura. The percent of cells in the culture that were Ura+ was determined. The percent plasmid bearing cells for TEL-ARS plasmid was tested under selective conditions (SC –Leu –Trp, transformation plates) without overnight growth. The vector plasmid used was pCN416 or pAS2 for cells bearing the CEN-ARS plasmid and pAS2 for cells bearing the TEL-ARS plasmid. The *STN1* plasmid was pCN421 or pCN188 for cells bearing the CEN-ARS plasmid and pCN188 for cells bearing the TEL-ARS plasmid. All experiments were performed at 23°C. Colonies tested in the wild-type, *cdc7-1*, *dbf4-1*, and *mcm7-1* were each from four different transformation plates while testing the CEN-ARS plasmid and were each from five transformation plates while testing the TEL-ARS plasmid. (WT= wild-type) Median with standard deviation was shown.
n= number of independent colony tested

* = Indicate the result was significant as the P value was less than 0.05
P value for WT/Vec/CEN-ARS vs. WT/STN1/CEN-ARS was 0.025
P value for *cdc7-1*/Vec/CEN-ARS vs. *cdc7-1*/STN1/CEN-ARS was 0.818
P value for *dbf4-1*/Vec/CEN-ARS vs. *dbf4-1*/STN1/CEN-ARS was 0.491
P value for *mcm7-1*/Vec/CEN-ARS vs. *mcm7-1*/STN1/CEN-ARS was 0.314
P value for WT/Vec/TEL-ARS vs. WT/STN1/TEL-ARS was 0.517
P value for *cdc7-1*/Vec/TEL-ARS vs. *cdc7-1*/STN1/TEL-ARS was 0.018
P value for *dbf4-1*/Vec/TEL-ARS vs. *dbf4-1*/STN1/TEL-ARS was 0.046
P value for *mcm7-1*/Vec/TEL-ARS vs. *mcm7-1*/STN1/TEL-ARS was 0.157

Another observation was that the TEL-ARS plasmid stability measured by percent plasmid-bearing cells was not significantly increased in the wild-type as the P value was greater than 0.05, 45% (wild-type/Vec) vs. 63% (wild-type/STN1). On the other hand, the CEN-ARS plasmid stability was significantly decreased after *STN1* over-expression as the P value was 0.025, 100% (wild-type/Vec) vs. 91% (wild-type/STN1). (Figure 3.6)

One additional finding while comparing the CEN-ARS and TEL-ARS plasmid stability was the effect of the added 2μ vector plasmid. The addition of the extra 2μ vector plasmid to the wild-type cells carrying the TEL-ARS plasmid seemed to destabilize the TEL-ARS plasmid. The median value for the percent of cells retaining the TEL-ARS plasmid dropped to 45% following the addition of the 2μ plasmid, compared to 76% median retention in cells without the extra 2μ plasmid. (Figure 3.7 B) Although this seems to be a huge difference, the addition of extra vector plasmid did not significantly affect the TEL-ARS plasmid stability in the wild-type as the P value from unpaired student t test was 0.0552. Therefore, I could possibly exclude the affect of the additional vector plasmid in the experiment testing the TEL-ARS plasmid stability. On the other hand, addition of a 2μ plasmid to the wild-type [CEN-ARS] cells seemed to enhance the CEN-ARS plasmid stability, as the percent plasmid-bearing cells measured

in the wild-type [CEN-ARS, 2µ] went up to 100%, as compared to an original value of 79% in cells without the extra vector plasmid. (Figure 3.7 A)

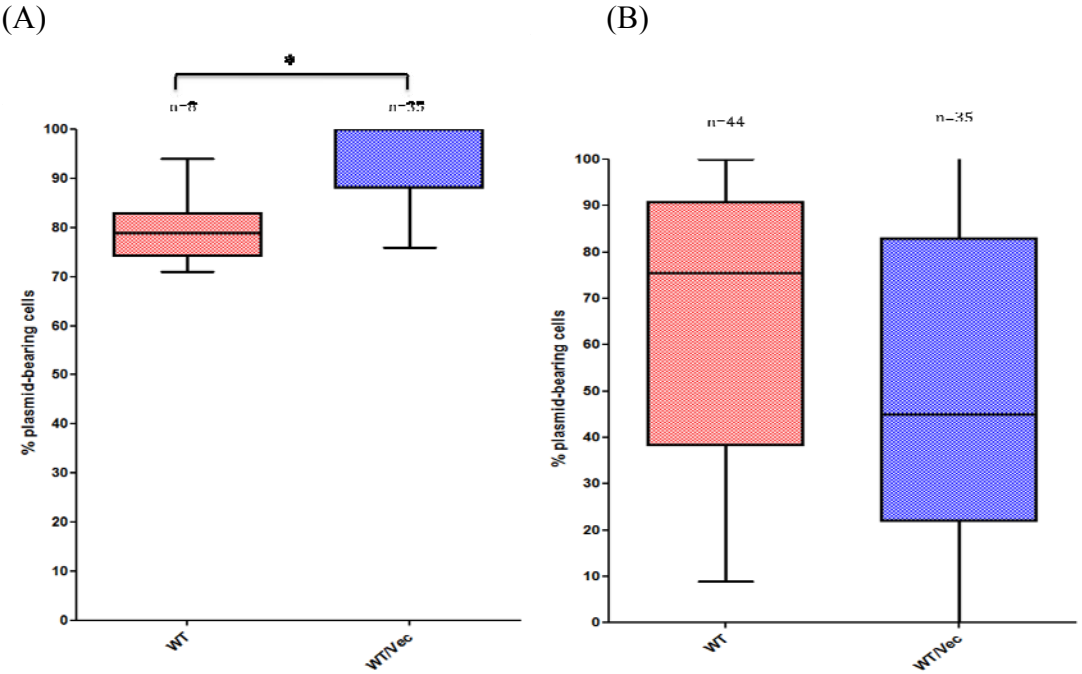


**Figure 3.7 Testing how additional vector plasmid affects the CEN-ARS plasmid and TEL-ARS plasmid stability in the wild-type strain.**
A) CEN-ARS plasmid stability with or without the vector plasmid. The percent of cells bearing only the CEN-ARS plasmid (PJBN218) or both the CEN-ARS plasmid and the vector plasmid (pCN416 or pAS2) was tested following overnight growth of cells under selective conditions (5 mls of liquid SC –Ura for WT and 5 mls of liquid SC –Leu –Ura for WT/Vec) at 23°C. The percent of cells in the culture that were Ura+ or Ade+ was determined. Colonies tested in WT were from one transformation plate, and were from four transformation plates in WT/Vec. The result for WT/Vec was from pooled data from different experiments under the same condition. (WT= wild-type)
n= number of independent colony tested
* = Indicate the result was significant as the P value was less than 0.05
P value for WT vs. WT/ Vec was <0.0001
B) TEL-ARS plasmid stability with or without the vector plasmid. The percent of cells bearing only the TEL-ARS plasmid (YRpRW41) or both the TEL-ARS plasmid and the vector plasmid (pAS2) was tested under selective conditions (SC –Leu transformation plates for WT and SC –Leu –Trp transformation plates for WT/Vec) at 23°C. The percent of cells in the culture that were Leu+ was determined. Colonies tested in WT and WT/Vec were each from five different transformation plates. The result for both WT and WT/Vec was from pooled data from different experiments under the same condition. (WT= wild-type)

n= number of independent colony tested
P value for WT vs. WT/Vec was 0.0552

*Overproduction of Stn1p had no affect on Segregation deficient mutants*

The low TEL-ARS plasmid stability in *cdc7-1*, *dbf4-1*, and *mcm7-1* suggested that the loss of DDK function or MCM function potentially compromises one of the followings: 1. The telomere based segregation mechanism. 2. Replication through telomere repeats and/or general presence of direct or inverted repeats. 3. Origin firing. Results from *STN1* overexpression in *cdc7-1* and *dbf4-1* bearing TEL-ARS plasmid further suggested that overproduced Stn1p was acting to restore the TEL-ARS plasmid stability. To investigate if overproducing Stn1p can enhance the TEL-ARS plasmid stability by improving the telomere based segregation mechanism, I tested TEL-ARS plasmid stability in *sir4Δ* and *rap1-5* with or without overexpressing *STN1*.

*SIR4* is shown to participate in silencing the mating type loci, HML and HMR, maintaining the heterochromatin structure near telomeres (Rine and Herskowitz 1987, Aparicio et al. 1991, Loo and Rine 1995, Brindle et al. 1990). The maintenance of the heterochromatin structure requires the binding of Rap1p, Abf1p, or Origin Recognition Complex to the dsDNA and then recruits Sir1p, Sir2p, Sir3p, and Sir4p to these protein bound transcription repression sties (Laurenson and Rine 1992, Kinnerly et al. 1988, Gardner et al. 1999, Triolo and Sternglanz 1996). Besides acting as a silencer, *SIR4* is also suggested to participate in telomere specific segregation mechanism as *sir4* mutants including *sir4Δ* is shown to reduce the plasmid stability of their TEL-ARS plasmid (Kimmerly and Rine 1987, Longtine et al. 1993). Later studies

propose that Sir4p can direct the telomere sequences to Esc1p which binds to the nuclear pore complex, thus anchor the telomeres onto the nuclear envelope at G1 phase (Taddei and Gasser 2004).

Another protein that participates in the telomere specific segregation mechanism is Rap1p. *RAP1* is characterized as a regulator that can both activate or repress transcription depending on the context of the binding site and is also fund to play a role in telomere maintenance (Elledge and Davis 1989, Shore and Nasmyth 1987, Buchman et al. 1988, Vignais et al. 1987). The role of Rap1p in telomere specific segregation mechanism is first identified in 1992 where the scientists use different *rap1* mutants, including *rap1-5*, to test the plasmid stability of a telomere repeat sequence (TRS) containing plasmid (Longtine et al. 1992). The results show that the TRS plasmid stability is greatly reduced in *rap1-5* and the loss rate is not affected with different type of plasmids such as 2 micron plasmid containing TRS or a CEN-ARS plasmid, suggesting *RAP1* is involved in telomere specific segregation mechanism (Longtine et al. 1992, Enomoto et al. 1994).

The unexpected observation reported here was that the TEL-ARS plasmid was not less stable in *sir4Δ*/Vec (2% plasmid loss per generation) than in wild-type/Vec (10% plasmid loss per generation). (Figure 3.8) Thus, it was not clear that *sir4Δ* could really be used to test my hypothesis. Nevertheless, after overexpressing *STN1*, TEL-ARS plasmid stability was not significantly enhanced in *sir4Δ*/p*STN1* (0.08% plasmid loss per generation). (Figure 3.8)

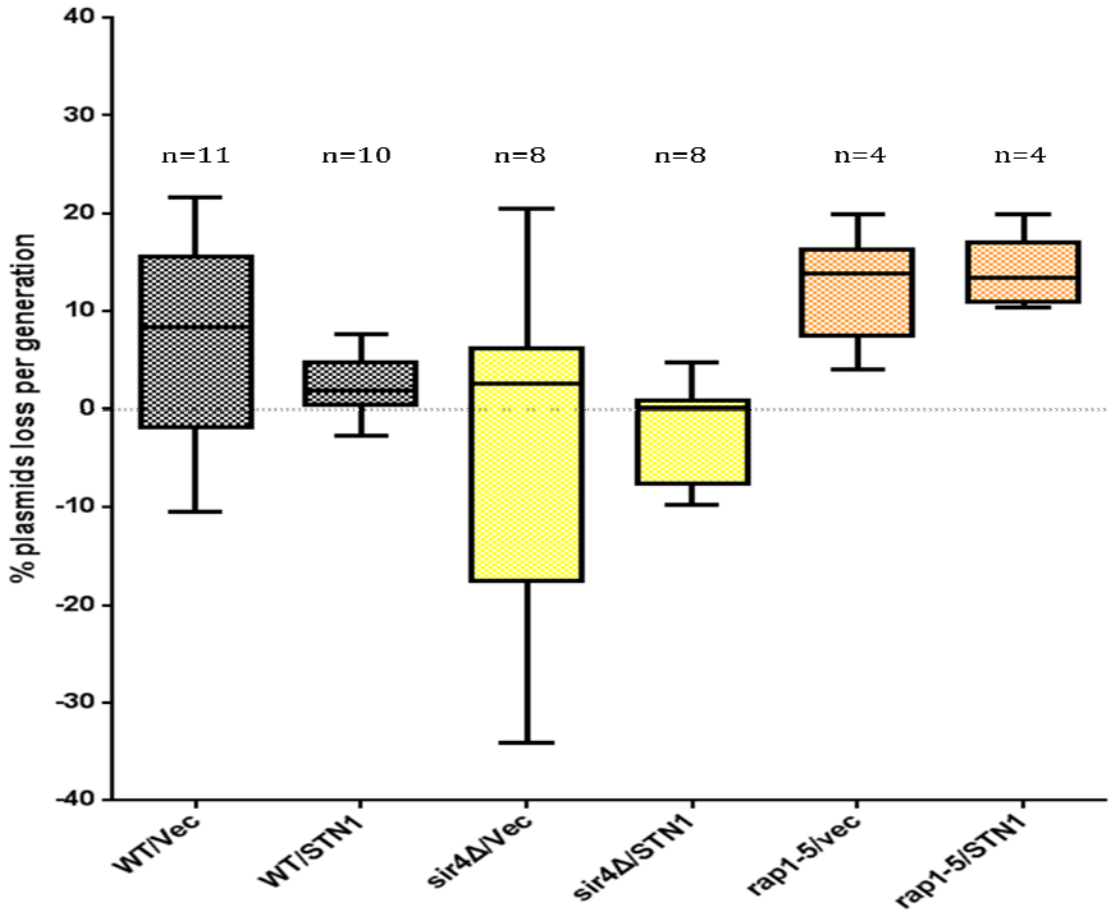


**Figure 3.8 TEL-ARS plasmid stability by percent plasmid loss per generation in plasmid segregation deficient mutants after *STN1* overexpression.**
The percent plasmids loss rate of the TEL-ARS plasmid (YRpRW41) was tested under nonselective conditions (10 mls of liquid YPD) overnight at 30°C in wild-type (hC160), *sir4Δ* (hC1654), and *rap1-5* (YJB208). The percent of cells in the culture that were Leu+ was determined. Colonies tested in the wild-type were from three transformation plates, in the *sir4Δ* strain were from two transformation plates, and in the *rap1-5* strain were from one transformation plate. Each independent colony from *rap1-5* strain was tested twice. (WT= wild-type)
n= number of independent colony tested
P value for WT/Vec vs. WT/p*STN1* was 0.214
P value for *sir4Δ*/Vec vs. *sir4Δ*/p*STN1* was 0.95
P value for wild *rap1-5*/Vec vs. *rap1-5*/p*STN1* was 0.486

On the other hand, the TEL-ARS plasmid was not well maintained in *rap1-5* cells. We determined that *rap1-5*/Vec (14%) showed an expected higher plasmid loss per generation compare to wild-type/Vec (10%). (Figure 3.8) However, over-expressing *STN1* did not significantly alter the TEL-ARS plasmid stability as the plasmid loss rate per generation was 13% in *rap1-5*/*STN1* compared to 14% in *rap1-5*/Vec. Moreover, the P value was much greater than 0.05. (Figure 3.8)

One additional observation was in agreement with the previous results showing a trend that *STN1* overexpression seem to stabilize the TEL-ARS plasmid. The percent plasmid-bearing cells was increased with overexpressed *STN1*, 36% in WT/Vec vs. 63% in WT/STN1 (Figure 3.6) In figure 3.8, the plasmid loss rate was decreased with overexpressed *STN1*, 8% in WT/Vec vs. 2% in WT/STN1. However, both comparisons were not statistically significant.

**Discussion**

One speculation for why *STN1* overexpression suppresses the temperature sensitivity of the replication deficient mutants could be because of the disruption of the S phase checkpoint. However, our lab observed that defects in S phase checkpoint did not suppress the *dbf4-1* temperature sensitivity in the *mrc1Δ dbf4-1* double mutants. (Gasparyan, unpublished data) Therefore, the reduced temperature sensitivity of the replication deficient mutant is not a result of S phase checkpoint interference by overexpressed *STN1*. Perhaps *STN1* overexpression is more likely to facility in DNA replication to improve replication mutants' viability.

If CST are involved in promoting replication at telomeres or at difficult regions, then CST mutants should have harder time maintaining plasmids that contain telomere sequences. *stn1-281t* and *ten1-105* were used because they were both suggested to have deficiency in interacting with Polα indirectly (Xu et al. 2009, Petreaca et al. 2007, P.C., data not shown) This is because both mutants have reduced interaction with Cdc13p which is shown to interact act with Pol1p directly (Xu et al. 2009, Petreaca et al. 2007, Qi and Zakian 2000). In addition, both mutants cannot maintain proper telomere length. *stn1-281t* shows elongated telomeres and internal ssDNA gap at permissive temperature (Petreaca et al. 2007). *ten1-105* accumulates long telomeres over generations at permissive temperature and results in long telomeric ssDNA at semi-permissive temperature (Xu et al. 2009). Thus, I was expecting higher instability of the TEL-ARS plasmid in both mutants.

Surprisingly, both *stn1-281t* and *ten1-105* reported higher TEL-ARS plasmid

stability than the wild-type, and such high plasmid stability was not due to fewer plasmid loss events from less cell cycles in both mutants, nor to integration of the TEL-ARS plasmid into the genome. Another speculation for the high TEL-ARS plasmid stability in *stn1-281t* and *ten1-105* is the delayed S/G2/M phase in these mutants (Petreaca et al. 2007, Xu, data not published) that cause the plasmid distribution more evenly between the mother and daughter cells. This idea is supported by the published data showing extending the duration of mitosis will result in higher plasmid stability. (Gehlen et al. 2011) Random distribution of the No-CEN plasmid could diffuse in between the mother and daughter cells, and the diffusion kinetics is controlled by the geometry of the nuclear and the duration of the mitosis. The larger the nuclear and the longer the diffusing time, the better the cell will retain the plasmid. (Gehlen et al. 2011) Since the *stn1-281t* and *ten1-105* both have prolonged mitosis, the TEL-ARS plasmid could have higher passive segregation efficiency and result in high plasmid stability. Any more defects in fully replicating the plasmids that would normally destabilize the plasmids may thus be masked by the pro-longed cell cycle.

While the TEL-ARS plasmid exhibits high stability in the CST mutants, I found that the TEL-ARS plasmid is extremely unstable in the replication deficient mutants, *cdc7-1*, *dbf4-1*, and *mcm7-1*. Such high instability could be due to the lack of the centromere, the repetitive telomere sequences, and the direct and inverted telomere repeats within the plasmid plus the replication defects within the mutants. One common defect in these mutants is the crippled initiation from the replication origin. Although the plasmid stability is very different for both plasmids in all replication deficient mutants,

the CEN-ARS plasmid and the TEL-ARS plasmid share the same origin of replication, thus one could suggest that the origin firing might not be the reason for the low TEL-ARS plasmid stability. However, we still cannot rule out the possibility of inefficient firing of the origin causing the instability of the TEL-ARS plasmid in the replication deficient mutants as both plasmids showed lower stability in the mutants than in the wild-type.

If the high TEL-ARS plasmid stability in the CST mutants is the result of prolonged mitosis, the replication deficient mutants should also maintain high TEL-ARS plasmid stability since they have delayed S phase at the semi-permissive temperatures. The outcome, however, was the total loss of the TEL-ARS plasmid in these mutants, suggesting that one main reason for the high TEL-ARS plasmid instability should be the result of replication deficiency. Further investigation will be required to confirm this interpretation.

Since *STN1* over-expression lessens the percent plasmid loss rate in *cdc7-1* and *mcm7-1*, I expected to see a similar result for the TEL-ARS plasmid. Interestingly, the extreme low stability of the TEL-ARS plasmid is significantly improved in the *cdc7-1* and *dbf4-1* but not in *mcm7-1*. One simple explanation to why *STN1* over-expression affects the *ddk* mutants but not on the *mcm7-1* allele could be because the overproduced Stn1p only helps restore the defect with origin initiation but not with the replication elongation. However, this idea is somewhat in conflict with the previous speculation saying the low TEL-ARS plasmid stability is because of the origin firing defect. If the previous speculation is true, I should see higher TEL-ARS plasmid stability in *mcm7-1* as

well. In fact, the TEL-ARS plasmid stability is improved in the *mcm7-1* mutant after *STN1* over-expression. Although the change is not statistically significant compared to the *mcm7-1* with the vector plasmid, it could still be possible that *STN1* over-expression does help to enhance the TEL-ARS plasmid stability in *mcm7-1*. If one believes that *STN1* over-expression could increase the TEL-ARS plasmid stability in all replication deficient mutants mentioned here, then the idea of *STN1* over-expression could promote late origin firing under replication stress could be applied. Even more, the telomere sequences could convert nearby ARS into late replication origin during S phase (Ferguson et al. 1992, Stevenson and Gottschling 1999), thus promoting late origin firing by *STN1* over-expression should be more likely to happen in the TEL-ARS plasmid.

*STN1* over-expression decreases the CEN-ARS plasmid stability in the wild-type even under selective condition. One speculation could be that overexpressed *STN1* might interfere with the replication machinery under normal cell condition, thus lower the CEN-ARS plasmid stability in the wild-type cells. The idea of *STN1* overexpression in helping DNA replication could probably only be applied when cells encounter replication stress.

On the surface, the observation that *STN1* over-expression does not significantly affect the fraction of cells containing the CEN-ARS plasmid in the replication deficient mutants could be seen as surprising. This observation contradicts that from chapter 1 showing *STN1* overexpression improve the CEN-ARS loss rate in some replication deficient mutants. This could be explained as here I am only looking at how much plasmid-bearing cells are in the whole population instead of looking at the percent plasmid loss per generation. Perhaps *STN1* overexpression is still enhancing the plasmid

stability in the replication deficient mutants, however, the result is not obvious because the expending rate of the plasmid-bearing cells is not much higher than the expending rate of the plasmid-loss cells. If the cells are allowed to grown in the selective condition for a longer period, we might be able to see the effect of *STN1* overexpression. This rationale is consistent with the idea that looking at the percent plasmid loss rate is more accurate than looking at the percent plasmid-bearing cells.

While analyzing the data, we found an interesting observation that additional vector plasmid increases the CEN-ARS plasmid stability in the wild-type. Normally, multiple plasmids within one cell will often reduce the stability of one or the other due to plasmid incompatibility (Novick 1987, Futcher and Cox 1984). The result of the TEL-ARS plasmid stability with the additional vector in the wild-type cell is in agreement with this idea. The incompatibility could due to the competition in the same segregation mechanism, in the origin firing frequency, or in the plasmid-bearing capacity (Novick 1987). Thus, the high-proliferating 2μ vector plasmid would out-compete the TEL-ARS plasmid in origin firing frequency and the same random segregation mechanism. Strangely, the high-proliferating vector plasmid increases the CEN-ARS plasmid stability instead of out-competing it. Perhaps the vector plasmid has elements we do not know and can stabilize the CEN-ARS plasmid. In any case, it will be more accurate to look at the percent plasmid loss rate of the CEN-ARS plasmid with or without the additional 2μ vector.

Besides the possibility of promoting late origin firing to improve the TEL-ARS plasmid stability, overproduced Stn1p may participate in helping segregating the TEL-

ARS plasmid. The idea was that, TEL-ARS plasmid tend to segregate through different mechanism than the CEN-ARS plasmid by anchoring the telomeres onto the nuclear pore complex via the help of Ku 70/80, Sir4p, and Rap1p (Hediger et al. 2002, Kimmerly and Rine 1987, Longtine et al. 1992). Thus Stn1p could potentially bind to the telomere sequences on the TEL-ARS plasmid and participate in the TEL-ARS segregation directly or indirectly. My results reveal that the TEL-ARS plasmid stability is not significantly increased or decreased after *STN1* over-expression, suggesting *STN1* over-expression probably does not help in TEL-ARS plasmid segregation. Nevertheless, a different experimental design will still be required to back up this interpretation as the negative results are not strong enough to support the idea.

In sum, my results suggest the following: 1) the high TEL-ARS plasmid stability could be because of the prolonged S phase in the *stn1-281t* and *ten1-105* alleles 2) *STN1* over-expression could enhance the TEL-ARS plasmid stability by promoting late origin firing, 3) overproduced Stn1p probably is not involved in helping TEL-ARS plasmid segregation. Since these interpretations are only based on the results of plasmid stability assay, more biochemical and/or molecular level of experiments will be necessary to further confirm these speculations.

**Conclusions**

The starting point of my thesis is to solve how does constitutive over-expression of *STN1* permit growth of *cdc7-1*, *dbf4-1*, and *mcm7-1* at higher temperatures. There are mainly three hypotheses where 1) disruption of the S phase checkpoint by *STN1* over-expression can simply accommodate the defects that cause the temperature sensitivity in these replication deficient mutants, 2) over-expressed *STN1* can improve replication at telomeres, and 3) over-expressed *STN1* can improve replication away from telomeres.

The first hypothesis is being examined by other members in the lab. Two independent lines of data suggest that suppression of the temperature sensitivity phenotype of these replication deficient mutants is not the result of S phase checkpoint disruption. Thus, we could rule out the first hypothesis.

Here, I examined the second hypothesis and showed that over-expressed *STN1* can improve replication at telomeric sites. *STN1* over-expression improves TEL-ARS plasmid stability in wild-type, *cdc7-1*, *dbf4-1*, and *mcm7-1*, suggesting that high levels of Stn1p could promote either replication or segregation of the TEL-ARS plasmid. I favored the possibility of excess Stn1p in improving telomere replication because the mutant strains being tested here carry defects that are focused on replication. Nevertheless, we still cannot rule out the possibility of over-expressed *STN1* in helping the telomere-based segregation. Thus, I examined if excess Stn1p could increase the TEL-ARS plasmid stability in telomere-based segregation mutants, *sir4Δ* and *rap1-5*. The data demonstrates that the high level of Stn1p does not improve

the plasmid stability in either *sir4Δ* or *rap1-5*, suggesting either 1) excess Stn1p does not improve telomere-based segregation or 2) the function of Stn1p in facilitating telomere-based segregation is dependent on both Sir4p and Rap1p. It will be interesting to use GFP marked TEL-ARS plasmid to examine if indeed *STN1* over-expression can facilitate telomere-based segregation.

I also investigated the third hypothesis to see if over-expressed *STN1* can improve replication away from telomeres or not. The CEN-ARS plasmid stability data demonstrate that *STN1* over-expression can suppress the plasmid instability phenotype in *cdc7-1* and *mcm7-1*. The results strongly suggest that excess Stn1p can function away from telomeres as the CEN-ARS plasmid contains no telomeric sequences. However, the ability of Stn1p to stabilize the plasmid could either be due to replication facilitation or segregation improvement. To clarify the function of excess Stn1p in stabilizing the CEN-ARS plasmid, I compare the plasmid stability data within the CEN-ARS and the TEL-ARS plasmid. The interesting outcome is that, both CEN-ARS and TEL-ARS plasmid stability is improved by excess Stn1p in *cdc7-1*. Since the two plasmids use two distinct segregation mechanisms, the likelihood for excess Stn1p in facilitating the centromere-based segregation is low. Thus, I conclude that over-expressed *STN1* could facilitate replication at non-telomeric sites.

As the data suggest, excess Stn1p helps to improve replication, thus if I use loss of function alleles of *STN1* or even *TEN1* that cause severe telomere defects, I should see decrease in the plasmid stability for TEL-ARS. Surprisingly, the loss of function alleles, *stn1-281t* and *ten1-105* both retain the TEL-ARS plasmid in almost 100%

percent of cells, and show a loss rate that is close to 0% even in the absence of selection for markers on the plasmid. This unexpected outcome could possibly be a result of prolonged mitosis in both of the mutants. The idea is backed up by a published literature arguing that because of the nuclear geometry and the kinetics of passively segregating TEL-ARS plasmid, the delayed mitosis will enhance the ability for daughter cells to retain the plasmids (Gehlen et al. 2011). However, other speculations remain possible. Another explanation could be that the timing of the origin firing is altered in *stn1-281t* or *ten1-105*. In yeast, origins that are placed next to the telomere sequences will fire late in the S phase (Ferguson and Fangman 1992), which might be a reason for the low stability of the TEL-ARS plasmid in wild-type cells. The ability to alter the timing of origin firing near telomere sequences could require the function of Stn1p, thus loss of function alleles might loss this ability and ultimately stabilize the TEL-ARS plasmid. It would be interesting to relocate the ARS on the TEL-ARS plasmid to see if there is any effect on the timing of origin firing. Also, it is tempting to construct the CEN-ARS plasmid with late firing origin and compare the stability with the early fired CEN-ARS plasmid see if excess Stn1p will have any effects.

Some possible experiments could be done in the future to have a more close understanding of how over-expressed *STN1* in improving replication. Besides alternating the timing of origin firing, excesses Stn1p could possibility interact with RPA complex in stabilizing the replication fork as Stn1p is structurally similar to Rpa2p. Co-immunoprecipitation of Rpa3p with Stn1p could be one test. Examining the fork progression by combing in Rpa2 mutant with *STN1* over-expression is

another tempting experiment. Also, as published articles suggesting that Stn1p might promote replication by recruiting Polα (Grossi and Shore 2004, Petreaca et al. 2007), one could examine the co-localization of RFP-tagged Polα and GFP-tagged Stn1p with hydroxyurea treated wild-type cells.

In sum, my work strongly suggests that excess Stn1p could improve replication at both telomeric and non-telomeric sites. This finding is significant as other groups of scientists recently discovered that Stn1p orthologs in humans can promote replication away from telomere by enhancing fork restart under replication stress (Stewart et al. 2012) as well as xStn1p can also improve replication by promoting priming of ssDNA in Xenopus egg extracts (Nakaoka et al. 2011). Together, these data propose that Stn1p has other role than telomere specific protection that has never been thought before.

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## Appendix I

Chapter 2 Strain list

| Strain | Relevant Genotype | Reference |
|---|---|---|
| hC160 | *MATα ura3-52 ade2-101 lys2-801 leu2-D1 his3-D200* | Gasparyan et al. 2009 |
| hC2403 | *MATa cdc7-1 ura3-52 his6 trp1-289 leu2-3,112 bar1* | This study (Nugent) |
| JBY999 | *MATα dbf4-1 ade2 -1 can1 -100 his3 -11,15 leu2 -3,112 trp1 -1 ura3 -1* | This study (Bachant) |
| DBY2029 | *MATα mcm7-1(cdc47-1) ade2-1 lys2-801 leu2-3,112 ura3-52* | Fitch et al. 2003 |

Chapter 2 Plasmid list

| Plasmid | Type/ Promoter/ Marker/ Gene/ CEN/ ARS | Reference |
|---|---|---|
| pJBN218 | *URA3 ADE2 CEN6 ARSH4*（pRS416 backbone) | This study (Bachant) |
| pCN416 | *2μ ADH promoter LEU2* | Gasparyan et al. 2009 |
| pCN421 | *2μ ADH promoter LEU2 STN1* | Gasparyan et al. 2009 |
| pAS2 | *2μ ADH promoter GAL4 DBD TRP1* | CLONTECH Laboratories, Inc |
| pCN188 | *2μ ADH promoter GAL4 DBD TRP1 STN1* | This study (Nugent) |

## Appendix II

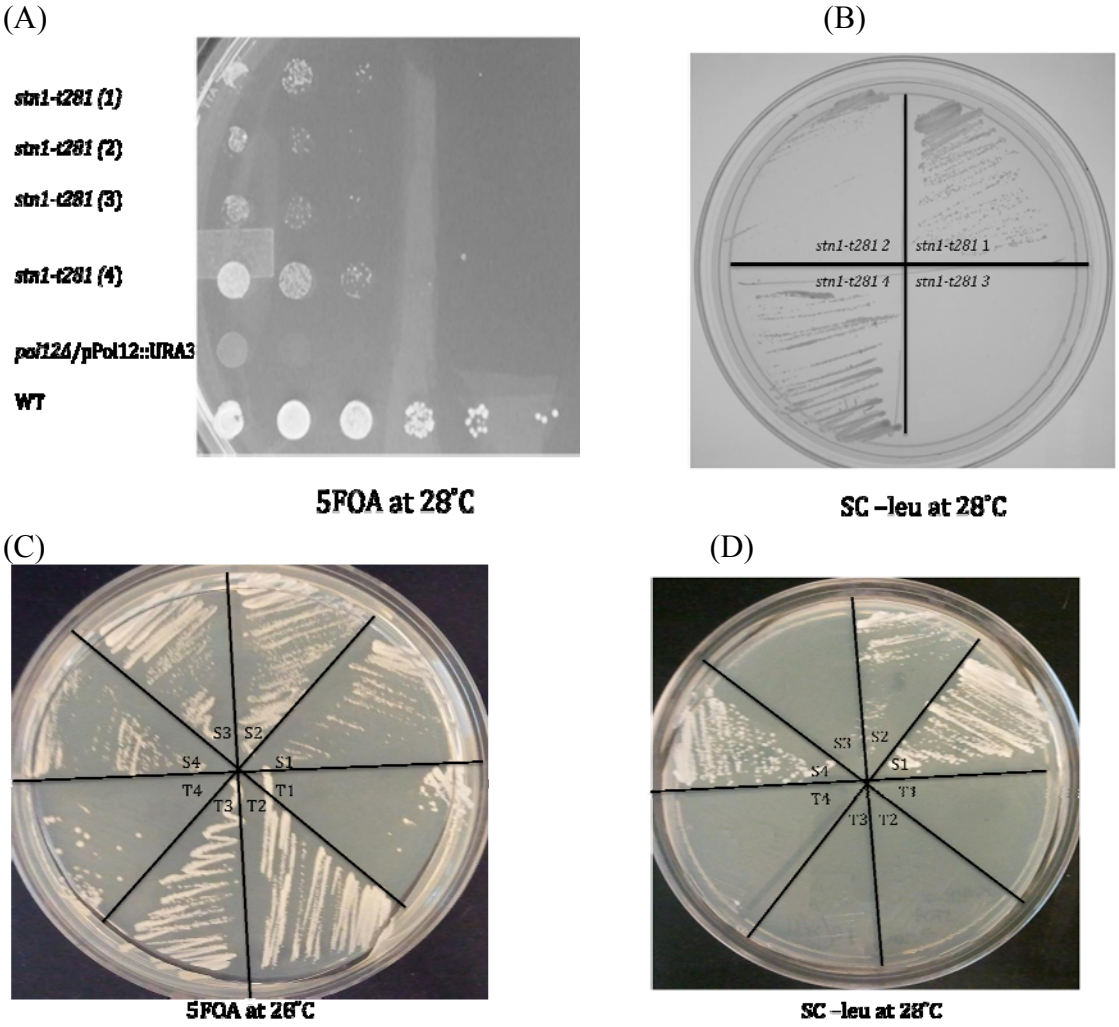
Chapter 3 Strain list

| Strain | Relevant Genotype | Reference |
|---|---|---|
| hC160 | *MATα ura3-52 ade2-101 lys2-801 leu2-D1 his3-D200* | Gasparyan et al. 2009 |
| hC2403 | *MATa cdc7-1 ura3-52 his6 trp1-289 leu2-3,112 bar1* | This study (Nugent) |
| jby999 | *MATα dbf4 -1 ade2 -1 can1 -100 his3 -11,15 leu2 -3,112 trp1 -1 ura3 -1* | This study (Bachant) |
| DBY2029 | *MATα mcm7-1(cdc47-1) ade2-1 lys2-801 leu2-3,112 ura3-52* | Fitch et al. 2003 |
| hC2241 | *MATα ura3-52 ade2-101 lys2-801 leu2-D1 his3-D200 ten1Δ::ten1-105* | This study (Nugent) |
| hc671 | *MATa ura3-52 ade2-101 lys2-801 leu2-D1 his3-D200 stn1-281t::kanMX2* $CF^{+}$ | Petreaca et al. 2007 |
| YJB208 | *MATa HMRa ade2-1 canl-lO0 his3-11,-15 leu2-3,-112 trpl-1 ura3-1 rap1-5* | Kurtz and Shore 1991 |
| hc1654 | *MATa ura3-52 leu2-3,112 his36 trpl-289 ade2Δ sir4Δ::HIS3* pJBN218 | Nugent lab |

Chapter 3 Plasmid list

| Plasmid | Type/ Promoter/ Marker/ Gene | Reference |
|---|---|---|
| pJBN218 | *URA3 ADE2 CEN6 ARSH4* (pRS416 backbone) | This study (Bachant) |
| YRpRW41 | *URA3 LEU2 KanMX ARSH4* (pRS305 backbone) | Dionne and Wellinger 1998 |
| pCN416 | *2μ ADH promoter LEU2* | Gasparyan et al. 2009 |
| pCN421 | *2μ ADH promoter LEU2 STN1* | Gasparyan et al. 2009 |
| pAS2 | *2μ ADH promoter GAL4 DBD TRP1* | CLONTECH Laboratories, Inc |
| pCN188 | 2*μ ADH promoter GAL4 DBD TRP1 STN1* | This study (Nugent) |

## Appendix III




**Examination of the TEL-ARS plasmid integration in *stn1-281t* (hC671) and *ten1-105* (hC2241).**

A) Four different *stn1-t281* colonies were picked from the SC -leu plates. Strains were grown to saturation in 2 mls of YPD and 10-fold serial dilutions were performed. Strains were then stamped onto 5FOA and incubated at 28°C for 4 days. *pol12Δ*/pPol12::URA3 was used as a negative control B) Four independent single colonies were picked from four different biological duplicates of *stn1-t281* from the 5FOA plate in (A) and were streaked onto a SC–leu plate. The plate was incubated at 28°C for 5 days. C) Four independent *stn1-t281* and *ten1-105* colonies were picked from the SC -leu plates and streaked onto 5FOA, then incubated at 28°C for 5 days. D) A single colony from each streak out from the 5FOA plate in (C) was streaked onto SC –leu plate and incubated at 28°C for 5 days. S1to S4 = *stn1-t281* (1) to *stn1-t281* (4) and T1 to T4 = *ten1-105* (1) to *ten1-105* (4)