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# Telomere Capping Protein Stn1p Require the RAD6-Mediated Ubiquitination Pathway for DNA Repair and Function in Global DNA Replication

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# DEDICATION

I dedicate this thesis to my parents, grandparents, friends, colleagues, and UCR faculties, who instilled in me, from a very young age, the virtues of hard work, diligence, respect, and integrity.

ABSTRACT OF THE THESIS

Telomere Capping Protein Stn1p Require the RAD6-Mediated Ubiquitination Pathway for DNA Repair and Function in Global DNA Replication

by

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Dr. Constance Nugent, Chairperson

In the budding yeast, *S. cerevisiae,* telomere integrity relies on the function of associated proteins. Current models posit that the Cdc13-Stn1-Ten1 (CST) complex has a dual role in the budding yeast, *S. cerevisiae*. First, it has a role physically capping the chromosome ends to prevent exonucleolytic degradation and inappropriate recombination. Second, it facilitates the efficient replication of chromosome ends through the activities of telomerase and Pol-α primase. The work in this thesis shows that compromising this complex, and the Stn1 member protein in particular, imposes a requirement for the *RAD6* post-replication DNA repair pathways. *RAD6* encodes an E2 ubiquitin conjugating enzyme that, through its interactions with distinct E3 ubiquitin ligases, promotes a number of cellular activities, including pathways for post-replication repair. Thus,

the hypothesis tested in this thesis is that the loss of *STN1* function leaves single-stranded DNA gaps that are repaired through Rad6/Rad18 or Rad6/Bre1 mediated pathways.

Here it is shown that the genetic interaction between *STN1* and *RAD6* is synthetically lethal. Interestingly, the *stn1-t281 bre1-*Δ double mutant but not the *stn1-t281 rad18-*Δ revealed a strong synthetic phenotype, indicating that the function of the Bre1 ligase and histone monoubiquitation is important when *STN1* is compromised. The question of whether *STN1* is important for genome integrity outside of telomeres was also explored using two different assays to detect aberrant chromosomal single-stranded regions. One assay assesses global chromosomal breaks and single-stranded regions, and the second directly tests repetitive telomere and rDNA regions. The initial data gathered here confirm that *stn1-t281* strains have high levels of single-stranded telomeric DNA, and provide initial data supportive of further examination of non-telomere related genome instability in strains compromised for *STN1* function.

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### Chapter 2



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# Chapter 1

# Introduction to Telomeres and the *RAD6* Pathway

## Essential capping function of telomere

*Telomere structure*

Eukaryotic cells have chromosomes with linear ends; these termini present a problem when replication occurs. For every replication cycle, the ends of the linear chromosomes resect or undergo recombination events. To understand the molecular events at the ends of linear chromosome, the budding yeast is a useful model system for deciphering the molecular events of telomere. In the budding yeast *Saccharomyces cerevisiae*, the chromosomes end with 300 bp of irregular $TG_{1-3}$ / $C_{1-3}A$ repetitive sequences that terminate with short single-stranded 3' overhangs of the G-rich repeats (Wellinger and Sen, 1997). The 3' overhang length varies among species, averaging around 10-15 nucleotides in yeast to >100 nt in mammals (Chakhparonian and Wellinger, 2003). Both the repetitive duplex and single-stranded overhangs of telomeres serve as binding sites for various proteins that protect the ends and allow complete or near-complete duplication.

*Capping – what it means to be "capped" by telomeres*

Linear chromosomes must be "capped" to prevent DNA damage leading to loss of chromosome integrity and cell viability. (Muller, 1938, McClintock 1938,

de Lange 2002). Capping is an essential function for the protection of chromosome ends from degradation, fusion, resection, and DNA damage. If there is no capping, the linear chromosome ends will degrade, resect, fuse end-to-end, and recognize as DNA damage. The essential capping function of telomeres was solved by the presence of complexes of repetitive DNA and protein that protect the ends of chromosomes. In eukaryotes, the majority of the telomere sequence is double-stranded DNA (dsDNA). To facilitate chromosome end protection, there are several classes of telomeric dsDNA binding proteins that bind to double stranded DNA (dsDNA). Single-strand DNA binding proteins also play an important role in the capping.

Without capping of the chromosome ends by telomere proteins, the ends would be recognized as damaged DNA, similar to a DNA double-strand break or to a degraded, single-stranded end. The loss of a telomere in wild-type yeast cells was shown to cause a DNA damage-mediated cell cycle arrest at G2/M, suggesting that telomere can help cells distinguish intact chromosomes from damaged DNA (Sandell and Zakian, 1993). In these experiments, they showed that the elimination of telomere repeats leads to a significant increase in the loss of that chromosome, suggesting that yeast telomeres are essential for maintaining the stability of chromosomes. In telomere deficient cells, the chromosome ends are recognized as being damaged and try to repair themselves via non-homologous end joining (NHEJ) (Sandell and Zakian, 1993). Interestingly, many cells recovered from the cell cycle checkpoint arrest without

repairing the damaged chromosome, and then underwent about ten cell divisions before the chromosome was eventually lost (Sandell and Zakian, 1993). In human cells, the loss of the protective telomere sequences or the proteins critical for mediating the cap function can lead to chromosome end-to-end fusions (Van Steensel & de Lange, 1998).

*Capping -mechanisms in ciliated protozoa*

The discovery that telomeres have capping protein was in the ciliate *Oxytricha nova* that has fragmented gene-sized molecules terminating with a $C_4A_4$ repeat in their genomes (Gottschling et al, 1984). The gene-sized molecules provide an abundance source of chromosomes ends. It was found that these chromosome ends contain a terminal complex involving about 100 bp with nucleosomes that function as telomere, and provide stability at the end of the linear DNA (Gottschling et al, 1984). Each *Oxytricha* telomere ends have short 3' overhang that is bound by a single-stranded DNA binding protein, TEBP, composed of an α and β subunit. TEBPα binds to telomeric repeat sequence in the overhang and the β subunit stabilizes the DNA-protein complex. The α and β form an extensive interface along the overhang and protect the 3' end of the telomere (Horvath, et al., 1998). The telomere complex provides an effective way to protect and hide from DNA repair enzymes. Another mechanism of capping in the hypotrichous ciliate involve a structure called a "t loop". A t-loop structure form when the 3' G-rich, single stranded overhang fold back and hybridizes with

the downstream sequence, analogous to a displacement loop formed during DNA recombination. The t-loop structures are found in micronuclear telomeres in micronucleus only. However, as intriguing as the capping mechanism in ciliates, they only provide a limited experimental system.

*Capping – mechanism in humans*

In humans, there are six proteins (POT1, TPP1, TIN2, TRF1, TRF2, and RAP1) that assemble together into one intriguing telomere capping complex called telosome/shelterin (Palm and de Lange, 2008). In mammals, this Shelterin complex has a key responsibility for capping and protecting chromosome ends. Two oligosaccharide binding (OB) fold containing proteins, TPP1 and Pot1, can form heterodimers that bind to the telomeric single-stranded DNA (ssDNA) for end protection or capping. In mammals, TRF1 and TRF2 proteins directly bind to the duplex DNA. Both proteins form homodimers, and associate with telomeric repeats through their C-terminal myb domains (Broccoli et al, 1997). Both TRF1 and TRF2 have roles in telomere capping and length regulation. Deletion mutation in TRF1 initiates a DNA damage response at telomeres because of the activation of the checkpoint kinases CHK1 and CHK2 (Martinez et al., 2009). The function of TRF2 is to prevent chromosome ends from being recognized as breaks and inhibits the fusions of chromosome. Deletion of TRF2 results in rapid and extensive chromosome end-to-end fusions and the accumulation of telomeric DNA damage foci (van Steensel et al, 1998).

When the telomeres in mammalian cells are dysfunctional or damages, it activates the ataxia telangiectasia mutated (ATM) kinase signaling pathway for DNA damage of telomeres. The loss of TRF2 causes an ATM dependent checkpoint response (Denchi and de Lange, 2007).

TRF2, which has affinity to double-stranded regions of telomere, is critical for capping chromosomes. It is thought that the TRF2 mechanism of action for telomere protection is by chromatin reorganization. TRF2 facilitates the remodeling of telomeric DNA such that the single stranded 3' overhang invades the double-stranded telomere repeats, forming a structure that has been termed a t-loop. The mechanism is the same as in the ciliate, although the ciliate t-lopp occur only in micronuclear cells (K.G. Murti & D. M. Prescott). The DNA sequence of mammalian telomeres has a precise TTAGGG repeat, allowing the ssDNA overhang to hybridize completely to the upstream duplex DNA aiding in the formation of the t-loop structure. The formation of the t-loop allows the interaction between ssDNA and dsDNA binding proteins of the Shelterin complex. Thus, TRF2 may prevent the ends of chromosomes from being recognized substrates for NHEJ (Celli et al. 2006; Okamoto et al. 2013).

While the telomere duplex binding protein TRF2 inhibits the ATM DNA damage response pathway, the single-stranded telomere binding protein POT1 prevents the activation of the Rad3-related (ATR) kinase signaling in cells with damage telomeres.

*Capping – mechanisms in S. cerevisiae*

In *S. cerevisiae*, the major dsDNA binding protein is the repressor activator protein 1 (Rap1p), along with its two interacting factors Rif1p and Rif2p (Lustig et al, 1990; Hardy et al, 1992; Wotton and Shore, 1997). Rap1p is a sequence-specific DNA binding protein that functions as both a repressor and activator of transcription that bind to the poly($C_{1-3}A$) repeats of telomeres in vitro (Hardy CF., et al. 1992). The role of Rap1p is protection of chromosome ends in fission yeast, and promoting gene silencing in *S. cerevisiae*. Rap1p also represses homology-directed recombination at telomeres in mammals (Chen Y. et al., 2011). Rap1p (ts) mutant was analyzed to have reduce telomere length in temperature-dependent manner. Plasmids containing the mutant *RAP1* binding sites were tested for their ability to function as substrate for poly (C1-3A) in vivo. Sure enough when there are mutations in the Rap1p binding sites, it will reduce the efficiency of the interaction with the telomere (Lustig AJ., et al. 1990). Genetic screen using the two-hybrid system have identified a gene encoding a RAP1-interacting factor (RIF1). Strains with mutation in RIF1 are defective in transcriptional silencing and telomere length regulation because of failure to bind to RAP1. Two hybrid system have confirmed that Rap1p missense mutation are defective in interaction with RIF1 (Hardy CF, et al., 1992). The data have suggested that RAP1 mutation failed to recruit Rif1p to telomere and that Rif1p is a mediator for RAP1 for telomere function. The Rap1p-Rif1p-Rif2p complex functions in both length regulation and capping at the telomere (Chen et al.,

2011). In addition to its role at telomeres, Rap1p functions in regulating transcription (Shore and Nasmyth, 1987). The function of Rap1p is that it interacts with both Rif1p and Rif2p (Marcand et al., 1997b). The mutants of Rap1p showed an increase in chromosome end-to-end fusions, suggesting Rap1p's role in inhibiting end-to-end fusion (Pardo and Marcand, 2005). It is observed that the loss of Rap1p results in nonhomologous end joining (NHEJ) between telomeres (Pardo and Marcand, 2005). These results have indicated that Rap1p is an essential protein to block NHEJ between telomeres (Pardo and Marcand, 2005). In *S. pombe*, the Rap1p protein does not directly bind to telomeric DNA, but it localizes to chromosome ends through an interaction.

In the budding yeast, there is a dsDNA binding protein called Ku70/80 heterodimer (Ku) (Gravel et al., 1998). Ku has numerous functions in DNA processes, including promotion of DNA repair by NHEJ, inhibition of homologous recombination, regulation of timing of origin firing, heterochromatin formation, telomere length regulation, and chromosome end capping (Fisher and Zakian 2005; Ribes-Zamora et al., 2007). Ku mutation showed increased telomeric ssDNA and have synthetic phenotype when combined with Cdc13p mutants (Nugent et al., 1998; Polotnianka et. al., 1998). Cdc13-1 mutant display extensive telomeric ssDNA accumulation in *yku70*-Δ cells, which is limited by the DNA damage checkpoint. Without checkpoint activity, resection can extend several thousand base-pairs into the chromosome (Maringele and Lydall, 2002). Ku also functions in an independent, parallel pathway with Rap1p complex to cap

telomeres in G1 (Bonetti et al, 2010; Vodenicharov et al., 2010). These results showed that Ku play a direct role in maintaining a normal DNA end structure on yeast chromosomes by functioning as a terminus binding factor (Gravel and Wellinger, 1998).

*Role of Cdc13p in protecting telomeres*

In the budding yeast *Saccharomyces cerevisiae*, the telomeres are capped by the well-known telomere protein Cdc13p. Cdc13p is a sequence specific single-stranded DNA binding protein with a strong affinity for telomeric repeats. *CDC13* was first identified in Hartwell's cell division cycle screens as a gene that when mutated, elicits a *RAD9*-dependent DNA damage checkpoint response (Garvik B. et al., 1995). Study have shown that *cdc13-1* mutants are temperature-sensitive; and at restrictive temperature, they can arrest in the G2 phase of the cell cycle as a result of DNA damage that activates the *RAD9* checkpoint response. *Cdc13-1* and *cdc13-1 rad9-*$\Delta$ double mutants were shown to accumulate aberrant amounts of single-stranded DNA (ssDNA) predominately at telomeres, and extends from the telomeres into unique subtelomeric sequences (Garvik B. et al., 1995;). Consistent with the presence of aberrant levels of ssDNA, *cdc13-1* strains also showed an increase in homologous recombination at chromosome end (Carson and Hartwell 1985; Garvik et al. 1995). Without Cdc13p, the 5' ends of chromosomes undergo exonucleolytic degradation in the telomere to centromere direction (Vodenicharov and

Wellinger, 2006). These observations have shown that the essential function of *CDC13* is to protect chromosome ends against inappropriate exonucleolytic activities. Both in vitro and in vivo studies have shown that Cdc13p has a very specific telomeric G-rich ssDNA binding activity (Nugent et. al., 1996; Lin and Zakian, 1996; Lin et al., 2001). Discovery of a second class of *cdc13* allele showed that it has two roles at the telomere. The *cdc13-2* mutation creates a telomerase deficient phenotype, indicating that the mutation negatively affects a function required in vivo for telomerase regulation (Nugent et. al., 1996). Together these data suggest that *CDC13* functions in telomere metabolism, either in the replication of telomeric DNA or in protecting telomeres from double-strand DNA damage (Garvik B., et al. 1995). Cdc13p acts together with two other telomeric binding proteins, Stn1p and Ten1p, to protect against nucleolytic degradation of telomeres in the budding yeast.

*Role of Stn1p and Ten1p in protecting telomeres*

In *S. cerevisiae*, the Cdc13, Stn1, and Ten1 proteins are essential for cell viability because they are critical for protection of chromosome ends against degradation and recombination. *STN1* was identified as a dosage suppressor of the *cdc13-1* mutation (Grandin et al., 1997), and *TEN1* was identified as a suppressor of a conditional *stn1* mutation (Grandin et al., 2001). Hypomorphic mutations in either *stn1* or *ten1* can lead to compromised end-protection and accumulation of terminal ssDNA, similar to *cdc13-1* mutant cells (Grandin et al.,

1997; Grandin et al,,2001; Petreaca et al., 2007; Puglisi et. al, 2008; Xu et. al., 2009). Compromising *stn1* function can also activate *RAD9* and *MEC3* G2/M checkpoints, confirming that DNA damage is occurring (Grandin et al., 1997).

The first 186 amino acids of Stn1p are required for the essential function of *STN1*; this domain is also necessary for interaction with Ten1p. In contrast, the carboxyl-terminal domain of Stn1 is necessary and sufficient for interaction with Cdc13 (Petreaca et al., 2006, Petreaca et al., 2007). Mutants that fail to interact with Cdc13 are viable, but have elongated telomeres that contain excessive single-strandedness (Petreaca et al., 2007). Another study has shown that overexpressing the amino terminus of Stn1p can complement the *stn1*-Δ allele (Gao et al., 2007). The study confirmed that the amino terminal end of Stn1p is necessary and sufficient for cell viability. Loss of function alleles of *STN1* and *TEN1* can also lead to the formation of extrachromosomal telomeric circles (t-circles) in budding yeast species, indicating that these telomeric proteins prevent recombination at telomeres (Iyer et al, 2005; Sun et. al., 2009; Basenko et.al., 2010).

All three proteins, Cdc13p, Stn1p and Ten1p, have been shown to interact physically by two-hybrid analysis (Grandin et al, 2001, Petreaca et al., 2006; Petreaca et al., 2007; Puglisi et. al., 2008, Xu et al., 2009). The predicted structure of Cdc13p-Stn1p-Ten1p has revealed conserved domain architecture to the replicate protein A (RPA) crystal structure (Rice & Skordalakes, 2016). This suggested that Cdc13p, Stn1p, and Ten1p form a heterotrimeric RPA-like

complex called the CST complex at telomeres that protects chromosome ends, and regulates telomerase elongation (Gao et. al., 2007; Paschini et. al., 2010). These three telomeric proteins interact with each other in specific domains for proper telomeric functionalities. Firstly, the amino terminus of Stn1p interacts with Ten1p, which is essential for capping function (Petreaca, et al, 2007, Puglisi et al., 2008). Secondly, the carboxyl terminus of Stn1p binds both Cdc13p, and the Stn1p-Cdc13p interaction negatively regulates telomerase (Petreaca et al, 2007, Puglisi et al., 2008). Studies have suggested that the binding of Cdc13p to telomeres helps to recruit Stn1p and Ten1p to the telomere (Pennock et al., 2001; Grandin et. al., 2001).

Ten1p is a protein that is known to regulate telomeric length and protect telomere ends as part of a complex with Cdc13p and Stn1p. A study showed that *ten1* temperature-senstive (ts) mutants display elongated telomeres at permissive temperatures. After a shift to non-permissive temperatures, the *ten1-ts* mutants accumulate extensive telomeric single-stranded DNA, suggesting that the *ten1-ts* strains are defective for telomere capping protection (Xu L. et. al., 2009). The study have shown that *ten1-ts* strains have a significant increase in telomeric single-stranded DNA and Rad52-YFP repair foci at high temperatures even though Cdc13p still binds to telomeres. This suggested that Cdc13p telomere binding is not sufficient for end protection and relied on Ten1p to carry out its essential function (Xu L. et al., 2009). Ten1p is also known to promote telomere addition.

*Resection of telomeres by MRX complex*

The ends of chromosomes are processed by the Mre11-Rad50-Xrs2 (MRX) complex to generate 3' overhangs (Larrivee et al., 2004; Takata et al., 2005). The MRX complex has endonuclease and helicase activities that process and modify the ends of chromosomes. Cells with a defective MRX complex cannot recruit Cdc13p or telomerase to chromosome ends generated by leading strand synthesis. In addition, telomere addition at a region adjacent to an HO induced double strand break also requires MRX processing (Diede and Gottschling, 2001). These data have indicated that MRX activity is a prerequisite to generate the terminal 3' overhang on telomeres synthesized by the leading strand.

*Cell cycle dependent regulation of telomere resection*

The chromosome end resection of both normal and uncapped telomeres is regulated in a cell cycle dependent manner by the Cdk1, the yeast cyclin-dependent kinase (Frank et al. 2006; Vodenicharov and Wellinger 2006). When telomeres are uncapped, they will undergo C-strand resection during G2/M phase, and inhibiting Cdk1 activity suppresses resection in these mutants. This data have suggested that Cdk1 kinase activates the nuclease for telomere C-strand degradation (Vodenicharov and Wellinger 2006, Xu L. et al., 2009). Cdk1 dependent processing is necessary to generate a functional 3' overhang. The inhibition of Cdk1 will prevent telomere addition at a critically short telomere

(Frank et al, 2006). The genetic analysis has shown that Cdk1 activity is required for telomere addition by generating the 3' overhang. Cdk1 kinase also phosphorylates Cdc13p to promote telomere addition (Li et al, 2009; Tseng et al., 2009). These data suggested that Cdk1 kinase is involved in regulating the end replication, C-strand resection, telomere elongation and capping.

*CST in humans*

In humans, six proteins of the Shelterin complex that is critical for telomere capping do not include homologs of Cdc13, Stn1 or Ten1. The OB fold-containing protein Pot1 is the only protein within the complex that directly binds to ssDNA; the other five members either bind to dsDNA directly, or associate with the complex through protein-protein interactions. However, large scale immunoprecipiations and mass spectrometry analysis of the TPP1 protein complexes in mammalian cells showed an association with the OB fold-containing protein 1 (OBFC1). OBFC1 was identified as a Polymerase α-accessory factor, called AAF44, in experiments using mice cell extracts (Wan & Liu, 2009). Bioinformatic sequence analysis revealed that the mammalian OBFC1 is a homolog of the yeast Stn1 protein. In human cells, OBFC1/AAF44 has been shown to localize to telomeres and bind to telomeric ssDNA in vitro (Wan & Liu, 2009). In addition, the over-expression of an OBFC1 mutant has resulted in telomere elongation and dysregulation in human cells, suggesting

OBFC1/AAF4 is a novel telomere-associated OB fold protein functioning in telomere length regulation (Wan M., et al. 2009).

In mammals, a complex analogous to the yeast CST complex has also been identified (Wan et al, 2009, Miyaki et al. 2009, Surovtseva et al., 2009). Specifically, the homologs of both the Stn1 and Ten1 proteins have been identified, and these interact with a larger DNA binding protein called Ctc1. In mammals, all three proteins within the mammalian CST complex physically interact and associate with telomeric ssDNA. Silencing of the expression of *CTC1* or *STN1* results in degradation of the telomeric C-strand, and stimulates formation of telomeric DNA damage foci (Miyake et al, 2009; Surovtseva et al, 2009).

In mammals, the CST complex appears to function independently of Pot1 in telomere capping because knocking down both Stn1 and Pot1 causes a synergistic induction of DNA damage foci (Miyake et al., 2009). Studies have shown that the human protection of telomeres 1 (Pot1) and STN1 interact physically with TPP1, the bridging factor within the Shelterin complex that connects ssDNA binding proteins to duplex DNA binding proteins (Hockemeyer et al, 2007; Wang et al., 2007; Xin et al., 2007; Wan et. al., 2009).

*Capping – mechanism in fission yeast S. pombe*

In the fission yeast *S. pombe*, study from Bauman and Cech found a distant ortholog of TEBPα called Pot1+ (protection of telomeres). Pot1p was

identified from the weak sequence similarity with the N-terminal OB-fold domain of TEBP α subunit (Baumann and Cech, 2001). The Pot1 (protection of telomeres) proteins bind to the G-rich strand of telomere repeat for the protection of chromosome ends (P. Bauman and T.R. Cech, 2001). Like Cdc13p, Pot1p binds to the single-stranded G-rich telomeric overhang of fission yeast (Bauman and Cech, 2001). Pot1 protein binds along the length of the single-stranded DNA tail of the telomere. When pot1+ is deleted in fission yeast cells, there is a complete loss of telomeric DNA due to exonucleolytic degradation and activation of DNA damage checkpoint (Baumann and Cech, 2001). Some pot1- cells are able to survive by circularizing their three chromosomes, where each chromosome fusing its ends together to survive the loss of the telomeres.

Pot1p is not an ortholog of the budding yeast Cdc13p (Theobald and Wuttke, 2004). Cdc13p is not an ortholog of TEPB α and β. Although Pot1p and Cdc13p do not share similar sequence homology, they each contain an OB-fold domain, which is a common protein domain that the proteins use to recognize telomeric G-rich ssDNA (Theobald and Wuttke, 2004). The respective OB folds, however, associate with the telomeric DNA a bit differently in each protein (Lei et. al., 2004). Based on sequence analysis, the Cdc13p DNA binding domain also shares weak sequence similarity to the N-terminal Pot1p OB-fold domain (Theobald et al., 2003a). Using sequence profile analysis directed at tandem copies of domains, there are evidence that both the vertebrate Pot1p proteins

and Cdc13p proteins are likely to contain additional multiple OB-fold domains (Theobald and Wuttke, 2004).

Stn1p and Ten1p homologs were identified in *S. pombe* by sequence alignment and structural prediction algorithms (Martin et. al., 2007). Multiple alignment of the N-terminal sequences of the Stn1p orthologs in *Homo sapiens, S. pombe, Aspergillus nidulans, Neurospora crassa, and S. cerevisias* were done simultaneously. Alignment of RPA is also provided. PSI-Blast indicates that the sequence similarities of the OB-fold domain are highly significant among the species indicating that the structures are conserved in evolution. The primary amino acid sequences have not diverged significantly through evolution. In an evolutionary aspect, both Stn1p and Ten1p from various species displayed remarkable conservation of their three-dimensional structures (Horvath, 2011). Similar to the budding yeast, Stn1p and Ten1p in *S. pombe* physically interact, and localize to telomeres. Mutation of either *stn1* or *ten1* leads to rapid loss of telomeric sequence, and end-to-end fusions that circularize chromosomes, similar to *pot1* deletion (Martin et. al., 2007). Stn1p and Ten1p do not physically interact with Pot1p, confirming the idea that Pot1p is not an ortholog of Cdc13p (Martin et al., 2007). There is no known Cdc13p homolog reported in *S. pombe*. These observations have indicated that fission yeasts have evolved to utilize two separate end-capping mechanisms to protect their telomeres.

There is a human ortholog of Pot1p identified by Baumann and Cech. Mammalian cells lacking Pot1p accumulate DNA damage foci containing γH2AX,

53BP1 and MDC1 at their telomeres and activate the ATR (Mec1) checkpoint response (Wu et. al., 2006; Denchi and de Lange, 2007; Hockemeyer et. al., 2007).

*Capping mechanisms - in plants*

CST-like proteins have been reported in plants (Song et al, 2008; Surovtseva et al, 2009; Miyake et al., 2009; Price et al., 2010). In plants, the *STN1* homolog appears to be fairly conserved. However, the *CDC13* has diverged significantly, and therefore have been termed *CTC1* (conserved telomere maintenance component 1). *CTC1* is not a sequence homologue of Cdc13p, but it has similar functionality. Similar to Cdc13p, *CTC1* physically interacts with *STN1* and with the lagging strand replication machinery (Casteel et al., 2009, Miyake et al., 2009, Surovtseva et al, 2009). There is also a *TEN1* homolog that appears to modulate telomerase processivity in the plant Arabidopsis (Shippen DE, 2013). In plants, *CTC1* forms a complex with *STN1* and *TEN1* and binds single-stranded DNA in sequence-independent manner (Miyake et al., 2009). CST also plays a key role in protecting telomere in plants. *Arabidopsis thaliana* can tolerate the absence of *CST,* but exhibit dramatic chromosome instability phenotypes. The *ctc1* and *stn1*- null mutants show dramatic telomere shortening, end-to-end chromosome fusions, increased G-overhangs, and elevated extrachromosomal telomeric circles, indicative of telomere recombination (Song et al., 2008, Surovtseva et al. 2009). Genetic

analysis has confirmed that *STN1* and *CTC1* are in the same pathway for chromosome end protection (Surovtseva et al., 2009).

Unlike mammalian cells, *Arabidopsis* only has a subset of the shelterin components. *Arabidopsis* encodes three POT1-like proteins, which associate with telomerase instead of telomere (Surovtseva et al., 2007, Rojas et al., 2011). Thus, it is thought that in plants, the CST functions as the major telomere protection complex (Price et al., 2010). Similar to the budding yeast, mutation in *STN1* and *CTC1* lead to loss of telomeric DNA sequences and generation of t-circles. Cells deficient in *STN1* and *CTC1* also display chromosome end-to-end fusions, which lead to the generation of di-centric chromosomes and anaphase bridges during mitosis (Song et al, 2008; Surovtseva et al., 2009).

When telomeres are compromised due to loss of essential capping, the unprotected telomeres trigger cellular DNA damage response (DDR) that mediate ATM (ataxia-telangiectasia mutated) or ATR (ATM and Rad3-related) (Sabourin and Zakian, 2008). ATM responds to double-strand breaks, whereas ATR is activated by excessive single stranded DNA (Nam and Cortez, 2011). *STN1* and *CTC1* deficient cells initiate the activation of an ATR-dependent DNA damage checkpoint and stimulate apoptosis (Amiard et al, 2011; Boltz et al., 2012). Plants deficient in both ATM and TERT, the telomerase catalytic subunit, show abrupt onset of genome instability compared with *tert* single mutants (Vespa et al., 2005). Analysis of telomere tracts has shown that ATM prevents “stochastic” intra-chromosomal recombination that shortens repeats, allowing

cells to maintain the length of telomere on the chromosome (Vespa et al., 2007). ATR appears even more crucial to telomere maintenance than ATM (Vespa et al., 2005). The double mutant *atr tert* showed greatly shortened telomeres compared to the single *tert* mutant.

## Replicative role of telomere

*The end replication problem*

In addition to the capping function, telomeres facilitate the replication of chromosome ends. DNA replication happens when two strands denature, and each strand serves as templates for the synthesis of two new complementary strands. During DNA replication, DNA polymerase synthesizes a new daughter strand. DNA polymerase synthesized new strand only in the 5' to 3' direction. Because DNA polymerase synthesized DNA in one direction 5' to 3' only, an RNA primer is needed to initiate new DNA strand synthesis. Due to this limitation of DNA polymerase, one of the strand of DNA will be replicated continuously called the leading strand. The other strand will be replicated discontinuously called the lagging strand. This type of replication is known as semi-conservative (Meselson and Stahl, 1985). In the leading strand, an RNA exonuclease will remove the primer after DNA replication is complete. DNA polymerase I and ligase will fill-in the gap and seal the nick respectively. In the lagging strand, the discontinuous new DNA is called Okazaki fragments and a problem occurs when replication reaches the end of DNA. After removal of the last primer, the DNA

polymerase and ligase cannot replace the RNA with DNA because there is no free 3' nucleotide present. Since there is no more DNA in the 5' direction after the final RNA primer, DNA polymerase cannot replace the RNA with DNA. Therefore, a gap is formed at the end of the chromosome. The gap in the lagging strand prevents new primer to synthesize new complementary strand at end of chromosome. So the daughter DNA strand has an incomplete 5' strand with 3' overhang. This DNA replication problem occurs in organisms with linear chromosomes, and is commonly called the "End Replication Problem". Bacteria with circular chromosomes do not have a similar problem. In every round of replication, the DNA ends shorten as each daughter DNA would become shorter than the parental DNA.

In one model by Leonard Hayflick, cells have a limited replicative capacity that is a key determinant of the number of division cycles they may carry out. In experiments carried out by Hayflick, he demonstrated that cells could only be cultured for 50 generations before the population would senesce (Hayflick, 1965). This observation suggested that the end-replication problem caused a gradual erosion of telomeres resulting in cell death. Not all cells became senescent, and some cells were able to evade the end-replication problem by maintaining their telomeres.

*Replication of telomeres by telomerase*

In 1985, the mechanism by which cells maintain their telomeres was discovered in the Blackburn lab at the University of California, San Francisco (UCSF). The Blackburn group was able to show that *Tetrahymena* cells contained an enzyme capable of adding terminal repetitive sequences (TTGGGG) to a linear DNA molecule (Greider and Blackburn, 1985). In her experiments, single-stranded DNA oligonucleotides $(TTGGGG)_4$ functioned as a primer for elongation at telomere, the synthesis of the TTGGGG repeats was independent of the endogenous DNA polymerase α, and the activity extending the primers required RNA. The data led them to propose that a novel telomere terminal transferase is involved in the addition of telomeric repeats essential for the replication of chromosome ends in eukaryotic cells (Greider and Blackburn, 1985). This terminal transferase is called telomerase, and functions as a reverse transcriptase. Telomerase uses an intrinsic RNA template to catalyze the addition of repetitive G-rich sequences to the 3' ends of chromosomes (Lingner et al, 1997b). The complementary C-rich strand is subsequently synthesized by DNA polymerase alpha (Pol-α). Telomerase and DNA polymerase alpha (Pol-α) are able to counteract the gradual erosion of chromosome ends, and overcome the end-replication problem.

In *S. cerevisaie*, the telomerase holoenzyme is composed of mainly three subunits (Est1, Est2, and Est3), and an RNA template (*TLC1*) (Lundblad and Szostak, 1989; Singer and Gottschling, 1994; Lendvay et al, 1996). The Est2

subunit contains the catalytic activity, whereas Est1 and Est3 are accessory factors (Lingner et al, 1997b; Evans and Lundblad, 1999; Hughes et al, 2000). These four components are all required for telomere maintenance in vivo. However, only Est2 and Tlc1 alone are needed for elongation of a telomeric seed in vitro (Lingner et al, 1997a).

*Recruiting and Regulating telomerase*

There are two genetically separable pathways in which telomerase gets recruited to the chromosome ends. The first pathway is through the Ku complex, where the Ku80 protein can recruit telomerase through an interaction with the stem-loop region of *TLC1* telomerase RNA (Fisher et al, 2004). When the interaction of Ku80-Tlc1 is disrupted, telomeres become moderately shorter (Stellwagen et al, 2003). In addition, the Ku80-Tlc1 mutant showed a reduction of DNA repair via telomere healing by 10 to 100-fold (Stellwagen et al, 2003). Thus, the interaction of Ku and TLC1 RNA enables telomerase to maintain chromosome ends, and to add new telomeres to DNA double-strand breaks with adjacent short telomere tracts. The second pathway of recruiting telomerase to the chromosome end is through an interaction between Est1 and Cdc13 (Nugent et al, 1996). The proposed mechanism of action is that Cdc13 recruits Est1 to the telomeres in late S phase. When the interaction of Cdc13 and Est1 is disrupted, this will lead to continual telomere shortening and senescence, as observed with the *cdc13-2* mutant strain (Nugent et al, 1996). The Cdc13-interacting

telomerase subunit is needed to bind to Est1 and this interaction mediate recruitment of telomerase complex (Pennock & Lundblad, 2001). Another study has confirmed through studying purified peptides that the Cdc13-Est1 interaction is mediated by an N-terminal region of Cdc13 and a recruitment domain of Est1 (Wu & Zakian, 2011).

It is known that telomerase does not seem to be recruited to every chromosome end within each S phase. Instead, telomeric repeats act as a buffer, and when they become short, structural changes occur that make telomerase more likely to extend that end. Studies in *S. cerevisiae* where telomeres are cloned and sequenced following a round of extension by telomerase concluded that telomerase does not act on every telomere in each cell cycle, and furthermore, that telomerase shows an increasing preference for shorter telomeres (Teixeira et al, 2004; Chang et al, 2007). In addition, by using two distinct telomerase RNA subunits with distinct templating regions, it was shown that the repeat addition processivity of telomerase (where processivity means the number of telomere repeats the enzyme adds before it dissociates) is greatly increased at extremely short telomeres. The processivity of telomerase appears to be regulated by the ATM-ortholog Tel1 (Chang et al., 2007).

It has been proposed that telomeres can adopt states that either promote or inhibit elongation by telomerase, and that the double-strand telomere DNA binding protein Rap1 and its binding partners are key to this switch between telomerase extendible and nonextendible states (Teixeira MT., et al. 2004). Thus

telomere length regulation is proposed to utilize a negative feedback mechanism that counts the number of Rap1 proteins bound to the telomere repeats, which ultimately generates a signal that regulates telomerase action (Marcand et al, 1997a; Shore and Bianchi, 2009). Consistent with this proposal, deletion of the Rap1 telomere associated proteins Rif1p or Rif2p results in longer telomeres and an increased frequency of telomerase elongation. Conversely, chromosome ends that are shorter have fewer bound Rap1 protein complexes, and, as measured by ChIP at a single shortened telomere, more efficiently recruit Cdc13p, Est1p, Est2p, as well as the checkpoint kinase Tel1p, which stimulates telomerase activity (Bianchi and Shore, 2007b; Chan et al. 2007; Hector et al, 2007; Sabourin et al, 2007).

In rare cases, telomerase adds telomere repeats to internal DNA double strand breaks (DSB). This event is termed telomere healing, and can stabilize chromosome fragments and truncations, and contribute to genomic instability (Diede and Gottschling, 1999). Typically, other repair pathways will act to repair DSBs, and, lacking telomere repeats, telomerase won't be bound nor recruited efficiently. Interestingly, it has been observed that *S. cerevisiae* Cdc13p can bind to double strand breaks and promote repair via telomere addition, particularly if the break has some flanking G rich sequences (Zhang and Durocher, 2010). Normally, the checkpoint kinase Mec1 phosphorylates Cdc13p to block its association with DSBs, thus promoting error-free recombination-dependent repair and inhibiting telomere healing (Zhang and Durocher, 2010). However, If the

broken ends are unable to be repaired by a recombination event or NHEJ pathways, the Pph3 phosphatase can eventually dephosphorylate Cdc13p, and repair will be promoted through telomere addition or healing.

*Alternate capping pathways in yeasts*

Not all organisms employ the same mechanisms to cap their telomeres, but do share many commonalities. In *S. cerevisiae,* a few variations have been identified that do not rely on Cdc13p to protect the ends (Larrivee and Wellinger, 2006; Petreaca et al, 2006; Zubko and Lydall, 2006; Ngo and Lydall, 2010). First, if cells lack key proteins involved in the detection and metabolism of terminal DNA damage, Sgs1p, Exo1p, and Rad9p, then cells divide with reduced or absent Cdc13p (Ngo HP and Lydall D., 2010). Pulse field gel electrophoresis showed that *cdc13-1 rad9-*Δ *sgs1-*Δ *exo1-*Δ strains are able to maintain chromosome ends despite the absence of telomere capping by Cdc13p. However, with continued cell division, the telomeres in the mutant cells became short and were maintained by recombination based mechanisms (Ngo HP and Lydall D., 2010). Second, recombination pathways that amplify telomere repeats (type II) or subtelomeric repetitive elements (type I) can help to ameliorate severely diminished or absent Cdc13p. In these cases, the normal checkpoint response is impaired as well (Larrivee and Wellinger, 2006). It was proposed that both type I and type II recombination pathways allow cells to adapt to the loss of the essential capping protein Cdc13p by inducing an alternate terminal

state. Finally, the telomere capping function of Cdc13p can be bypassed by altering the expression of the two Cdc13-interacting proteins, Stn1p and Ten1p. Co-overexpressing the *TEN1* gene with a truncated allele of *STN1* encoding just the first 186 amino acids bypasses the essential function of *CDC13* (Petreaca et al. 2006). In this case telomeres could be maintained along with fully functional DNA damage checkpoint and repair systems. However, the lack of Cdc13p did eventually lead to telomere shortening and senescese, likely because the recruitment function of Cdc13p was lost. Altogether, these data suggest that the eukaryotic telomere cap is flexible and allows for alternative mechanistic strategies for telomere capping.

## Intersection of telomere capping proteins and DNA replication

CST complex recruits Pol α to the telomere when Cdc13p interact directly with the catalytic subunit, Pol1 (Qi and Zakian 2000; Sun et al, 2011), and Stn1p binds to Pol12p via the regulatory B-subunit (Grossi et al, 2004; Petreaca et al, 2006). Pol12p and Stn1p have been shown to interact together by two hybrid and biochemical assays. Pol12p and Stn1p mutant displayed lethal synthetic interaction caused by the loss of capping. The data show that Pol12p interacts with Stn1p and both play a role in telomerase action and capping (Grossi et al., 2004). Study have shown that when the function of Pol-α and its interaction with the CST complex is disrupted, it will lead to telomere elongation along with increased terminal ssDNA (Carson and Hartwell, 1985; Qi and Zakian 2000;

Grossi et al, 2004). When there is no C-strand fill-in synthesis, telomerase can over-extends the G-strand leading to elongated, single-stranded chromosome ends.

In mammalian system, there are growing evidences that telomeric proteins CST not only function in facilitating chromosome end replication and capping, but they also function as a general replication factors throughout the genome. *CTC1* and *STN1* were shown to stimulate Pol α processivity and affinity for ssDNA templates (Casteel et al., 2009). There are evidences that the CST complex has a role in promoting Pol-α dependent DNA synthesis at non-telomeric sites in mammalian cells. Ctc1p and Stn1p from mammalian cells were identified as DNA polymerase alpha accessory factors, even before they were recognized as orthologs of the yeast CST complex components (Goulian and Heard, 1990; Goulian et al, 1990). These studies have suggested that in vitro, Ctc1p and Stn1p function in both stimulating the primase and polymerase activities of Pol-α, and increase its affinity to DNA template. In fact, experiments with immunostaining from live cells have shown that these protein co-localize with PCNA during S phase, suggesting that Ctc1p and Stn1p have a role in general DNA replication (Casteel et al, 2009). In mammalian, CST was found to be involved in telomere maintenance and the depletion of it lead to longer G overhang and loss of telomere (Chen et al. 2012; Miyake et al., 2009; Steward, 2012). Like the budding yeast CST (ScCST), mammalian CST (HsCST) has *STN1* and *TEN1* subunits conserved, both CST complexes resemble RPA, and

both CST bind ssDNA (Miyake et al., 2009; Chen et al., 2012; Price et al. 2010). Unlike yeast CST (ScCST) capping role, mammalian cells have an alternate structure termed the shelterin complex that is mainly responsible for telomere protection or capping. In mammalian cells, the HsCST plays a role in replication both at the telomere and elsewhere in the genome. However, mammalian CST (HsCST) is not a common replication factor as it does not co-localize with replication foci (Miyake et al. ,2009). During replication stress, mammalian CST function in duplex DNA replication at the telomere (Stewart, 2012). Mammalian CST facilitates replication at the telomere duplex by rescuing replication after fork stalling. In mammals, HsCST function in the restart of DNA synthesis via new origin firing elsewhere in the genome. In budding yeast, it is known that the ScCST complex controls G-strand extension through positive and negative regulation of telomerase (Giraud-Panis et al., 2010). It is also known that Cdc13p and Stn1p in budding yeast interact with Pol *α* (Chandra et al., 2001, Puglisi et al., 2008; Qi and Zakian, 2000; Sun et al. 2011). The ScCST in yeast is also proposed to recruit pol α for complimentary C-strand synthesis. However, there is no direct evidence demonstrating this role of C-strand synthesis in yeast. In mammals, both the *CTC1-STN1* and *Xenopus* CST stimulate Pol α activity (Goulian et al., 1990; Nakaoka et al., 2012). Given this study, mammalian CST is a likely candidate to direct telomeric C-strand fill-in. Depletion of *STN1* will lead to a defect in C-strand fill-in during late S/G2 phase. Data from mammalians study have indicated that HsCST functions in two distinct aspects of telomere

replication: 1) passage of the replication fork through telomeric duplex and 2) C-strand fill-in synthesis after telomerase action. It was shown that Stn1p depleted cells showed a delay in overhang shortening in late S/G2 phase and does not effect on the timing or extent of overhang elongation in early to mid S. The study showed that *STN1* is involved in the overhang shortening, which occurs as cells exit S phase (Wang et al. , 2012).

*STN1* depletion causes a delay in C-strand fill-in by monitoring the overhang maturation on lagging daughters strands. To do this, overhang density analysis in which the density of overhang was examined by BrdU incorporation. The change in density is detected by CsCl density gradient of the overhangs that have been released from telomeric duplex by DSN (Duplex Specific Nuclease) digestion. During telomere duplex replication, the parental G-strand does not incorporate BrdU. Therefore, the parental G-overhangs is unlabeled and is low density until they are extended by telomerase. The telomerase incorporates BrdU and the lagging strand is ~50% unlabeled DNA and ~50% BrdU-labeled. Telomerase synthesized DNA are of intermediate density. After C-strand fill-in, the remaining overhangs are fully BrdU labeled and become high density (Wang et al., 2012). When the overhangs that were isolated from normal cells and mutant *STN1* cells were compared, the G-overhangs in the wildtype cells were converted to higher density due to C-strand fill-in, and became fully BrdU labeled after 9-12 hrs post release. In contrast, the overhangs from the mutant *STN1* cells remained at intermediate density and showed small increase in density by

12 hrs. This data suggested that cells defective in *STN1* have a delay in C-strand fill-in during late S/G2 phase and suggest that *STN1* may participate in the fill-in process (Wang et al., 2012). The data also suggested that human STN1/CST is required for C-strand synthesis after lagging strand extension of the telomere by telomerase. The delay overhang shortening observed in this data also implies that CST is needed for C-strand synthesis at the leading strand telomere. Other study have also shown that *STN1* depletion causes elongation of overhang at both leading and lagging daughter strands and delayed in overhang shortening in late S/G2 (Huang et al., 2012). It has been shown that CST interacts with the shelterin complex in the 3' overhang and these interactions may deliver CST to the G-strand where it is position to recruit Pol α. It has been shown that HsCST may assists in the restart of replication after fork stalling during telomere duplex replication (Stewart, 2012). This function is likely to involve the ability of HsCST to modulate pol α activity. It has been hypothesized that HsCST may recruit Pol α to assist restart stalled forks where replisome has become damaged and lost in the polymerase. It is also shown that HsCST may facilitate firing of dormant replication origins (Drosopoulos et al., 2012). There is another study that supports the idea that HsCST may promote genome-wide origin firing during recovery from HU-induced fork stalling (Stewart, 2012). The study has demonstrated that when *STN1* has been knocked down in mammalian cells, there is a reduction in the recovery of DNA replication after exposure to hydroxyurea HU (Stewart et al, 2012). This compromised recovery results from a

failure to activate late-firing and dormant origins, rather than from problems in restarting stalled forks. This findings support a model in which HsCST complex facilitates Pol-α dependent priming both at telomeres and other challenging regions to promote efficient and complete replication of the genome. These results have indicated that mammalian CST may be a novel replication factor that is utilized to fix or repair replication problem.

It has been shown that overexpressing of Stn1p in HU- treated cells can cause a defect in the S phase checkpoint (Gasparayan et al. 2008). In *S. cerevisiae*, the S-phase checkpoint mutant showed premature extension of the mitotic spindle after HU treatment, leading to abortive segregation of partially replicated chromosomes (Bachant J. et al, 2005). Overproducing Stn1p cells showed severe uncoupling of DNA replication and spindle extension in presence of HU. The S-phase checkpoint is responsible to delay late replication origin firing and prevent active replication forks from lethal collapse (Branzei D., Foiani M., 2005). To determine whether Stn1p overproducing cells in the presence of MMS activates the S phase checkpoint and permit limited replication, BrdU was incorporated into DNA, recovered by IP with anti-BrdU antibodies, and hybridized to microarray. This experiment showed in regions of active DNA synthesis, late replication origins have been seen to fire abnormally in Stn1p overproducing cells during 30-60 min BrdU incorporation interval after release from α-factor. In the control cells, there was no evidence of these inappropriate firing from these origins. The replication fork progression from the origins during the 30-60 interval

showed that DNA synthesis associated with the progressing fork is less extensive in Stn1p overproducing cells compare to wild type cells. The analysis of these data showed that replication forks may appear to progress less efficiently in the MMS-treated Stn1-overproducing cells. These data showed that Stn1p overproducing cells have defects in the S-phase checkpoint because of the hypothesis that overexpressed Stn1p can altered an upstream step in the checkpoint signaling pathway.

Stn1p is known to associate with Pol12p, the regulatory subunit of Polα. It has been shown that two different *pol12* loss of function mutants (*pol12-40* and *pol12-216*), can dramatically attenuate both the S phase checkpoint spindle extension and HU sensitivity. The hypothesis to explain why the Stn1p override of the S phase checkpoint is sensitive to Pol12p is that the interaction between Pol12p and other proteins causes overproduced Stn1p to become mislocalized to chromosomal regions outside of telomeres (Gasparayan et al. 2008). This hypothesis was tested by performing a spreading analysis to examine the Stn1p association with chromosomal DNA. The spreading analysis revealed that overproduced Stn1p localized throughout the spread nuclei and concentrating into punctate foci (Gasparayan et al. 2008). In the budding yeast *S. cerevisiae*, these observations have shown that overproduced Stn1p can associate with chromosomes at nontelomeric sites, and that *pol12* mutants can restore the S phase checkpoint defects due to the reduce Stn1p binding to chromosome. This analysis showed that Stn1p interaction with Pol12p is on the replicating

chromosomes, and that overproducing Stn1p deregulates the S-phase checkpoint (Gasparayan et al. 2008).

Studies have shown that other fungal Cdc13p is not as telomere specific as *S.cervisiae* Cdc13p. In the fungus *C. albicans*, the CST complex may have a genome wide role in DNA replication rather than a telomere specific function, in part because Cdc13p can bind to non-telomeric DNA templates (Mandell et al, 2011). This observations have suggested again that CST may have a role in general DNA replication in other organisms other than the budding yeast *S. cerevisiae*. There are more evidences that suggested the CST complex can facilitate global DNA replication and non-telomeric role. Recent work from the African clawed frog *Xenopus laevis* have shown that immunodepletion of Stn1 in egg extracts have severely compromised the synthesis of a single-stranded template (Nakaoka et al, 2012). However, the synthesis of primed template is unaffected, indicating that the CST complex has a role in priming single-stranded templates.

Many telomeric proteins have acquired secondary functions in genome maintenance and DNA metabolism. As of this writing, it is not clear if Stn1p, Ten1p, or other telomeric proteins function in double strand breaks repair or recombination at non-telomeric sites. However, we do not know everything about Stn1p or Ten1p, and it is not surprising to discover that the telomeric proteins Stn1p or Ten1p have adopted these secondary roles.

## Roles of the *RAD6* pathway

DNA, the carrier of genetic information, is vulnerable to DNA damage during the process of DNA replication. During DNA replication, the replication forks may collapse and give rise to chromosomal abnormality leading to disease like cancer. To protect the genome against DNA damage, many organisms have evolved DNA damage tolerance mechanisms that promote cell survival (Karras G.I. and Jentsch S., 2010). These DNA damage tolerance (DDT) mechanisms help the cells survive even when the DNA damage cannot be removed completely from the genome. One of the genes involved in cell survival is *RAD6. RAD6* plays essential roles in recombination repair. Rad6p is an E2 ubiquitin-conjugating enzyme that interacts with three E3 ubiquitin ligases (Bre1p, Rad18p, and Ubr1p) each known to be involved in different DNA repair pathways (Robzyk and Osley, 2000). In eukaryotes, the DNA damage tolerance requires the *RAD6* pathway that consists of two main branches: 1) error-free involving sister-chromatid recombination; 2) error-prone involving specialized translesion synthesis (TLS) polymerases. These two mechanisms control the modification of PCNA, an essential replication fork component, by ubiquitin on the replication fork after DNA damage. In contrast to the conventional DNA repair pathways, DNA damage tolerance (DDT) does not directly repair the primary DNA lesion (Ganesan, 1974). DDT is activated as a result of a replication block-induced uncoupling of DNA unwinding and synthesis (Chang and Cimprich, 2009; Janion,

2008). This replication block-induced uncoupling of DNA leads to the formation of ssDNA, which is a key trigger of DDT (Broomfield et al., 2001).

Studies in prokaryotes have indicated that bacterial DDT promotes restart of stalled replication forks, which involves the re-priming at the damaged template (Courcelle and Hanawalt, 2003). Eukaryotes utilize two distinct DDT modes: an error prone mechanism that involves translesion polymerases that can bypass bulky DNA lesions by catalyzing DNA synthesis across the damaged template and an error-free pathway that engages recombination proteins (Friedberg, 2005). Eukaryotic DDT involves the ubiquitin protein modifications pathway. Many enzymes of the ubiquitin protein modification system are involved in eukaryotic DDT in the *RAD6* pathway (Broomfield et al.,1998b; Jentsch et al., 1987; Ulrich and Jentsch, 2000). The substrate for the *RAD6* pathway is the replicative polymerase processivity clamp PCNA (Hoege et al., 2002). The switch between the error-prone or the error-free branch of the DDT pathways depends on the types of ubiquitin modifications on the substrate PCNA that become induced upon DNA damage. Error-prone DDT is triggered by conjugation of a single ubiquitin molecule (monoubiquitylation) to PCNA at lysine-164 (K164) by the Rad6 ubiquitin-conjugating (E2) enzyme and Rad18, a RING-finger ubiquitin ligase (E3) that binds PCNA (Karras & Jentsch, 2010; Hoege & Jentsch, 2002). The monoubiquitylation of PCNA promotes TLS through recruitment of TLS polymerase with ubiquitin-binding motifs. In contrast, error-free DDT involves the

modification of the same residue of PCNA by a non-canonical polyubiquitin chain that linked via K63 of ubiquitin (Hoege et al., 2002). In other words, the error-free PRR requires that the monoubiquitination of the PCNA lysine-164 (K164) that is mediated by RAD6-RAD18, and becoming a polyubiquitin chain. Additional proteins, including E2 Ubc13-Mms2, and the RING-finger E3 ubiquitin ligase Rad5, which binds PCNA and Rad18, are needed for the polyubiquitin chain synthesis (Hoege et al, 2002; Ulrich & Jentsch, 2000). In other words, the polyubiquitination chain modification of PCNA triggers the template switching damage avoidance that involves using the undamaged template (template switching) to repair the damaged site (Branzei et al, 2008; Zhang and Lawrence, 2005). PCNA ubiquitylation is known to be physically coupled to the stalled fork (Ulrich, 2009). PCNA modification were believed to promote the progression of the replication fork in frog egg extracts, yeast, and humans (Bi et al, 2006, Leach and Michael, 2005). In addition to Rad6p, the helicase activity of Rad5p is believed to catalyze fork regression or reversal in vitro (Blastyak et al., 2007). It is suggested that Rad5p promotes template switching directly at the replication fork. Together, this data suggested the model that DDT employed error-prone pathway in which TLS polymerases promote bypass replication across lesion, and the error-free template-switching mode where either sister chromatid junctions or fork regression may lead to a DNA structure called a “chicken foot” acting near replication fork to promote replication restart.

DNA damage or lesions remain a major problem during replication in eukaryotic cells. An example of these bulky DNA lesions is UV-induced cyclobutane pyrimidine dimers that can block replicative DNA polymerases. If the DNA lesion is left unrepaired, it may stall the replication fork, and cause eventual fork collapse resulting in chromosome breaks and genomic instability (Karras & Jentsch, 2010). In addition to homologous recombination, a major pathway for dealing with damage at stalled forks or gaps left after fork passage, is the *RAD6* pathway. The *RAD6* pathway is required for post replicative repair (PRR) ensuring cell survival. The *RAD6* post replicative repair (PRR) pathway is activated as a result of the formation of single-stranded DNA (ssDNA) and stalled replication fork.

As mentioned above, the error-prone mechanism involves translesion polymerases that can bypass bulky DNA lesions by performing DNA synthesis across the lesion template. How the translesion polymerase does this involves inserting correct or incorrect nucleotide across the damage site in a mechanism known as translesion synthesis (TLS). This translesion synthesis (TLS) promotes "bypass replication" across the gaps or lesion at the replication fork. In contrast, the error-free pathway avoids the use of damaged region as a template for DNA synthesis, but takes advantage of genetic information encoded by the undamaged sister chromatid to restore the sequence opposite the lesion. In other words, the error free pathway involves a temporary reversal of the replication fork that allows pairing of two newly synthesized strands often called

"chicken foot" structure (Ulrich et al., 2005). This "chicken foot" structure involves a template switching mode either by sister chromatid junctions or fork regression near the replication fork.

*Rad18 E3 ligase function*

DNA damage tolerance (DDT) mechanism are essential for maintaining replication hindered by unrepaired DNA lesions that can block DNA polymerase and stall replication. Post-translational modifications, such as ubiquitylation/deubiquitylation,plays a major role in both DNA repair and the regulation of DDT. DNA lesions can activate the E3 ubiquitin ligase to prevent the blocking of DNA polymerase and stalled replication forks. As we mentioned earlier, the primary role of Rad6p in the PRR pathway is to interact with Rad18p, an E3 ubiquitin ligase, to promote ubiquitinylation of PCNA, a DNA encircling clamp that functions as a DNA polymerase processivity factor and aids in DNA replication (Jentsch et al, 2010; Hoege & Jentsch, 2002). Rad18 is an E3 ubiquitin ligase that associates with its E2-conjugating enzyme Rad6. The Rad18-Rad6 complex catalyzes the monoubiquitylation of proliferating cell nuclear antigen(PCNA) on lysine K164.2-4 (Zlatanou & Stewart, 2015). It is this post-translation modification of PCNA that facilitates the binding of specialized translesion synthesis (TLS) DNA polymerases that can synthesize across bulky DNA lesions. However, the lesion bypass mechanism of Rad18 is inherently prone to replicative error (Zlatanou & Stewart, 2015). Rad18-mediated lesion

bypass must not be used frequently to prevent mutations (Zlatanou & Stewart, 2015).

Besides the post replicative repair (PRR) function of RAD6-Rad18 pathway, there are other roles of the RAD6 pathway. The other roles of RAD6 pathway is the N-end rule ubiquitination by the E3 ligase Ubr1p and the ubiquitination of histones by the E3 ligase Bre1p. Here, we will explore both of these E3 ubiquitin ligases in more detail.

*Bre1 E3 ligase function*

Histone H2B ubiquitination on K123 is mediated by the ubiquitin conjugase *RAD6* and the E3 ubiquitin ligase gene *BRE1.* Downstream of *BRE1*, the histone H3 methylation at K79 residue is controlled by Dot1; this methylation influences DNA damage checkpoints and X-ray sensitivity in yeast. This suggested that deleting *RAD6* would abolish both the ubiquitination of histone residue K123 and H3-K79 methylation (Game et al, 2006).

The mono-ubiquitylation of histone H2B at Lys-123 that is mediated by the Rad6p and Bre1p enzymes has been shown to be associated with transcriptional elongation, and the association of RNA polymerase II with the active genes (Hwang et al., 2003; Song et al., 2010; Wood et al., 2003). Studies have shown that deletion of *RAD6* had significantly decreased the association of polymerase II with the coding sequence of the *GAL1* gene following transcriptional induction in galactose containing growth medium, consistent with the role of histone H2B

ubiquitylation in transcriptional elongation. In the absence of this mono-ubiquitinylated H2B-K123, the RNA Pol II is destabilized and inhibits the recruitment of the CTK1 kinase that is crucial for the elongation of transcription.

The ubiquitylation of histone by Bre1p may also have a role in DNA replication (Trujillo et al., 2012). Cells that are not able to add monoubiquitin to H2B-K123 become sensitive to hydroxyurea, implicating replication stress. FACS analysis showed that the *htb-K123R* mutants exhibited a delay in the completion of S phase after HU was removed, while control cells took about 20 minutes faster to complete S-phase. In these *htb-K123R* mutant strains, reductions in replisome factors required for DNA synthesis are observed, potentially suggesting that the transcriptional defect may at least partially underlie this replication defect. The lower levels of replication factors lead to a defect in progression of the replication fork and destabilization of the replisome (Trujillo et al. 2012). Defects in the ubiquitylation by Bre1p can lead to replisome instability at stalled replication forks during a G1 to HU shift as shown by ChIP experiment to monitor the association of replisome components with replication origins in both wildtype and *htb-K123R* cells (Trujillo et al. 2012). In this experiment, Mcm4 and Cdc45 were monitored for their amount at the origins an both wildtype and *htb-K123R* mutant cells. They have shown that in *htb-K123R* cells defective in Bre1p ubiquitylation, there is only as small amount of Mcm4 and Cdc45 widely distributed downstream of the origins. The data suggests that cells defective in Bre1 ubiquitylation have fewer Mcm4 and Cdc45 association with the DNA as

they entered S phase, which will lead to fewer stable replisomes at origin-distal positions (Trujillo et al. 2012). Bre1p ubiquitylation of histone also regulate the stability of the nucleosome during replication. Firstly, the amount of DNA replicated at the origins is decrease in the mutant htb-K123R cells as shown by quantitative PCR assay and BrdU ChIP. This suggested that *htb*-K123R mutant cells have a defect in the progression of fork replication at origin-distal positions. Secondly, the level of histone H3 is measured on replicated DNA near the origin. In the mutant htb-K123R cells, the level of H3 was reduced to ~25% at these origins compared to the wildtype. Furthermore, there was a slower kinetics in H3 deposition and reduced H3K56 acetylation (Trujillo et al. 2012). These data suggested that there is a defect in the assembly of the nucleosome at the origins and that the nucleosomes assembly is not stabilized in the absence of Bre1p modification of histone (Trujillo et al. 2012).

*Ubr1 E3 ligase function*

The third major E3 ubiquitin ligase in the *RAD6* pathway is Ubr1p. Its major role is in the polyubiquitinating proteins to target them for the degradation based on the N-end rule (Dohmen et al. 1991) (Figure 1.9). The *UBR1* pathway is not specific to DNA repair, although *ubr1* deficiencies lead to chromosome stability indirectly by affecting sister chromatid cohesion. The Scc1 cohesin protein is targeted for degradation by the ubiquitin/proteasome-dependent N-end rule pathway of *RAD6-UBR1*. Cohesin is a protein complex that established

cohesion between sister chromatids during DNA replication. In *S. cerevisiae*, the subunit of cohesin Sccl, is cleaved at the metaphase-anaphase phase by the Esp1 separin. Separin is a protease activated at anaphase by ubiquitin E3 Ubr1p. Thus, in *ubr1*Δ cells, there is a significance increase in the frequency of chromosome loss because Scc1 fragment becomes stabilized, and not being cleaved by the protease separin of the Rad6-Ubr1 pathway (Rao & Varshavsky, 2001). The accumulation of the Scc1 fragment is lethal due to the accumulation of chromosome loss in ubr1Δ cells. In addition, the overexpression of the Met-SCC1$^{269\text{-}566f}$ fragment was lethal in both wildtype UBR1 and ubr1Δ cells (Rao & Varshavsky, 2001). Another experiment is to explore what phases of the cell cycle is the accumulation of the Met- SCC1$^{269\text{-}566f}$ toxic to the cells. They overexpressed the Met- SCC1$^{269\text{-}566f}$ fragment from the $P_{GAL1}$ promoter in cells that were arrested in S phase with α-factor or hydroxyurea, or in metaphase like state with nocadozole. They found that the transient overexpression of the Met-SCC1$^{269\text{-}566f}$ fragment was nontoxic to cells in G1 and S phases but highly toxic to mitotic (nocoadazole-arrested) cells. This finding suggested that the accumulation of the SCC1$^{269\text{-}566}$ fragment may disrupt the process that take place at metaphase-anaphase. This fragment is the first substrate of the N-end rule pathway of Ubr1. These results suggested that the N-end rule pathway of Rad6-Ubr1 is critical for cell survival.

The sensitivity of *UBR1* to X-ray dose was tested by making double and triple mutants involving *ubr1*Δ*, rad18*Δ*,* and *bre1*Δ (Game et. al, 2006). The

*bre1*Δ *ubr1*Δ double mutants have little sensitivity to X-ray dose compared to *bre1*Δ single (Game et. al, 2006). However, the *rad18*Δ *ubr1*Δ doubles have increase in sensitivities to X-ray dose compared to the single *rad18*Δ (Game et. al, 2006). This implies that Ubr1p may have a role in HRR along with its role in chromosome stability and cohesion degradation (Rao et al., 2001). The *rad18*Δ *bre1*Δ *ubr1*Δ triple mutants resemble *rad18*Δ *bre1*Δ double and *rad6-*Δ single mutant suggesting that *ubr1*Δ have a minor role in the *RAD6* ubiquitination pathway (Game et. al, 2006). We expect the triple mutant phenotype to resemble *rad6-*Δ mutant since it lacks all three ubiquitination pathways that *RAD6* contain.

*Connections between telomeres and the Rad6 pathway*

Until recently, no publications had tied the *RAD6* pathway with telomere function. However, a study has revealed that one of the recombination-dependent pathways that allows senescent cells to maintain telomeres in the absence of telomerase does require the RAD6 pathway.

In telomerase defective *S. cerevisiae* mutants, most cells undergo an event called senescence after about 50-100 divisions when telomeres become shorten. However, there are few cells that are able to bypass the senescence short telomere event via a Rad52-dependent recombination pathway. These survivors of short telomere are grouped into two types, Type I and Type II, that each possess different telomeric DNA structures resulting from recombination of

either terminal subtelomeric repeats or the telomere repeat sequences (Hu and Zhou et al., 2013). A recent study has suggested that Type II survivors may depend on Rad6p (Hu and Zhou et al., 2013). In double mutant *rad6*-Δ *tlc1*Δ cells that lack telomerase and the *RAD6*-dependent paths, only Type 1 survivors were generated, suggesting that Rad6p is required for Type II survivor formation (Hu and Zhou et al., 2013). *RAD51* is required for Type I survivors formation. When *RAD51* is also knocked-out, which blocks the recombination pathway from generating Type I survivors, the *tlc1*-Δ *rad6*-Δ *rad51*-Δ mutant cells underwent senescence without generating any survivors (Hu and Zhou et al., 2013). This result suggested that Rad6p is required for Type II survivors. The downstream pathway of Rad6p during Type II survivor was determined by constructing both the *tlc1*Δ *ubr1*Δ *rad18*Δ and the *tlc1*Δ *ubr1*Δ *bre1*Δ. The *tlc1*Δ *ubr1*Δ *rad18*Δ mutants showed no significant amplification of Y'-subtelomeric elements whereas the *tlc1*Δ *ubr1*Δ *bre1*Δ showed significant Y'-element amplification, suggesting that Rad18p is important for type I survivor formation. (Hu and Zhou et al., 2013).

Rad6p also affects telomere recombination because it impacts the break-induced-replication (BIR) mechanism (Hu and Zhou et al., 2013). BIR requires one free DNA end to take place and require for survivor generation and telomere recombination. BIR efficiencies were measured in the *rad6*-Δ mutants to determine whether the Rad6p participate in telomere recombination via Rad51-dependent BIR process (Hu and Zhou et al., 2013). In *rad6*-Δ mutants, the BIR

efficiencies were greatly reduced (Hu and Zhou et al., 2013). In contrast, wildtype cells have sufficient BIR efficiencies. The results show that Rad6p is required for high BIR efficiencies and telomere recombination repair in cells (Hu and Zhou et al., 2013).

# Chapter 2

## Stn1p requires the Rad6p-dependent ubiquitin ligase pathways for cell viability

## Abstract

Previous work from the Nugent Lab suggested that cells with impaired *stn1* function require functional *RAD6* to survive. This finding struck us as potentially important because Rad6p is an E2 ubiquitin conjugating enzyme that, through its interactions with E3 ubiquitin ligases, promotes DNA post-replication repair via Rad18p, histone modification via Bre1p, and protein degradation via Ubr1p. At the time the project was initiated, no reports of the *RAD6* pathway being important for telomere function were in the literature, so our central goals in these experiments were to test whether Rad6p is important for telomere repair, and whether impaired Stn1p leaves damage, presumably single-stranded gaps, that necessitate post-replication repair by the Rad6 pathway.

## Introduction

Telomeres are the physical repetitive DNA sequences located at the termini of linear chromosomes of both eukaryotic and prokaryotic organisms. The primary function of telomeres is to aid in the protection of chromosome ends through several telomeric binding proteins. There are three intriguing interacting proteins, Cdc13p, Stn1p, and Ten1p whose role is to prevent excessive

nucleolytic degradation of the C-rich telomere strand in *Saccharomyces cerevisiae*. Each of these proteins is essential for the viability, and partial loss of function results in unstable chromosome ends and activation of DNA damage checkpoint.

An interesting recent observation from the Nugent lab is that Rad6p, a ubiquitin E2 ligase critical for post-replication repair, is important for viability of cells deficient in *STN1* or *TEN1* function. The single *stn1* and *ten1* mutants have greatly elongated telomeres with single-stranded regions (Petreaca, et al 2007). *RAD6* is known to interact with three different E3 ligases which promote N-end rule protein degradation, control post-replication repair through PCNA ubiquitination and stimulate homologous recombination repair (HRR) through histone H2B ubiquitination. The purpose of this study is to determine which of these Rad6-dependent pathways is required for cell viability when *STN1* is compromised, by testing the phenotype of E3 ligase-*stn1* double mutants. In addition, we explore whether the *RAD6* PRR pathway is required when *TEN1* is compromised.

# Results

Dissection of a diploid strain doubly heterozygous for *rad6-Δ::LEU2* and *stn1-t281::KanMX2* alleles revealed a near synthetic lethal phenotype (Table 1.1). A total of 50 tetrads were dissected, and the drug and nutritional markers

were scored in the viable progeny to determine the genotype. The expectation was that 50 spores, one quarter of the progeny, would be the *stn1-t281 rad6-*Δ double mutant; however, only one such spore (0.5%) was identified out of the expected 25%, indicating a strong synthetic phenotype (Table 1.1 and Figure 1.1). It was noted as well that, as we have seen previously, the single *stn1-t281* mutant strain is sick; only 6% of the viable spores were the *stn1-t281* single mutant, as opposed to the expected 25% (Table 1.1 and Figure 1.1). Both the wild-type and *rad6-*Δ strains were recovered much closer to expected levels (23% and 19%, respectively), indicating that the diploid was not defective and there are not general problems with chromosome segregation (Figure 1.1). The difference between the viable spore counts of the single mutant *stn1-t281* (6%) as compared with the double mutant *stn1-t281 rad6-*Δ (0.5%) is statistically significant, with a p-value 0.001 using the "Simple Interactive Statistical Analysis SISA T- test software tool".

Since Rad6p interacts with three different E3 ligases (Rad18p, Bre1p, Ubr1p), the next set of experiments tested whether one particular E3 ligase is key to the synthetic phenotype. First, we tested whether *RAD18* is required for *stn1-t281* viability. If the PRR pathway is required in *stn1-t281,* for example to repair single-stranded gaps, we would expect this double mutant strain to show a similar phenotype as *rad6-*Δ *stn1-t281.* If this is the case, it could suggest that *stn1-t281* mutation accumulates single stranded DNA damage, and this single stranded DNA damage required the *RAD6* DNA repair pathway for cell survival.

Dissection of a diploid strain doubly heterozygous for *rad18-Δ::LEU2* and *stn1-t281::KanMX2* alleles did not reveal a synthetic phenotype (Table 1.2) Again, we dissected 26 tetrads and the drug and nutritional marker were used to score the viable progeny to determine the genotypes. Since four different genotypes were segregating in the cross, 26 spores were expected for each progeny (*wildtype, stn1-t281, rad18-Δ,* and *stn1-t281 rad18-Δ*). We observed that double mutant *stn1-t281 rad18-Δ* had a low percentage of viable spores 8.6% (9 spores), as opposed to the expected 25% (13 spores) (Table 2.1). However, the percentage of viable spores for the *stn1-t281* single was 12.5% out of expected 25%, which suggested that *stn1-t281* affected the survival of cells (Figure 1.2) on its own. The other viable progenies had observed proportions of 15.38% and 14.4% for *wild-type* and *rad18-Δ*, respectively (Table 1.2, Figure 1.2). This data suggested that the double *stn1-t281 rad18-Δ* is not synthetically lethal; whether the surviving spores are more sick is addressed later (Figure 1.5). Thus, in contrast to *stn1-t281 rad6-Δ* double mutant, the *stn1-t281* and *rad18-Δ* mutations do not combine to show a lethal synthetic interaction (Table 1.2).

The viable spore counts for the double mutants *stn1-t281 rad6-Δ* and *stn1-t281 rad18-Δ* have been compared to each other. The tetrad analysis has shown that the double mutant *stn1-t281 rad6-Δ* has a very low survival count of 1/200 or 0.5%. In contrast, the double mutant *stn1-t281 rad18-Δ* is 9/104 or 8.6%, which is not consistent with a synthetic interaction. We see clearly that the double *stn1-t281 rad6-Δ* has a significantly stronger synthetic phenotype (Figure 1.3). In fact, using

the two-sided t-test on the “Simple Interactive Statistical Analysis SISA T-test software, the p-value is 0.0114, which is statistically significant when comparing the frequency of viable spores for the two double mutants. This showed that the *stn1-t281* mutant is more dependent on *RAD6* for cell viability than *RAD18*.

A diploid strain heterozygous for *stn1-t281* and *rad30-*Δ was constructed and dissected. *RAD30* encodes a translesion polymerase, polymerase eta, that is in the pathway downstream of Rad6/Rad18p-mediated ubiquitination of PCNA. As observed in Table 1.3, similar to our results with *rad18-*Δ*,* the loss of *RAD30* does not significantly further comprise spore viability of *stn1-t281* mutants. The lack of synthetic interaction is consistent with the conclusion that the synthetic phenotype of *stn1-t281* and *rad6-*Δ is not due to a loss of post-replicative repair function mediated through the *RAD18* branch.

We next tested whether the E3 ligase Bre1p is important for viability of *stn1-t281* mutants. Tetrad analysis of a diploid strain triply heterozygous for *rad18-*Δ*::LEU2* and *stn1-t281::KanMX2* and *bre1-*Δ*::URA* alleles revealed that the double *stn1-t281 bre1-*Δ as sick as the *stn1-t281* alone and more sick than *stn1-t281 rad18-*Δ*.* The *stn1-t281 bre1-*Δ spore viability was 2.2% compared to the 9% spore viability of *stn1-t281 rad18*Δ (Table 1.4, Figure 1.4). This indicated that Bre1p may be needed to help maintain viability of the *stn-1-t281* truncation strains. The triple mutant *stn1-t281 bre1-*Δ *rad18-*Δ showed a higher spore survival rate of 8.5%, which is better than observed for *stn1-t281 bre1-*Δ*.* This may indicate that *rad18-*Δ suppresses the synthetic phenotype of *stn1-t281 bre1-*

Δ (Table 1.4). The single mutants *bre1*-Δ and *rad18*-Δ have spore viability of 5.1% and 10.2%, respectively. The *bre1*-Δ mutant has a more synthetic phenotype than *rad18*Δ (Table 1.4, Figure 1.4). In addition, the single mutant *stn1-t281* has spore viability of 2.2% suggesting it alone has a severe impact on viability (Figure 1.4).

Because of the low spore recovery overall for the diploid used for Figure 1.4, there was some concern that the triple mutant diploid strain may have had genome instability that was contributing to the low spore viability. So, a diploid heterozygous for *stn1-t281/STN1 bre1-Δ/BRE1* was created and dissected. This diploid gave results more in line with expectations, with 95 wild-type and 84 *bre1*-Δ single mutants spores being recovered, when 103 were expected for each. Only 52 *stn1-t281* spores were obtained, and 16 *stn1-t281 bre1*-Δ double mutants, revealing a strong synthetic phenotype. Together, this data have indicated that the *RAD6/BRE1* pathway is more essential for cell viability and genome stability than the *RAD6/RAD18* pathway. In other words, Bre1p is more essential for cell viability when Stn1p function is compromised.

Tetrad analysis can reveal synthetic lethal defects, but subsequent analysis of cell growth can also show whether growth is compromised in double mutants. Here, serial dilution cell viability assays were done to compare growth of the double mutant *stn1-t281 rad18*-Δ to the single *stn1-t281* and *rad18*-Δ single mutants. The double mutant *stn1-t281 rad18*-Δ has the same phenotype as the single mutant (Figure 1.5). Although the double mutant *stn1-t281 rad18*-Δ

is somewhat synthetically sick compared to the single *stn1-t281* at 30°C and higher temperature. The data conferred that Rad18p is not as essential as Rad6p for cell survival.

In contrast, serial plating dilution on the double *stn1-t281 bre1-*Δ shows a severe growth defect of cells grown from the dissection plates (Figure 1.6). The single *stn1-t281* and single *bre1-*Δ strains do each show less growth than wild-type cells (Figure 1.6). This data further conferred that the *RAD6/BRE1* pathway is more essential than the *RAD6/RAD18* pathway when *STN1* is compromised. It suggested that the double mutant *stn1-t281 bre1-*Δ is more sick than the double mutant *stn1-t281 rad18-*Δ*.*

Testing the growth of the triple *stn1-t281 rad18-*Δ *bre1-*Δ mutant by the serial plating dilution viability assay showed that its pattern of growth appears most similar to the *stn1-t281 bre1-*Δ double mutant strain, with particularly slow growth at 23˚C (Figure 1.7). Neither *stn1-t281* nor *bre1-*Δ grew well in this assay. In contrast, the *stn1-t281 rad18-*Δ double mutants and *rad18-*Δ single mutants appear to grow least well at high temperatures. Thus, here again the data are consistent with loss of the *BRE1* versus *RAD18* branches having different impact in *stn1-t281* mutant strains.

Since the Stn1p amino terminus is necessary for the interaction with Ten1p in the CST complex (Petreaca et al, 2006), and *STN1* and *TEN1* seem to play the similar roles in maintaining telomere integrity by preventing inappropriate terminal resection. It would be interesting to see if the loss of Ten1p requires the

*RAD6* PRR pathway for cell survival. We constructed the double mutant of *Ten1-105 rad6-*Δ and produced serial plating dilution at different temperatures (23°C, 30°C, 32°C, 36°C). We observed that both the surviving single *ten1-105* and double mutant *ten1-105 rad6-*Δ strains have very sick growth and are temperature sensitive as compared to *rad6-*Δ (Figure 1.8).

## Discussion

The general model of this project is that the function of Stn1p, and hence likely CST, is required to maintain DNA integrity such that the *RAD6* post-replication repair pathways are not needed. The partial loss of *stn1* functions that have been characterized, and *stn1-t281* used here, develop greatly extended telomeres, where the telomere repeat sequences are greatly amplified (Petreaca et al, 2007). Both telomerase and homologous repair pathways seem to be involved in generating these amplified terminal tracts. The greatly amplified ends may help act as a buffer for the *stn1-t281* strains, because the mutant *stn1* fails to provide adequate protection to the chromosome termini, and they become greatly single-stranded.

We first hypothesized that the synthetic phenotype of *stn1-t281* with *rad6-*Δ reflects a requirement for post-replication repair of the single-stranded tracts by either accurate or error prone lesion bypass pathways. Our data showing that

there is not a synthetic interaction with either *rad18*-Δ or *rad30*-Δ is not consistent with this hypothesis. The double mutant combinations of either *stn1-t281 rad18*-Δ or *stn1-t281 rad30*-Δ are recovered as well as the *stn1-t281* single mutants. Instead, we found that the *BRE1* branch downstream of *RAD6* is instead important for survival and growth of *stn1-t281* (Figure 1.9). Far fewer double mutant *stn1-t281 bre1*-Δ double mutants are recovered as compared with the single mutants. The growth of the surviving double mutants is poor.

It was a bit surprising that *stn1-t281* triple mutants carrying null alleles of both *bre1*-Δ and *rad18*-Δ survived better than the *stn1-t281 bre1*-Δ double mutants. Given the rarity of recovering viable double mutant *stn1-t281 rad6*-Δ spores, we would have expected a phenotype at least as severe as *stn1-t281 bre1*-Δ. It is possible that the third Rad6 branch of E3 ligases, mediated by Ubr1, also contributes to the *stn1-t281 rad6*-Δ phenotype. Since the Ubr1 E3 ubiquitin ligase is important for targeting proteins for degradation, it is important that an imbalance of critical proteins or presence of misfolded proteins contributes to the *stn1-t281 rad6*-Δ synthetic phenotype. This E3 ligase remains to be tested.

While a synthetic lethal interaction could occur due to loss of interacting proteins, we do not know exactly why the Rad6/Bre1 function is important following loss of *STN1* function. Based on what we do know of Stn1p function, it seems unlikely that Stn1p is a critical downstream target of the ubiquitin ligase pathway. Instead, we would favor the hypothesis that the synthetic phenotype is more related to the role of *BRE1* in promoting homologous recombination

through the *RAD51* pathway, or potentially promoting DNA damage checkpoint function. Previous work has shown that *RAD52*-mediated homologous recombination is critical for viability of *stn1-t281* mutants. Impairing the *RAD51* recombination pathway or break-induced replication, as in *bre1-*$\Delta$ mutants, would thus be expected to be likely to also be important for *stn1-t281* telomere replication and amplification.

Overall, our results have indicated that some of Rad6p functions are DNA repair, induced mutagenesis, sporulation, replication elongation, and regulation of genome stability when *STN1* is compromised. Further experiments to explore this connection should test more *STN1* alleles, as well as test how generally the synthetic phenotype extends to *TEN1* and *CDC13* alleles.

# Materials and Methods

## Strains and plasmids

All strains used in this chapter are listed in Table 4.1, and all plasmids used are listed in Table 4.2. Yeast strains were grown and propagated following standard procedures (Sherman, 2002).

## Serial dilution plating assays

Cells of the indicated genotype were inoculated from single colonies and grown on rich YPD medium. Cells were incubated for 3 days at 30°C (or 4 days at 23°C for temperature sensitive strains). For each strain, 10-fold serial dilutions from the same initial concentration of cells were done in a 96-well microtiter dish, and stamped onto appropriate plates. Plates were incubated for 3-4 days at the indicated temperatures (23°C, 30°C, 32°C, 36°C).

## Tetrad Analysis

Haploid yeast cells of the indicated genotypes were mated for approximately 8-hours to make the diploid. Colonies from the diploid strain were streaked onto KOAc plate for approximately 3-4 days to allow for sporulation. Cells from the sporulated diploids were streaked onto YPD plates for tetrad isolation. Tetrad spores produced from meiosis of the diploid were picked and layed onto 4 rows and 12 columns for each strain.

Table 1.1: Tetrad analysis of *stn1-t281*, *rad6-*Δ cross

Parental genotype: *rad6-*Δ*::LEU2* / *RAD6* *stn1-t281::KanMX* / *STN1*

Number of tetrads dissected: 50
Number of total spores: 200
Number of viable spores: 97

*Tetrad Analysis*
*stn1-281 x rad6*Δ
*50 tetrads ( 4 spores/tetrad) = 200 progenies*

| | *wildtype* | *stn1-281* | *rad6*Δ | *stn1-281 rad6*Δ |
|---|---|---|---|---|
| Expected | 50/200 | 50/200 | 50/200 | 50/200 |
| % Expected | 25 | 25 | 25 | 25 |
| Observed | 46 | 12 | 38 | 1 |
| %Observed | 23 | 6 | 19 | 0.5 |

Table 1.1: Double mutant *stn1-t281 rad6-*Δ displays a synthetic lethal interaction. Tetrad analysis of 50 tetrads indicates that the *stn1-t281 rad6-*Δ shows a low viability rate. The single *stn1-t281* mutant also shows a low viability rate, but not as extreme compared to the double mutant. The difference between the survival rate between the *stn1-t281* and the *stn1-t281 rad6-*Δ is statistically significant, with p-value of 0.001.

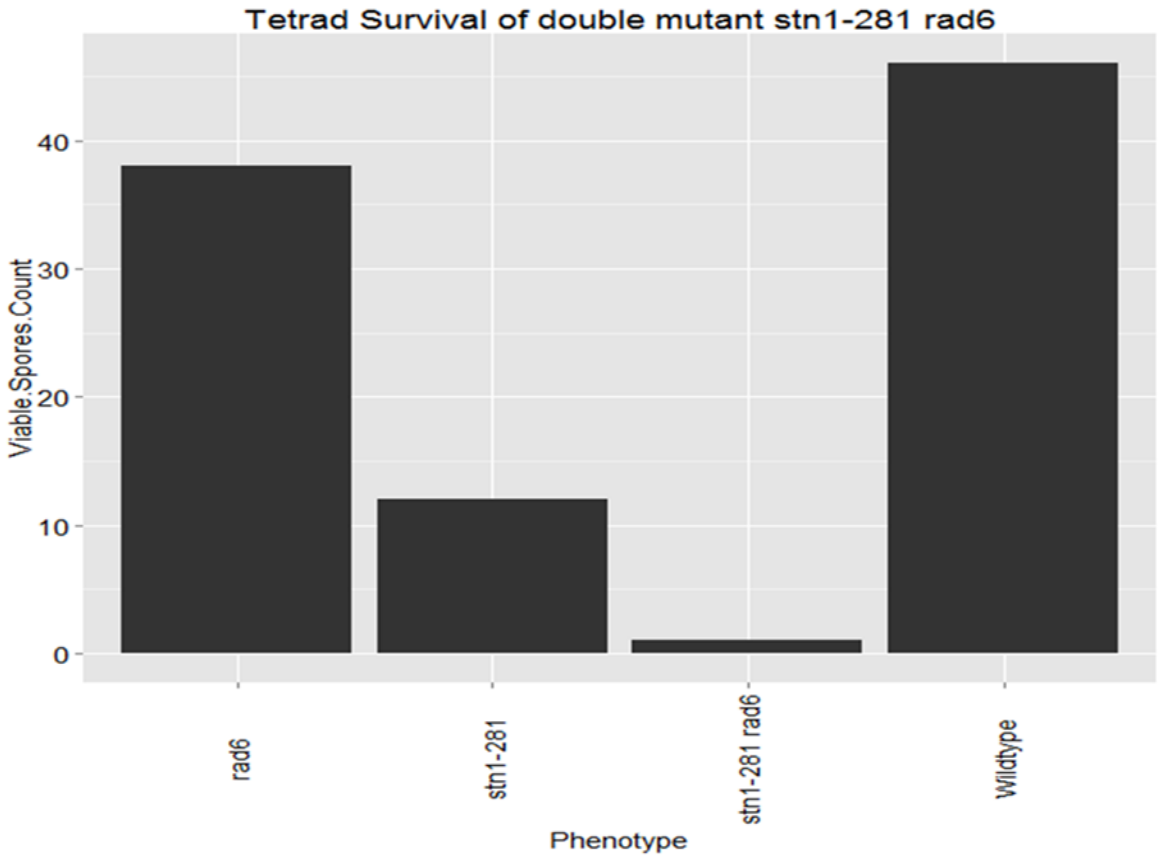


Figure 1.1 Double mutant *stn1-t281 rad6-*∆ displays a synthetic lethal interaction. Bar graph display showing the number of viable spores from dissection of 50 tetrads. Since *STN1* and *RAD6* are unlinked, 50 spores of each genotype were expected to be recovered. The single *stn1-t281* mutant and the *stn1-t281 rad6-*∆ double mutant showed low viability counts, far below expectations, with 12 spores for the single and 1 spore for the double. The difference between the survival rate between the *stn1-t281* and the *stn1-t281 rad6-*∆ is statistically significant with p-value of 0.001 using the online "Simple Interactive Statistical Analysis SISA T- test software. This software compares two independent count by testing for the significant of a difference between two proportions or percentages. Figure 1.1 was constructed using R- statistical software.

## Table 1.2: Tetrad analysis of *stn1-t281, rad18-*Δ cross

Parental genotype: *rad18-*Δ*::LEU2* / *RAD18* *stn1-t281::KanMX* / *STN1*

Number of tetrads dissected: 26
Number of total spores: 104
Number of viable spores: 53

*Tetrad Analysis*
*stn1-281 x rad18*Δ
*26 tetrads (4 spores/tetrad) = 104 progenies*

| | *wildtype* | *stn1-281* | *rad18*Δ | *stn1-281 rad18*Δ |
|---|---|---|---|---|
| Expected | 26/104 | 26/104 | 26/104 | 26/104 |
| % Expected | 25 | 25 | 25 | 25 |
| Observed | 16 | 13 | 15 | 9 |
| %Observed | 15.38 | 12.5 | 14.4 | 8.6 |

Table 1.2: Double mutant *stn1-t281 rad18*Δ is synthetically sick, but not lethal. The *stn1-t281* mutation does not appear to require the *RAD18* arm of the post-replication repair pathway. The difference in survival rate of the single *stn1-t281* and *stn1-t281 rad18-*Δ are not statistically significant with p-value of 0.396, suggesting the double mutant does not change the survival rate significantly. However, the difference between the survival rate between *stn1-t281 rad6-*Δ and *stn1-t281 rad18-*Δ is statistically significance with p-value of 0.0072.

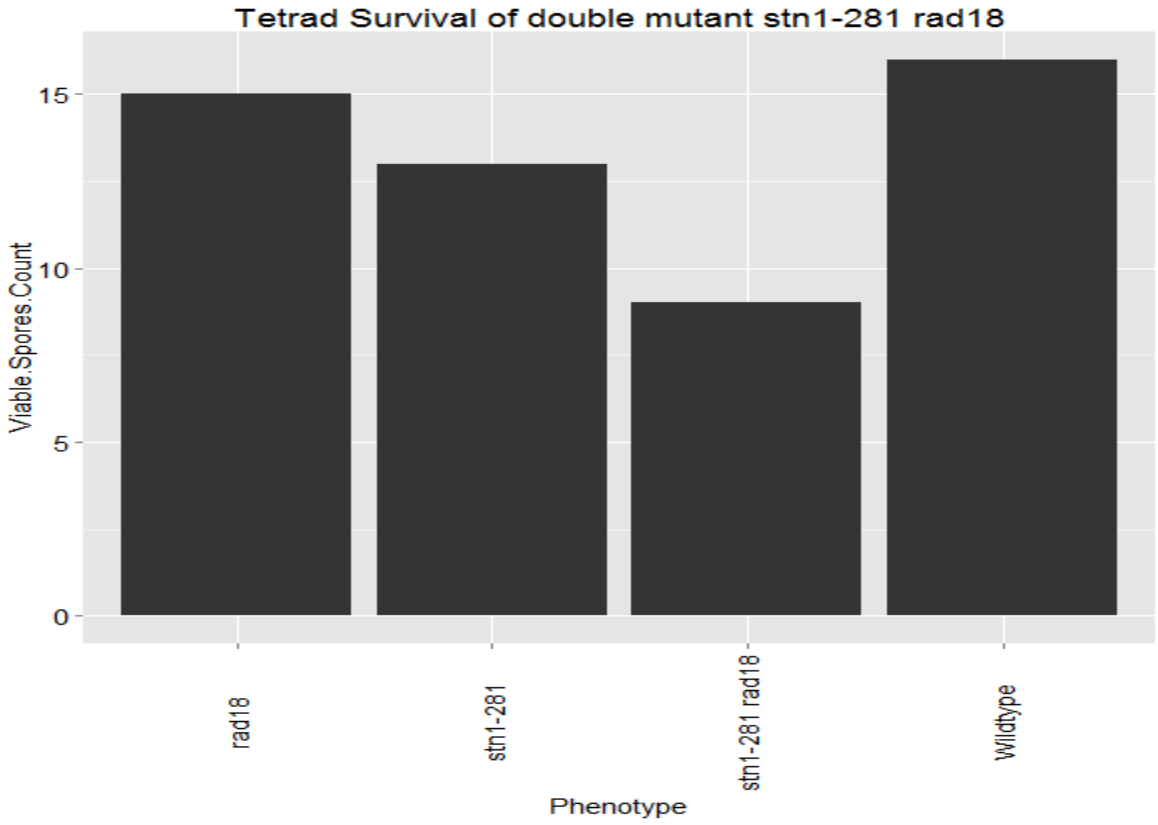


Figure 1.2. Double mutant *stn1-t281 rad18-*Δ is recovered at a somewhat reduced frequency as compared to *stn1-t281.* Twenty-six tetrads were dissected. Since *STN1* and *RAD18* are not linked, it was expected that 26 spores of each genotype would be recovered. The survival count for *stn1-t281* is *13/25.* The survival rate for the double *stn1-t281 rad18-*Δ is *9/25.* The difference in survival rate of the single *stn1-t281* and *stn1-t281 rad18-*Δ are not statistically significant with p-value of 0.396, suggesting the double mutant does not change the survival rate significantly. Thus, the *stn1-t281* mutation does not appear to require the *RAD18* arm of the post-replication repair pathway. However, the difference between the survival rate between *stn1-t281 rad6-*Δ and *stn1-t281 rad18-*Δ is statistically significant, with p-value of 0.0072.

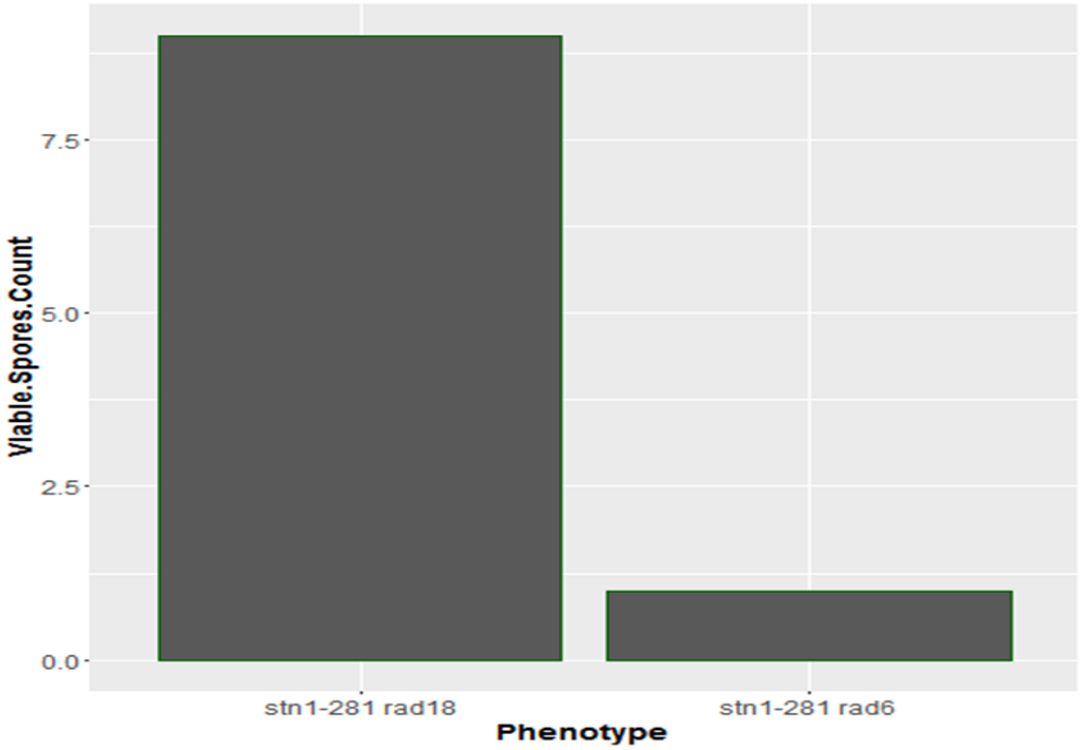


Figure 1.3. Percentage of surviving double mutant spores from tetrad dissections. After dissection of the respective diploid strains, 9/26 *stn1-t281 rad18*-Δ spores were viable as compared with 1/50 *stn1-t281 rad6*-Δ spores. Using the two sided t-test on the online "Simple Interactive Statistical Analysis SISA T- test software, the p-value is between these two double mutants is 0.0114, which is statistically significant. This showed that the *stn1-t281* is more dependent on *RAD6* for cell viability than on *RAD18.*

## Table 1.3 Tetrad analysis of *stn1-t281 x rad30-*Δ cross

Parently genotype: *stn1-t281::KanMX* *rad30-*Δ
*STN1* *RAD30*

Number of tetrad dissected: 25
Number of total spores (expected): 100
Number of viable spores: 43

| | ***Wild-type*** | ***stn1-t281*** | ***rad30*Δ** | ***stn1-t281, rad30*Δ** |
|---|---|---|---|---|
| **Expected** | 25 | 25 | 25 | 25 |
| **% Expected** | 25 | 25 | 25 | 25 |
| **Observed** | 16 | 7 | 13 | 7 |
| **% Observed** | 16 | 7 | 13 | 7 |

Table 1.3. Tetrad analysis of spores recovered from dissection of *stn1-t281/+ rad30-*Δ*/+* diploid. *RAD30* encodes a translesion polymerase that would be downstream of the *RAD18* pathway. As observed in other crosses, spores with the *stn1-t281* genotype are recovered at a low frequency. The recovery of viable *stn1-t281 rad30-*Δ spores is similar to the single *stn1-t281,* with just 7% viable.

## Table 1.4: Tetrad analysis of *stn1-t281 bre1-Δ, rad18-Δ* cross

Parental genotype: *rad18-Δ::LEU2* / *RAD18* *stn1-t281::KanMX* / *STN1* *bre1-Δ::URA3* / *BRE1*

Number of tetrads dissected: 44
Number of total spores: 176
Number of viable spores: 140

Total Tetrads= 44
4 spores/ tetrad = 44 X 4 = 176 progenies

| | ***Wild-type*** | ***Stn1-t281*** | ***bre1Δ*** | ***rad18Δ*** | ***stn1-t281, rad18Δ*** | ***stn1-t281, bre1Δ*** | ***bre1Δ, rad18Δ*** | ***stn1-t281, bre1Δ, rad18Δ*** |
|---|---|---|---|---|---|---|---|---|
| **Expected** | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 |
| **% Expected** | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 |
| **Observed** | 8 | 4 | 9 | 18 | 16 | 4 | 26 | 15 |
| **% Observed** | 4.5 | 2.2 | 5.1 | 10.2 | 9.0 | 2.2 | 14.7 | 8.5 |

Table 1.4. Tetrad analysis of spores recovered from dissection of *stn1-t281/+ bre1-Δ/+ rad18-Δ/+* diploid. For certain genotypes, the recovery of viable spores was unexpectedly low, with *stn1-t281* and *stn1-t281 bre1-Δ* showing the lowest cell viability rates of 2.2%. There were more viable *stn1-t281 rad18-Δ* and *stn1-t281 bre1-Δ rad18-Δ* spores, with 9% and 8.5% respectively. It is not clear why so few wild-type spores were recovered.

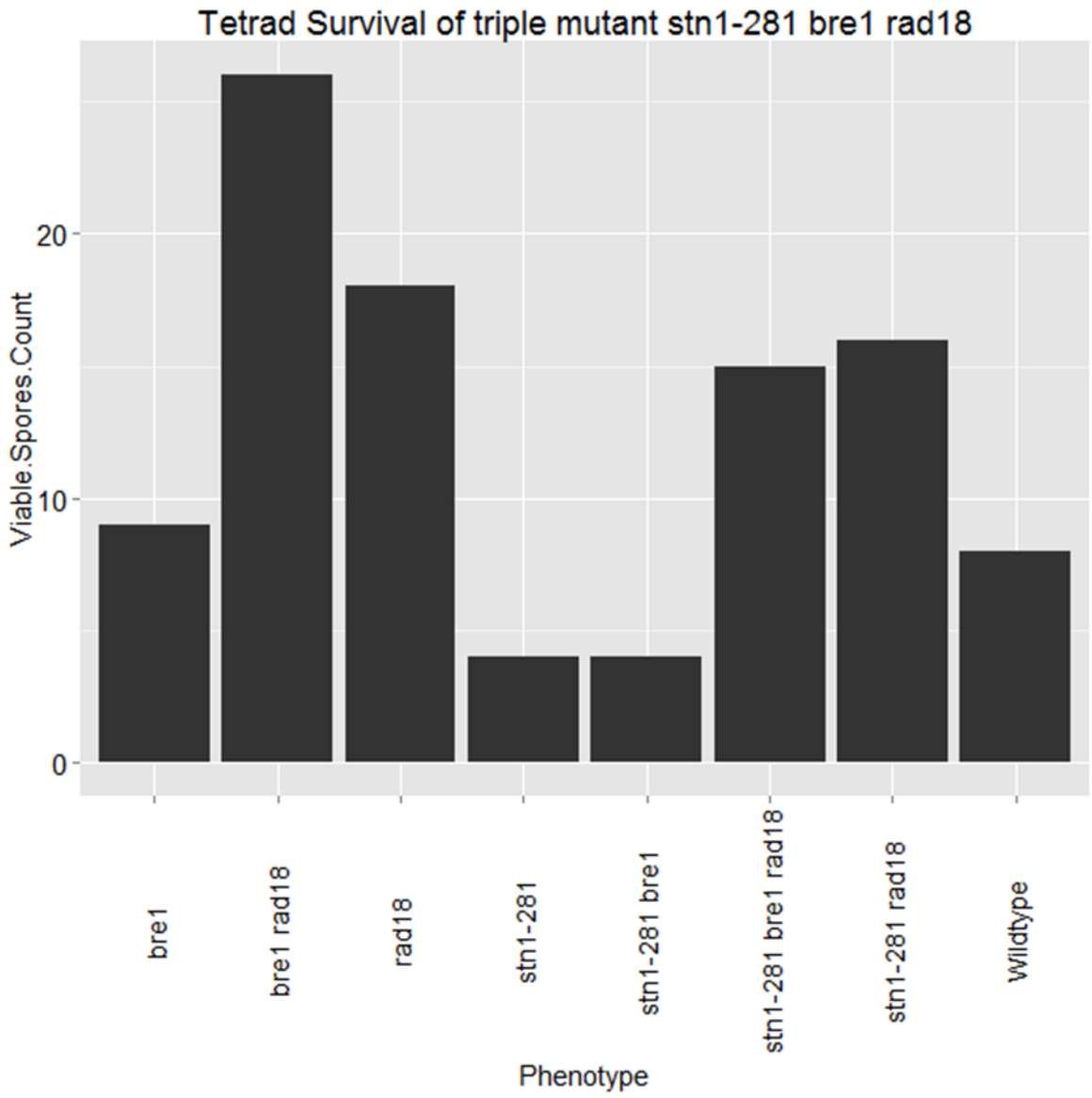


Figure 1.4. Bar graph showing percent recovery of spores from dissection of a diploid heterozygous for *stn1-t281/+ rad18-Δ/+ bre1-Δ/+*. *STN1, BRE1,* and *RAD18* are each unlinked from each other. The absence of *RAD18* appears to rescue the inviability associated with *stn1-t281* and *bre1-Δ* mutations, at least in this diploid.

## Table 1.5: Tetrad analysis of *stn1-t281 bre1-*Δ cross

Parental genotype: $\frac{\textit{stn1-t281::KanMX}}{\textit{STN1}}$ $\frac{\textit{bre1-}\Delta\textit{::URA3}}{\textit{BRE1}}$

Number of tetrads dissected: 103
Number of total spores expected: 412
Number of viable spores: 247

| | ***Wild-type*** | ***stn1-t281*** | ***bre1-*Δ** | ***stn1-t281, bre1-*Δ** |
|---|---|---|---|---|
| **Expected** | 103 | 103 | 103 | 103 |
| **% Expected** | 25 | 25 | 25 | 25 |
| **Observed** | 95 | 52 | 84 | 16 |
| **% Observed** | 23 | 12.6 | 20.3 | 3.8 |

Table 1.5. Tetrad analysis of spores recovered from dissection of *stn1-t281/+ bre1-*Δ*/+* diploid. The recovery of wild-type and *bre1-*Δ single mutant spores was closer to the 25% frequency expected. However, the frequency of obtaining viable double mutant spores is significantly reduced compared even to the single *stn1-t281* mutant.

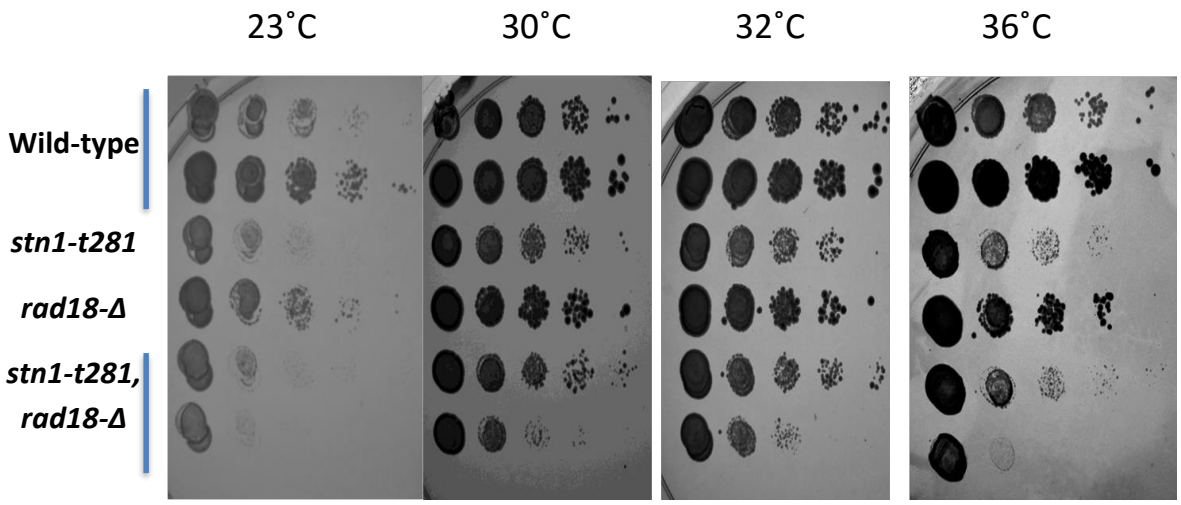


Figure 1.5. Double mutants carrying *stn1-t281* and *rad18*-Δ produce the same phenotype as the *stn1-t281* single mutant. The double *stn1-t281 rad18*-Δ do not appear to show synthetic growth defects as compared to the single *stn1-t281* and *rad18*-Δ mutant strains. Cells of the indicated genotype were grown to saturation in YPD for 3 days at 23°C, then 10-fold serial dilutions were stamped on YPD plates. Plates were incubated at the indicated temperatures for 3-4 days, then pictures were taken.

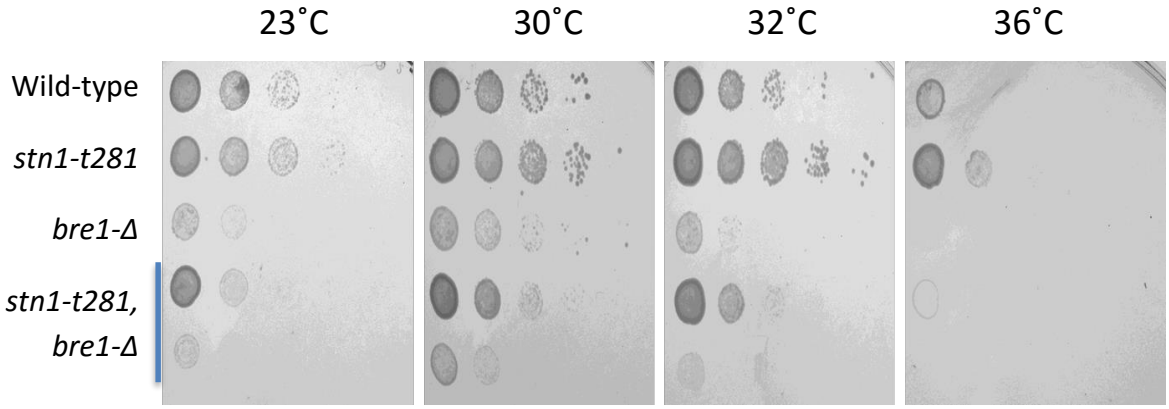


Figure 1.6. Serial dilution viability assay comparing the *stn1-t281* and *bre1-*Δ gene interactions. The growth of single mutant *bre1-*Δ cells is quite poor at all temperatures. The growth of the double *stn1-t281 bre1-*Δ strain is comparable to the single *bre1-*Δ. Cells of the indicated genotype were grown to saturation in YPD media for 3 days at 23°C, then 10-fold serial dilutions were stamped on YPD plates. Plates were incubated at the indicated temperatures for 3-4 days, then pictures were taken.

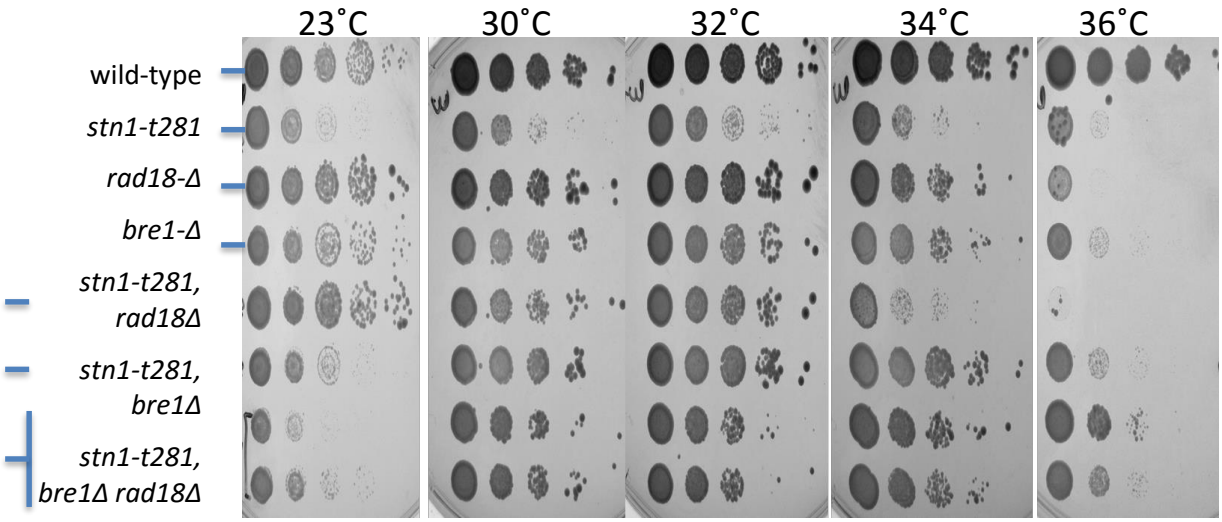


Figure 1.7. Serial dilution viability assay to test genetic interaction of the single, double, and triple mutants among *stn1-t281, rad18*-Δ and *bre1*-Δ alleles. Cells of the indicated genotype were grown to saturation in YPD media for 3 days at 23°C, then 10-fold serial dilutions were stamped on YPD plates. Plates were incubated at the indicated temperatures for 3-4 days, then pictures were taken. The single *stn1-t281* mutant as well as the *stn1-t281 bre1*-Δ double and *stn1-t281 rad18*-Δ *bre1*-Δ triple mutants appeared to have particularly slow growth at 23˚C. None of the double or triple mutant combinations appeared to have more severe growth as compared with the single *stn1-t281* strain.

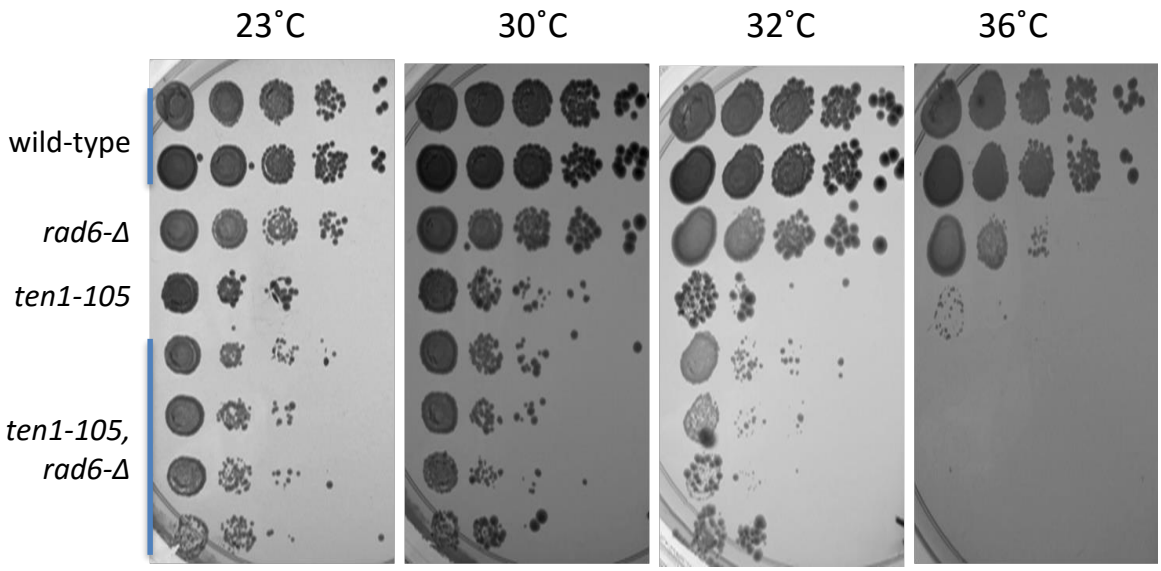


Figure 1.8. Serial plating dilution assay of the single mutant *ten1-105* and the double *ten1-105 rad6-*Δ. Mutation of both the *ten1-105* and *rad6-*Δ genes shows a slightly worse growth defect than the single *ten1-105*. The double mutant appeared similar to the single *ten1-105* strain. Cells of the indicated genotype were grown to saturation in YPD media for 3 days at 23°C, then 10-fold serial dilutions were stamped on YPD plates. Plates were incubated at the indicated temperatures for 3-4 days, then pictures were taken.

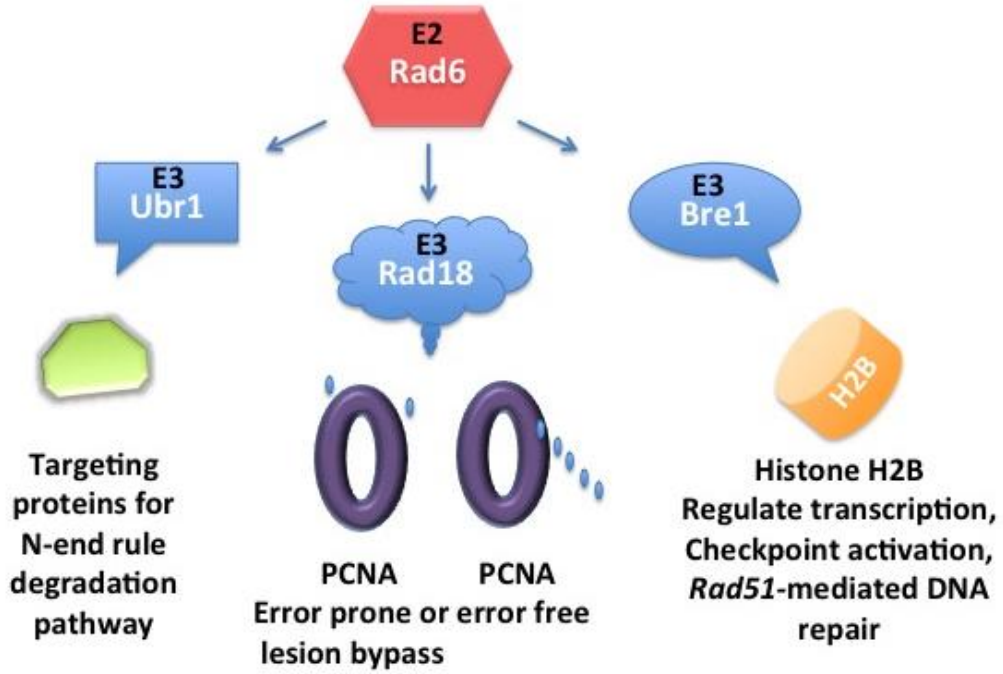


Figure 1.9. The loss of Stn1, and potentially the CST complex, requires the *RAD6*-dependent ubiquitination pathway. The diagram shows how Rad6, an E2 ubiquitin ligase, interacts with three distinct ubiquitin E3 ligases, which in turn impact cellular processes. The Ubr1 E3 ligase targets misfolded proteins for polyubiquitination and degradation by the 26S proteasome. The Rad18 E3 ligase targets PCNA for either mono- or polyubiquitination in response to DNA lesions blocking replication. The ability to stimulate this DNA repair or tolerance pathway is critical for maintaining genome stability in proliferating cells. The Bre1 ligase targets the histone H2B for monoubiquitination. The covalent addition of ubiquitin to this nucleosome component has broad impacts on transcription, DNA repair (affecting homologous recombination), and maintenance of DNA damage checkpoint activation. The data in this thesis support the hypothesis that the function of the Rad6/Bre1- branch is critical for cell viability when *STN1* function is compromised.

# Chapter 3

## *STN1* mutation produces single stranded DNA damage at non-telomeric regions

## Abstract

It has been well established that *stn1* mutants accumulate single stranded DNA damage at the telomere. Our finding that *stn1-t281* requires Rad6/Bre1 for viability could indicate a requirement for robust homologous recombination or DNA damage checkpoint responses to handle the damage present in the mutant strains. At the time of this study, no previous examples were found indicating a specific functional need for *RAD6* to handle telomere generated damage, opening the possibility that damage in *stn1* mutants is not restricted to telomeres and telomere proximal regions. It is unclear if *STN1* mutation can produce single stranded DNA damage at non-telomeric repeated regions known as rDNA. This chapter explores whether compromising *STN1* function can lead to the accumulation of single-stranded DNA gaps at non-telomeric loci such as rDNA regions. As part of this, we wanted to explore whether the fragmentation of DNA happens in a genome-wide manner throughout the chromosomes rather than telomeric in *stn1* mutants.

# Introduction

## *STN1* depletion leads to internal gaps at telomeres

One of the underlying questions asked in this chapter is if the loss of Stn1p can lead to single stranded DNA damage in non-telomeric regions. Stn1p plays a role in telomere end protection known as capping and also negatively regulate telomere extension by telomerase (Grandin et al. 1997; Chandra et al. 2001). Partial loss of Stn1p function displays extensive single-stranded telomere G strands (Grandin et al. 1997; Petreaca et al, 2007). In addition, studies have shown that when the function of Stn1p is compromised, telomere length can greatly increase (Grandin et al., 1997; Grossi et al. 2004; Petreaca et al, 2007). The negative regulation of telomerase by Stn1p could be mediated by the interaction of Stn1p and Cdc13p, and this interaction can compete with the Cdc13p-Est1p.

The amino terminus of Stn1p contributes to both the essential telomere capping and length regulation functions of Stn1p. The carboxyl terminus of Stn1p interacts with Cdc13p that contribute to the length of telomere. *Stn1-t186* and *stn1-t281* mutant strains displayed excessive levels of single stranded $TG_{1-3}$ in the native gels as compared to wild-type strain, and the telomere in this strain are elongated (Petreaca et al. 2007). The excessive G-rich single stranded signal in *stn1-t186* and *stn1-t281* strains was not entirely present as 3' overhangs at the telomere end because digestion by *E. coli* Exo1 only partially reduced the signals

of ssTG$_{1\text{-}3}$ (Petreaca et al. 2007). However, if the single stranded DNA ssTG1-3 in these strains were digested with a single strand DNA endonuclease such as mung bean nuclease, the ssTG$_{1\text{-}3}$ were completely digested (Petreaca et al. 2007). This data was interpreted to indicate that single-stranded gaps could be present at the yeast telomeres. How these single stranded DNA gaps would be created in the *stn1* mutant strains is not clear. One possibility is that the replication of the lagging telomere strand may be compromised, such that single strand gaps are produced (Petreaca et al. 2007). Another idea was that long terminal single strand gaps could have initially been created by nuclease resection with subsequent partial repair or fill-in synthesis that produces single-stranded gaps.

Further indirect evidence for potential non-terminal single-stranded DNA in *stn1* strains comes from comparing the response to loss of the DNA damage checkpoint in *cdc13-1* versus *stn1-t281.* Prior studies have shown that *cdc13-1* cells can strongly elicit the DNA damage checkpoint in response to single stranded TG$_{1\text{-}3}$ that appears above 23˚C in the mutant cells (Weinert and Hartwell 1993; Garvik et al, 1995). Interestingly, removal of the DNA damage checkpoint by mutating key genes allows *cdc13-1* cells to survive at higher temperatures. Because the single-stranded DNA in the *cd13-1* cells seems to be exclusive to the chromosome termini, the interpretation is that the checkpoint was arresting the cell cycle due to abnormally high levels of single-stranded DNA, but at temperatures where *cdc13-1* function is not completely inactivated,

the terminal damage is not lethal. In contrast, the DNA damage checkpoint is essential when the *stn1-t186* and *stn1-t281* strains double mutants were constructed with *rad9* or *rad24* checkpoint mutations (Petrecea et al., 2006), there were synthetic lethal interactions. One possibility then is that the DNA damage present in these *stn1* mutant strains is distinct from that in *cdc13-1,* and indeed lethal without the ability of the checkpoint to be able to properly activate a response. Because the loss of Stn1p is known to produce telomeric single stranded gaps, we are interested in exploring whether the loss of Stn1p can also produce single stranded gaps at the non-telomere regions (rDNA locus) when Bre1p has been compromised.

rDNA is a highly repetitive sequences in most eukaryotes containing thousands of tandem repeats copies (Ganley and Kobayashi et al., 2013). In *S. cerevisiae*, the rDNA is encoded by the ribosomal RNA gene known as *RDNA*, which is a 1.5 Mb regions containing tandem repeats copies of 9.1 kb unit on chromosome XII (Kim et al., 2006). A single 9.1 kb unit of rDNA consists of two transcribed regions, the 35S precursor rRNA and 5S rRNA, and two non-transcribed regions, NTS1 and NTS2. The repeated structure of rDNA causes it to be unstable and lose its copies by homologous recombination of the repeats (Ganley & Kobayashi et al., 2013). Because of the repetitive nature of the rDNA, it is known to be the most unstable structure in the yeast genome. The instability of the rDNA repeats in the genome have led us to investigate whether Stn1p may have a role in repairing single stranded gaps caused by the rDNA repeats.

It is known that rDNA repeats can cause replication fork stalling leading to replisome instability and production of single stranded DNA. The variation in the copy number of rDNA depends on two genes called SIR2 and FOB1. The Sir2 protein suppresses meiotic and mitotic recombination within rDNA repeat. The Fob1 protein is required for replication fork blocking activity at the replication fork barrier (RFB) site in the rDNA and stimulate recombination within the rDNA repeats (Kim et al., 2006). They may play an essential role in rDNA expansion/contraction. Fob1p also caused the recombination of repeated sequence that lead to the accumulation of extrachromosomal rDNA circles (ERCs) (Kim et al. 2006). The binding of Fob1p to the RFB caused DNA double strand breaks (DSBs ) at stalled forks, and Fob1-dependent DSBs that induce unequal sister chromatid recombination (Ganley & Kobayashi et al., 2013).

To look at chromosome abnormality, we are employing the pulse field gel electrophoresis (PFGE) to look at whole chromosomes and chromosome numbers in the *stn1-t281*, *bre1*-Δ, and *stn1-t281 bre1*-Δ double mutants. We are investigating for the presence or absence of chromosome aberrations or deformities using the pulse field gel electrophoresis (PFGE). We hypothesized that in the absence of Stn1p and Bre1p, cells undergo irreparable damage leading to the accumulation of ssDNA lesions or gaps. In the *bre1*-Δ mutants, replication forks tend to stall leading to long stretches of ssDNA lesions that are not repair because of defects in homologous recombination repair (HRR) (Game et al., 2006). There have been studies that showed that Bre1p is epistasis to

Rad52 homologous recombination pathway (Game et al., 2006), which means that they are in the same pathway. Since Bre1p is associated with homologous recombination (HRR), a defect in Bre1p function may compromise HRR and lead to accumulation of ssDNA lesions if Stn1p is compromised.

Study have shown that homologous recombination repair (HRR) deficient cells are sensitive to methyl methanesulfonate (MMS) (Lundin et al., 2005), and experimental data produced evidences that MMS causes DNA fragmentation. Previous reports have shown that a low dose methyl methanesulfonate (MMS) treatment to mutant *rad18* cells generates single stranded DNA lesions that are irreparable (Huang et al., 2013). When the post replicative *rad18*-$\Delta$ cells were treated with the single-strand specific S1 endonuclease that cuts DNA regions containing ssDNA nicks or gaps, the *rad18*-$\Delta$ DNA is converted to double strand breaks (DSBs) that can be visualized as fragmentation by PFGE (Huang et al., 2013). The presence of chromosomal fragmentation following MMS and S1 treatment is indicative of the presence of ssDNA gaps left unrepaired in the Rad6/Rad18 mutants (Huang et al. 2013). Here, we will conduct a similar experiment, asking the question of whether *stn1* truncation mutant exhibits ssDNA gaps when treated with S1 endonuclease. In particular, is Stn1p required to prevent single stranded DNA gaps?

# Results

The primary goal of this project is to determine if there are internal DNA lesions occurring outside of telomeric regions when Stn1p is compromised. More importantly, does Stn1p prevent accumulation of single stranded gaps or lesions at non-telomeric regions under stress? The first approach that was taken to see whether chromosomes have aberrant levels of single-stranded regions was to use S1 nuclease to digest single-stranded regions of chromosomes immobilized in agarose plugs, followed by separation of the chromosomes on PFGE. The expectation is that chromosomes with single-stranded regions become fragments following S1 digestion (Huang et al. 2013). The single stranded specific S1 endonuclease will hydrolyze single stranded regions in between gaps and loops in the duplex DNA.

As seen in Figure 2.1, the single *stn1-t281* mutant did not show a shift in chromosomes or breakage of chromosomes. However, both the *bre1*-Δ and the double *stn1-t281 bre1*-Δ lanes showed a blurry smear in the lanes from plugs both treated and untreated with S1 nuclease. This could indicate the breakage of chromosomes due to long single stranded DNA lesions that were left unrepaired by defects in Bre1p. The results showed that Bre1p is required for the repair of single stranded DNA gaps or lesion left unrepaired under *stn1-t281* background. Although the result does not support whether Stn1p is required to repair single stranded DNA gaps, it did showed that *stn1-t281* mutant seemed to have more fragmented chromosomes when Bre1p is compromised (Figure 2.1, Lane 6). As

an aside, we also observed possible evidence of abnormal chromosomes number in *stn1-t281* mutant indicating possible defects in segregation leading to aneuploidy (Figure 2.1, Lane 3 compare to Lane 2).

In a second experiment, the mutants were grown in YPD media and arrested in G2 phase with nocodazole prior to collection and DNA plugs preparation. By arresting in nocodazole, cells should have largely completed DNA replication, with only problematic regions potentially left incomplete. Treated cells were collected and embedded in agarose DNA plugs. We treated the DNA plugs with S1 endonuclease for 2 hr and ran the DNA plugs on PFGE. The PFGE gels were stained with ethidium bromide (EtBr) to observe for chromosome fragmentation.

Figure 2.2 shows that the positive control of *rad18*-Δ 0.001% MMS and 0.01% MMS treatment did indicate fragmentation of chromosomes because of their shift in chromosomes. The *rad18*-Δ defective cells with the 0.01% MMS treatment had a more severe smear downward indicating higher levels of chromosome fragmentation resulting from single stranded DNA gaps left unrepaired by defective *RAD18*. The low dose 0.001% MMS treatment of *rad18*Δ shows a slight shift in chromosome indicating possible chromosome fragmentation, but to a lesser extent. The single *stn1-t281* mutant does not show a shift in chromosomes or breakage of chromosomes, suggesting that if there are single stranded gaps, they either aren't sufficiently frequent to be detected here, or are being repaired. However, the double *stn1-t281 bre1*-Δ again shows a

breakage of chromosomes indicating the likely presence of single stranded DNA lesions that were left unrepaired when Bre1p is compromised. In this experiment, this double mutant phenotype is more severe than the fragmentation and smearing observed in the single *bre1*-Δ mutant. The results showed that Bre1p is required for the repair of single stranded DNA gaps or lesions. Surprisingly, we have seen the same pattern of results in the absence of S1 treatment, suggesting that chromosomal damage when the Bre1p E3 ligase is absent is severe enough to be observed without S1 treatment.

Since the S1 nuclease treatment did not appear to be having an impact on the pattern of chromosome migration in the PFGE, next an overnight incubation with S1 nuclease was tried. In addition, both the *stn1-t281* and *stn1-t186* alleles were tested. Figure 2.3 showed that this extensive incubation with S1 was likely too much. All of the chromosomes appeared to become degraded for all strains tested. The *stn1-t281* mutants that were not treated with S1 endonuclease showed no obvious chromosome fragmentation, although one of the lanes was more smeary (Figure 2.3).

We know that defects in *STN1* can accumulate single-stranded DNA at the telomere. Our hypothesis is that *stn1* truncations mutants can accumulate single stranded DNA gaps at the rDNA repeat regions outside of telomere. To test this hypothesis, the use of the Klenow fragment polymerase to fill in single stranded gaps in the Stn1p truncation mutants was demonstrated in Figure 2.4. Klenow polymerase is a large fragment of DNA polymerase I that has

5’ → 3’ polymerase activity and can randomly fill in single stranded gaps by incorporating nucleotides. Our preliminary data have shown that the *stn1* truncations mutants can accumulate single stranded DNA gaps at the rDNA repeat regions (Figure 2.4). Figure 2.4 showed Klenow signals in the *stn1* truncation mutants and not as much apparent in the wildtype lane, suggesting that *stn1* truncation mutants produced single stranded gaps or lesions. This also suggested that Stn1p may have a global role in DNA damage repair of single stranded lesions. Since this single stranded lesion is outside of the telomere regions, it suggests that Stn1p may have non-telomeric function.

In addition, the presence of single stranded DNA damage at the telomere for the *stn1* truncation mutant has also been explored by the Klenow synthesis. Because the Klenow polymerase can bind to single stranded DNA regions, extend and elongate thereafter, Klenow synthesis assay is used to detect for the presence of single-stranded DNA. A time course experiment was done where cells were arrested in G1, then time points taken as they go through the cell cycle following release from the G1 block. Consistent with expectations for high levels of telomere single-stranded DNA in both *stn1-t186* and *stn1-t281,* strong signal was seen for both single mutants on a Southern blot probing telomere sequence fragments synthesized by Klenow (Figure 2.5). The wild-type lanes show signal most clearly at two time points, 25 and 100 minutes. Whether these time points correspond to DNA replication is not clear. Nevertheless, there is a clear

difference in signal intensity between the wild-type and *stn1* mutants over the time courses. (Figure 2.5).

## Discussion

Our primary objective in this study is to decipher whether the RAD6/RAD18 post-replicative repair (PRR) pathway is required when Stn1p is compromised. As mentioned earlier, the double mutant Stn1-t281 Rad6-Δ displayed somewhat of a synthetic phenotype with only 0.5% of viable spore recovered from the tetrad analysis. Previous reports have shown that *stn1-t281 rad52*-Δ also displayed synthetic lethal interaction, requiring both the DNA damage checkpoint and homologous recombination for survival (Petreaca et al. 2007). Our data have indicated that *stn1-t281* likely requires homologous recombination repair or checkpoint function via histone modification of the RAD6/BRE1 pathway (Chapter 2). This suggested that *stn1* mutants have single-stranded lesions left unrepaired under defective *RAD6* pathway.

Here we found that the double mutant *stn1-t281 bre1*-Δ leads to fragmented chromosomes on pulse field gel electrophoresis (PFGE), potentially more fragmented than in *bre1*-Δ single mutant strains. This data needs confirmation, potentially by an alternate method because the level of damage in the single *stn1* mutant may not be sufficient for detection using this approach. The Klenow approach to detect single-stranded DNA by synthesis of complementary sequences to fill in gaps and extensions indicated that *stn1*

mutants not only carry excess telomeric single-stranded regions, as expected, but also may have excessive single-stranded regions at rDNA. This finding also merits further investigation.

In eukaryotic cells, DNA damage or lesions remain a devastating problem during replication. If a DNA lesion is left unrepaired, it may cause a halt in DNA replication, stalling the replication fork, and eventually collapsing the fork, which causes chromosome breaks and genomic instability. It is well known that *STN1* mutation produce single stranded DNA damage at the telomere ends that leads to genomic instability. If indeed it also normally helps to maintain genome integrity outside of telomeres in *S. cerevisiae,* this would alter our current models for *STN1* function.

It is not known if the Stn1p, Ten1p or other telomeric proteins function in double strand breaks repair or recombination at nontelomere regions. Since there are evidences in the literature that telomeric proteins also function as a general DNA replication factors throughout the genome. For example, the CST complex have a role in promoting Pol-α dependent DNA synthesis at non-telomeric sites. For example, mammalian Ctc1p and Stn1p were identified as DNA polymerase alpha accessory factors stimulating the primase and DNA polymerase Pol-α. Ctc1p and Stn1p colocalize with PCNA during S phase, suggesting that they have a role in DNA replication (Casteel et al, 2009). There is also study that Cdc13p may bind to non-telomeric DNA templates (Mandell et al., 2011). When Stn1p is nonfunctional in mammalian cells, there is a reduction in

the recovery of DNA replication after exposure to hydroxyurea. The reduction in recovery of DNA replication is a result of a failure to activate late-firing and dormant origin.

Our data as well as others have supported a model that CST complex facilitates Pol-α dependent priming both at telomeres and other challenging difficult-to-replicate regions to promote efficient and complete replication of the genome. It would been interesting in our future goal of deciphering the details of the downstream players of repair pathways required for cell viability when the capping protein Stn1p is compromised. At the time of the experiment, we did not have the Ubr1p of the RAD6 pathway available to study the RAD6/UBR1 pathway. Further study may be to look at specific non-telomere regions where Stn1p may bind to using a genome wide approach such as Chip-Seq or RNA-seq. Bioinformatic analysis of these Stn1p binding sites on the Chip-Seq or RNA-Seq may be accomplish.

# Conclusion

Telomeres are very important for the maintenance of linear chromosomes. Telomere act to cap the ends of chromosomes, protect against degradation, recombination, end-to-end fusions, and help overcome the end replication

problem. In budding yeast, the CST complex is essential for telomere capping. The CST loss of function mutations can lead to elevated levels of telomeric ssDNA, indicating that CST is required for blocking against exonucleolytic degradation activities.

The replicative function of the CST complex has been that Cdc13p recruits telomerase to elongate the G-rich strand. Next, the Cdc13-Pol1 and Stn1-Pol12 fill in the complementary C-strand. However, we still do not know the details of how this happen. An interesting new direction in the field of telomere is to elucidate whether the telomeric Stn1p protein affect DNA replication throughout the genome at nontelomeric regions. Mammalian *STN1* and *CTC1* were initially identified as DNA polymerase-alpha accessory factors (Goulian and Heard, 1990; Casteel et al, 2009). A study has shown that knocking down *STN1* in mammalian cells inhibit recovery from replication stress by preventing the activation of late firing and dormant origins (Stewart et al., 2012). Mammalian cells can acts as a general DNA replication factor (Stewart et al., 2012). An interesting finding from *S. pombe* is that both Rif1 and Taz1 control the timing of replication origin firing globally (Hayano et al., 2012; Tazumi et al., 2012). In *Xenopus* egg extracts, work have been done that have shown that Stn1p is required for replication of a single-stranded plasmid (Nakaoka et al. 2012). These findings reinforce our understanding that the function of telomeric proteins may not be restricted to chromosome ends.

Our work has demonstrated that *S. cerevisaie* Stn1p protein has a role in promoting DNA synthesis at nontelomeric regions. We show that loss of *STN1* result in accumulation of single stranded DNA at nontelomeric regions. Much of our understanding in this thesis is derived from genetic experiments. It will be more interesting to explore the DNA replication function of Stn1p using more directed, biochemical assays. Furthermore, it would be more interesting and exciting to take a more genomic approach using Chip-Seq or RNA-Seq to determine the binding locations of Stn1p in nontelomeric regions. These genome-wide experiments will bona fide our objective that STN1p binds to nontelomeric regions. The function of CST in global DNA replication is just poorly understood. Our study will be of interest to future investigators studying telomeres, DNA replication, and DNA repair. With mutations in *CTC1* and *STN1* leading to a variety of human diseases, a detailed understanding of Stn1p's roles in telomere capping, replication, checkpoint control, and global DNA metabolism will help us find the cause of these diseases.

# Materials and Methods

## Strains and plasmids

All strains used in this chapter are listed in Table 4.1, and all plasmids used are listed in Table 4.2. Yeast strains were grown and propagated following standard procedures (Sherman, 2002).

## Pulse field gel electrophoresis

Pulse field gels assays were carried out following previously described protocols ( Iadonato and Gnirke, 1996), with minor modifications. Strains of the indicated genotype were grown to logarithmic phase at 23°C, and synchronized in G1 by arresting with alpha factor pheromone. Cells were then washed and released into fresh media at 26˚C. At the indicated time points, cells were collected and counted by the hemocytometer. For each sample, $10^8$ cells were collected, and the volumes were adjusted to 50 µl. The cell suspensions were warmed to 55˚C, and mixed with 50 µl of low melt agarose (Invitrogen) dissolved in 1X TBE at 2% concentration. The cells and agarose mixture were pipetted into plug molds, and allowed to solidify at room temperature for 15 minutes. The solidified plugs were then treated with 1ml of spheroplasting solution (1M sorbitol, 20mM EDTA, 10mM Tris pH7.5, 14mM BME, 0.5mg/mL Zymolyase 20T) for 4 hours at 37C. Plugs were then washed with SDS solution (1% SDS, 100 mM EDTA, 10 mM Tris pH8) twice for 15 minutes each, and incubated with SDS solution at 37°C

overnight with gentle rocking. The plugs were washed with NDS solution (1% sarkosyl, 10mM Tris base, 0.5M EDTA pH9.5) 3 times for 30 minutes each, followed by 5 washes with TE for 30 minutes each. Plugs were then loaded onto a 1% low melt agarose gel, and run out in an electrophoresis chamber (CHEF DRII System from BioRad) at 14˚C for 24 hours, at 5.5 V/cm voltage, and switch times of 60-120 seconds. The gel was then stained with ethidium bromide (10 mg/ml in 1X TBE) for 10 minutes, and imaged in a UV light box (EpiChemi II Darkroom for UVP) with the Labworks image acquisition software (UVP).

Strains embedded in agarose forming plugs were wild-type, *stn1-t281, bre1-*Δ*, rad18-*Δ*,* and the double *stn1-t281 bre1-*Δ cells*.* Our mutants were grown in YPD medium for exponential growth and arrested in G2/M cell cycle phase by treatment with nocodazole for 4h at 30°C prior to DNA plugs isolation. G2-arrested cells were verified microscopically as large-budded cells before collection. Cells were collected and embedded in agarose plugs using 2% low-melting agarose at 50°C. We treated the DNA plugs with S1 endonuclease for 2 hours and the treated plugs were loaded into a 1% Megabase agarose gel (Bio-Rad), and genomic DNA was resolved by pulse field gel electrophoresis (PFGE). PFGE gels were then stained with ethidium bromide (EtBR) to observe chromosome fragmentation. For a positive control, prior to embedding in the agarose, *rad18-*Δ mutants were treated with 0.001% and 0.01% MMS, according to data from Huang et al. 2013.

## Klenow synthesis assay

Klenow synthesis experiments were run following previously published protocols (Feng et. al., 2011) with minor modifications. Strains of the indicated genotype were grown to logarithmic phase at 30°C in YPD, and arrested with alpha factor pheromone for 2 hr as indicated. Cells were harvested, washed with 50 mM EDTA, and the concentration of each sample was determined by hemocytometry. For each sample, about $10^9$ cells were collected and the volume adjusted to 500 µl. The cell suspensions were warmed to 55°C and mixed with 500 µl of low melt agarose (Invitrogen) dissolved in 1X TBE at 1 % concentration. The mixture was pipetted into plug molds, and allowed to solidify at room temperature for 15 minutes. The solidified DNA plugs were treated with 5 ml of spheroplasting solution (1M sorbital, 20 mM EDTA, 10 mM Tris pH 7.5, 14 mM BME, 0.5 mg/ml Zymolyase 20T) for 4 hours at 37˚C. DNA plugs were then washed with SDS solution (1 % SDS, 100 mM EDTA, 10 mM Tris pH 8) twice for 15 minutes each, and incubated with SDS solution at 37°C overnight with gentle rocking. Plugs were washed with NDS solution (1% sarkosyl, 10mM Tris base, 0.5M EDTA pH9.5) 3 times for 30 minutes each, followed by 5 washes with TE for 30 minutes each, then stored in 4°C overnight. The agarose plugs were then pre-equilibrated in 5ml of TMB buffer (50 mM Tris,ph 6.8, 5 mM MgCl2, 10 mM BME) for 30 minutes at room temperature, then samples were split into two 0.5 plugs from each set. The two sets of plugs were mixed with 400 µl TMB buffer,

10 µl of dNTPs at 10 µM concentration each, 10 µl of random hexamer primers at 10 µM concentration (Thermos Scientific), 100 units of exo-Klenow polymerase (New England BioLabs) and 50 µl of 10x Klenow buffer. The other 5 were treated identically but no Klenow polymerase was added. Samples were incubated at 37°C for 2 hours, then washed with TE. DNA plugs were then pre-equilibrated with 1X B-agarase buffer for 30 minutes on ice, heated to 65°C to melt agarose, and treated with 5 units of B-agarase (New England BioLabs) for 1 hour at 42°C. Salt concentration was adjusted to 0.5M NaCl, 0.8M LiCl, 0.3M NaO-Ac, and samples were cooled on ice for 15 minutes, and DNA was precipitated with isopropanol. The DNA plugs were then washed with cold 70% ethanol, dried, and resuspended in 40 µl of TE. The DNA samples were then denatured by addition of 10µl of 1M NaOH and 1 µl of 0.5 M EDTA followed by boiling at 95°C for 5 minutes. Samples were cooled on ice for 5 minutes, the sample were then loaded and run out on a large 1% agarose gel overnight at 50 volts. The gel was then transferred to a nylon membrane (Hybond-XL from Amersham) by southern blot method for overnight, and DNA was cross-linked with 120 mJ of UV light (Stratagene) after the Southern blot. The membrane was blocked with Church's buffer (1% BSA, 1 mM EDTA, 0.5M phosphate buffer, 7% SDS) overnight at 55°C with gentle rocking. 25 µl of P-32 radiolabeled TG1-2 probe was added, and the blot was incubated overnight at 55°C with gentle rocking. The blot was washed 3X with 1L of washing solution (4X SSC, 0.1% SDS), and exposed to X-ray film for 5 days in -80°C. The film was developed on a developer (Mini-

medical). After development, the membrane was stripped of telomeric probe by immersing in boiling 0.1% SDS three times for 15 minutes each, then blocked with Church's buffer as before. 25µl of P-32 radiolabeled rDNA probe was added, and the blot was processed as before.

## Yeast Two Hybrid Assay

We use the Gal4 system that identified the interaction between two proteins Stn1 and Dbf4. *STN1* gene is on a plasmid pCN366 that has the GAL4 activation domain (AD). *DBF4* is a bait gene that is expressed on plasmid pAS1 as a fusion to the GAL4 DNA-binding domain (DNA-BD). The two proteins are expressed as fusion proteins either with the Gal4-BD or AD. When the bait and fusion protein interact, the DNA-BD and AD bind to each other forming a DNA-BD and AD complex. The DNA-BD-Bait protein- AD complex binds to the GAL UAS upstream of the promoter to activate transcription of reporter genes. The strain we use has three reporter genes: *ADE2, HIS3*, and *E.coli* LacZ. The two-hybrid technology is used to identify protein interactions. The two plasmids containing these constructs DNA-BD Dbf4 and Stn1-AD are co-transfected into the yeast strain containing the upstream activation sequences from the GAL1-GAL10 regions, which promote transcription of the reporter gene. The Stn1-AD fusion plasmid is marked with *LEU2,* while the DNA-BD Dbf4 bait plasmid carries *TRP1*. After co-transforming these two plasmids DNA-BD/Dbf4 and AD/Stn1 into the yeast strain pJ694a. The appropriate genotypes (Stn1-AD/Dbf4-BD, Stn1-AD/Cdc13-BD, Stn1-AD/PAS1-

BD) were diluted into selective media and stamped onto selective plates: Low stringency: Plate culture on -Leu/-Trp to select for all cotransformants; Medium stringency: Plate culture on Leu-Trp-Ade, and Leu-Trp-His to select for cotransformants. The colony growth will indicate an interaction between the two-hybrid proteins.

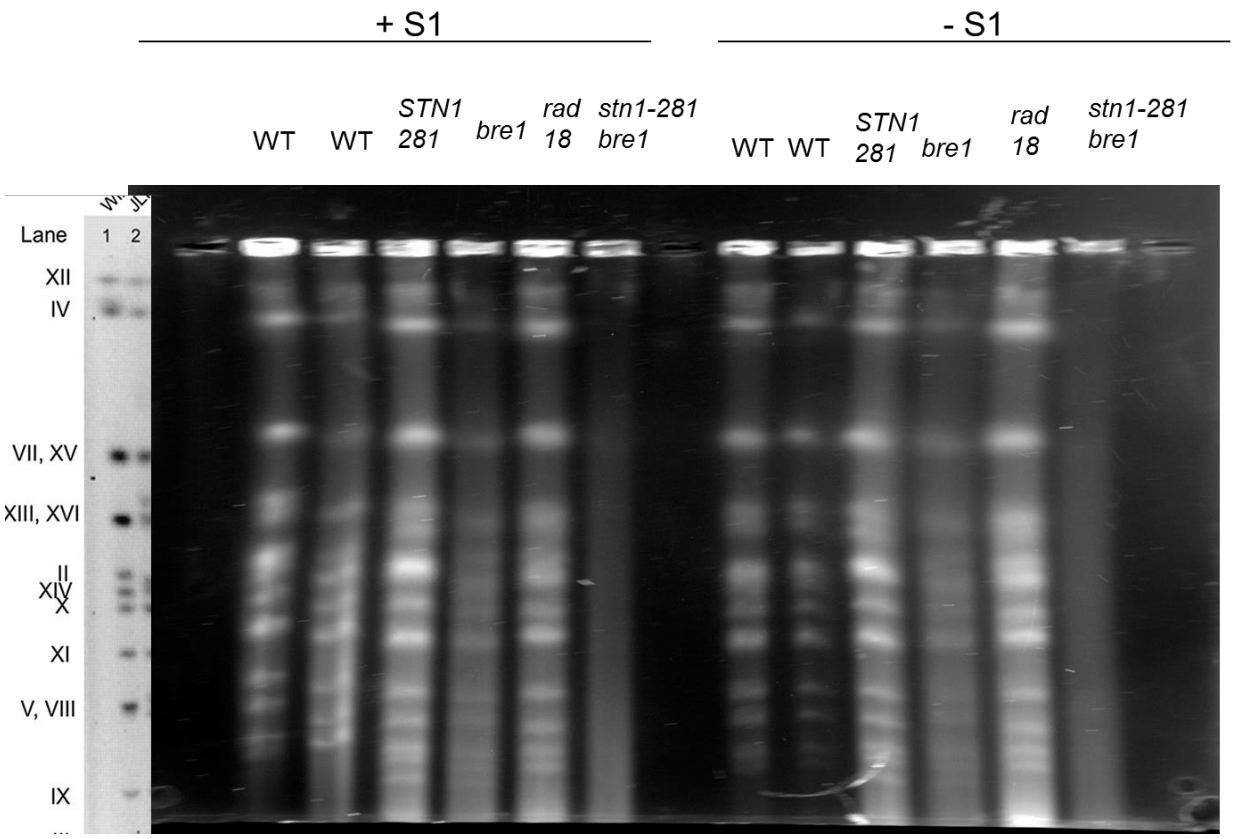


Figure 2.1. Analysis of chromosome integrity by pulsed field gel electrophoresis (PFGE). Wild-type, *stn1-t281, rad18-*Δ*, bre1-*Δ*,* and *stn1-t281 bre1-*Δ cells were arrested in G2 phase by nocodazole treatment for 4 hr. Arrested cells were collected and embedded in 1% agarose plugs. Half of the DNA plugs were treated with S1 endonuclease for 2 hours. The *rad18-*Δ mutant was treated with 0.01% MMS, a method previously reported to generate or reveal single stranded DNA gaps. The PFGE gel was stained with ethidium bromide. The *bre1-*Δ single and *bre1-*Δ *stn1-t281* double mutants showed significant smearing on the gel even without S1 nuclease treatment, consistent with chromosome fragmentation.

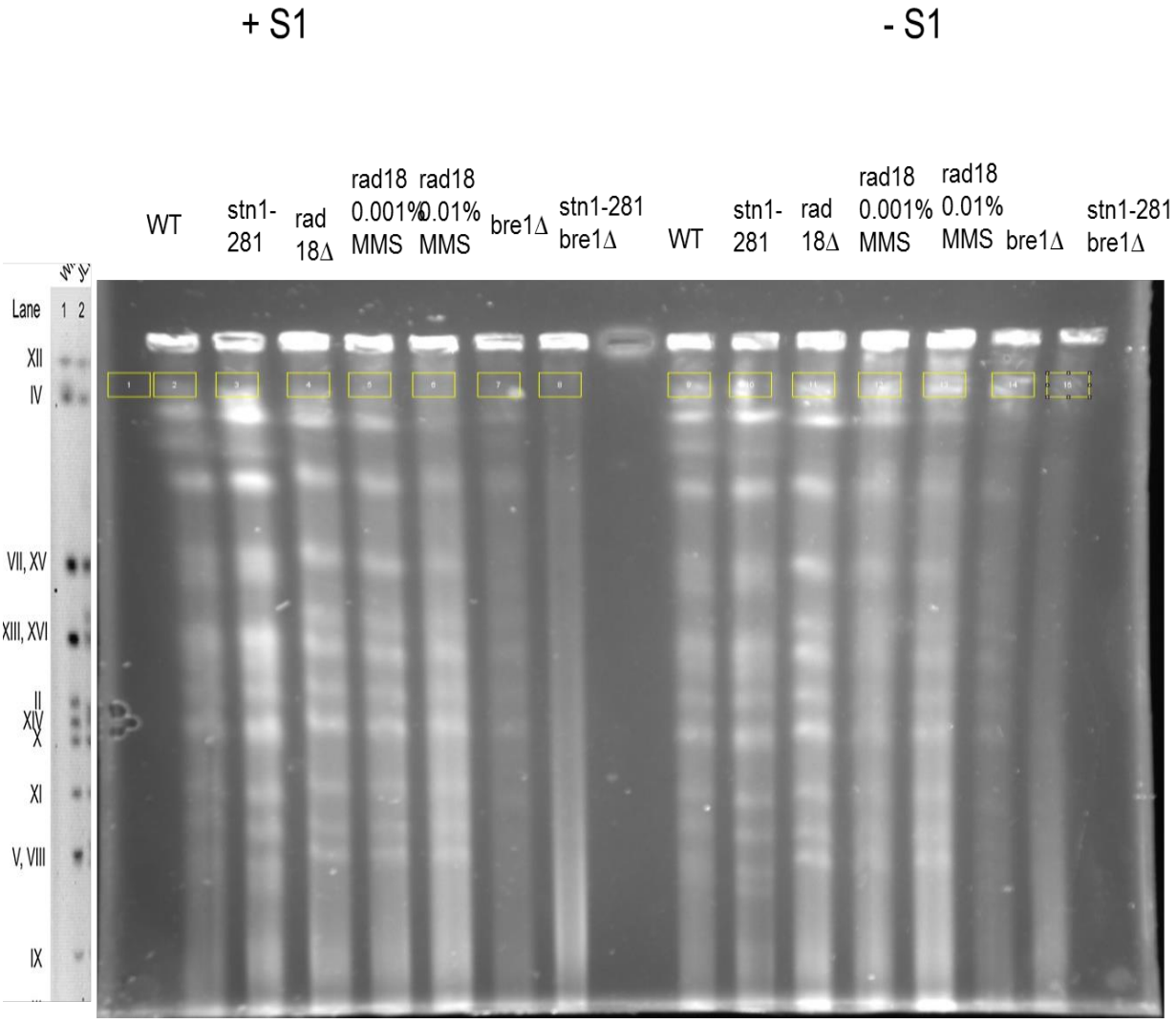


Figure 2.2. Analysis of chromosome integrity by PFGE. Wild-type, *stn1-t281, rad18-*Δ*, bre1-*Δ, and *stn1-t281 bre1-*Δ cells were arrested in G2 phase by nocodazole treatment for 4 hr. Arrested cells were collected and embedded in 1% agarose plugs. Half of the DNA plugs were treated with S1 endonuclease for 2 hours. The *rad18-*Δ mutants were treated with two doses of MMS (0.001% and 0.01%) and incubated for 2h as a positive control for single stranded DNA gaps. The PFGE gel was stained with ethidium bromide. The *bre1-*Δ single and *bre1-*Δ *stn1-t281* double mutants showed significant smearing on the gel even without S1 nuclease treatment, consistent with chromosome fragmentation.

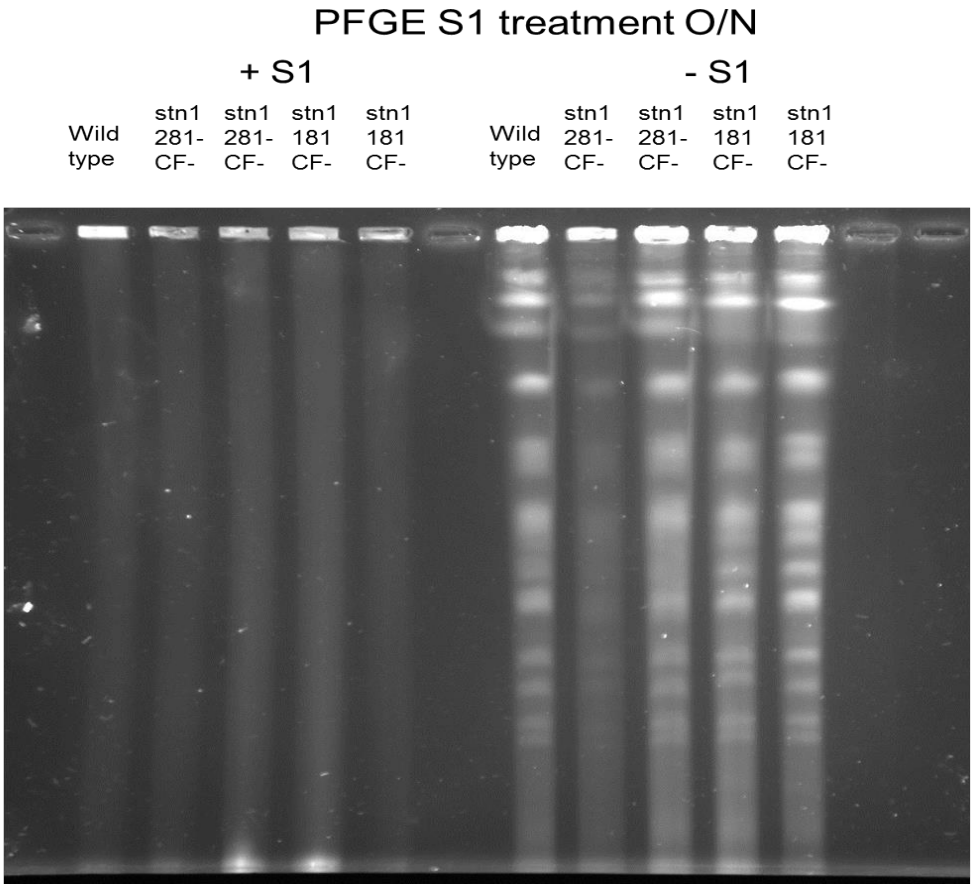


Figure 2.3. PFGE gels showing chromosomes in *stn1-t281* and *stn1-t186* mutants with overnight S1 treatment. DNA plugs were made from wild-type, *stn1-t281, stn1-t186* genotypes. Half were treated with S1 nuclease treatment for overnight (O/N) before loading onto PFGE agarose gel and staining with EtBr. All of the S1 treated plugs seem to have degraded the DNA (left side of gel). S1 nuclease cleaves single stranded gaps in the duplex DNA, but it appears that this overnight incubation led to overdigestion. Chromosomes in one of the *stn1-t281* strains appears to have a smeary migration pattern, but the others look similar to wild-type.

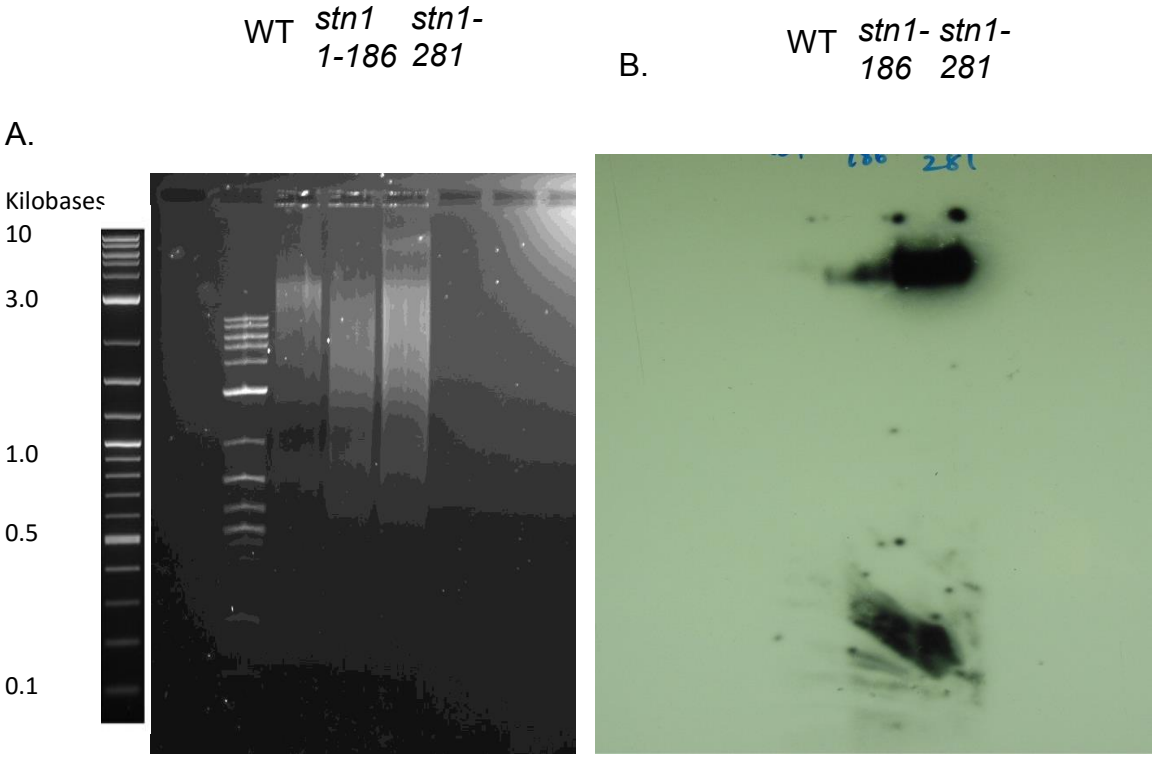


Figure 2.4 Klenow synthesis to detect single stranded gaps in *STN1* mutants.

Cells were arrested in G1 phase with alpha factor and then embedded in agarose plugs. The agarose plugs were treated with 100 units of exo$^-$Klenow polymerase, along with random hexamers and dNTPs to synthesize complementary single-stranded DNA in any gapped regions. Treated samples were run on an agarose gel. (**A**) Ethidium-bromide stained gel of Klenow synthesis products for wild-type, *stn1-t186* and *stn1-t281* strains. (**B)** Southern blot of gel in (A). The gel was blotted onto nitrocellulose and probed with P-32 radiolabeled rDNA probe for the hybridization method. Blot of rDNA for *wild-type, stn1-t186,* and *stn1-t281* indicates increase single stranded DNA at rDNA site.

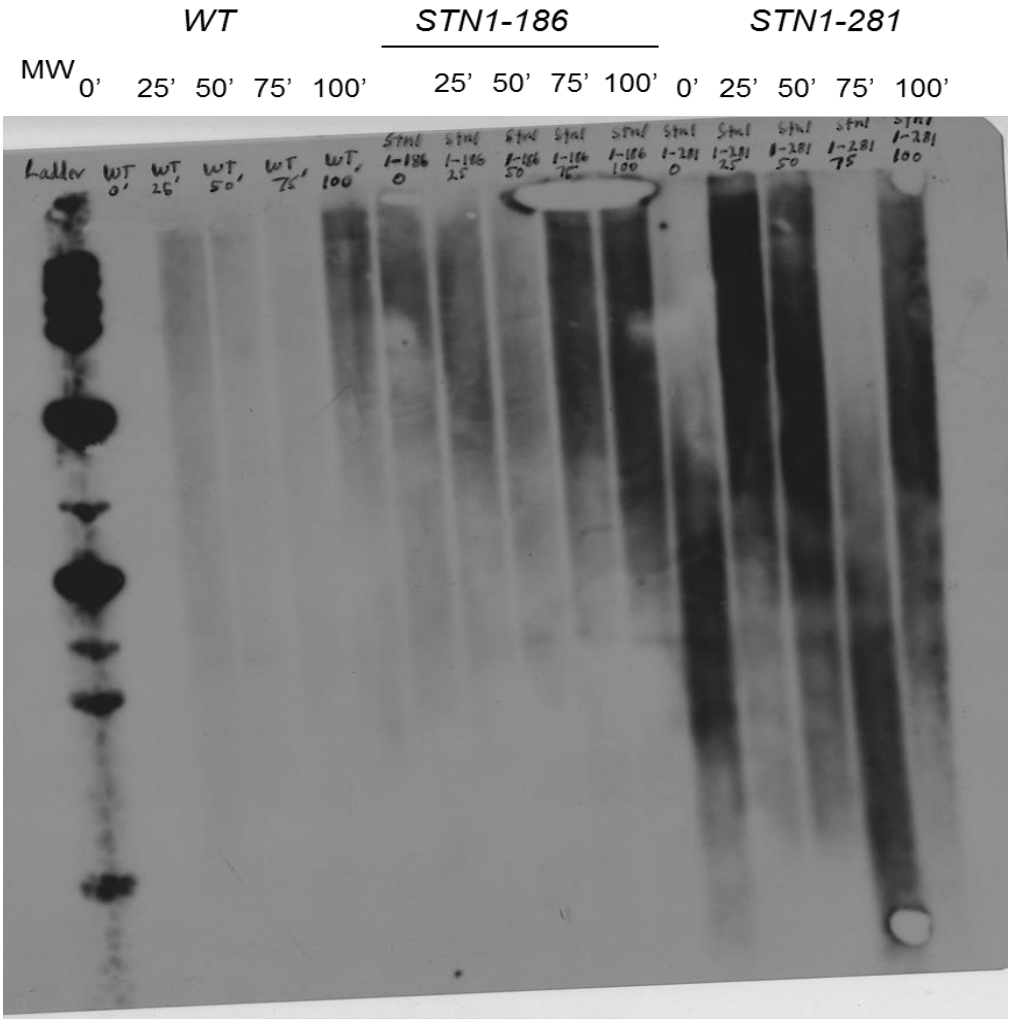


Figure 2.5 Truncation alleles of the telomere capping protein *STN1* accumulate single stranded DNA at the telomere by the Klenow method. Cells were arrested in G1 phase, then released and samples taken at the following time points: 0, 25, 50, 75, and 100 minutes. Cells collected at these time points were embedded in agarose plugs. The plugs were treated with 100 units of exo$^-$Klenow polymerase along with random hexamers and dNTPs. Treated samples were denatured, and run on an agarose gel. The large chromosomes will be too large to move from the plugs into the gel. The gel was transferred to a nitrocellulose membrane, and the blot was probed with P-32 radiolabeled telomere probe for the hybridization method. The *stn1-t186* and *stn1-t281* lanes have visible smear of bands indicating accumulation of single stranded telomere DNA. Smears of hybridizing DNA is seen in wild-type cells at 25 min, and 100 min time points.

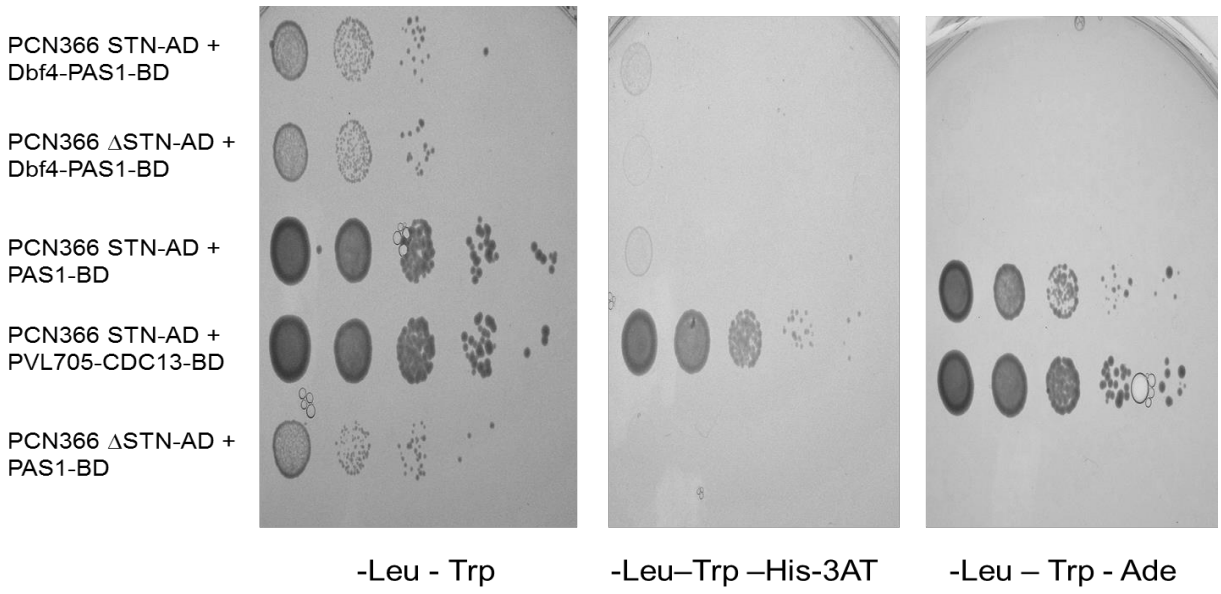


Figure 2.6 Yeast two hybrid assay between *STN1* and *DBF4*. pCN366 STN1-AD plasmid and pCN366 STN1ΔWH1-AD containing defective *STN1* lacking the first winged-helix were transformed into *S. cerevisiae* pJ694a two-hybrid strain. The yeast transformants were selected on proper nutritional media for interaction analysis using the yeast two hybrid assay. No interaction of STN1 and DBF4 on –Leu-Trp-His and –Leu-Trp-Ade were observed in this experiment.

## Table 1.6: List of Strains

| Strain | Description | Reference |
|---|---|---|
| hc671 | *MAT**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 stn1-t281::KanMX2* | *Petreaca et al, 2007* |
| hc672 | *MAT**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 stn1-t186::KanMX2* | *Petreaca et al, 2007* |
| hc1090 | *MAT**α** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 stn1-t281::KanMX2* | *Petreaca et al, 2007* |
| DCN 560 | *stn1-t281::KanMX2/STN1, bre1-Δ::URA3/BRE1* | *This study* |
| DCN 586 | *stn1-t281::KanMX2/STN1, rad6-Δ::LEU2/RAD6* | *This study* |
| DCN 588 | *stn1-t281::KanMX2/STN1, rad18-Δ::LEU2/RAD18* | *This study* |
| DCN 590 | *stn1-186::KanMX2/STN1, bre1-Δ::URA3/BRE1* | *This study* |
| DCN 558 | *stn1-186::KanMX2/STN1, rad18-Δ::LEU2/RAD18* | *This study* |
| DCN 561 | *stn1-t281::KanMX2/STN1, rad18-Δ::LEU2/RAD18, bre1Δ::Ura/BRE1* | *This study* |
| DCN 562 | *ten1-101::KanMX2/TEN1, rad6-Δ::LEU2/RAD6* | *This study* |
| DCN 563 | *ten1-105::KanMX2/TEN1, rad6-Δ::LEU2/RAD6* | *This study* |
| DCN 567 | *ten1-105::KanMX2/TEN1, rad18-Δ::LEU2/RAD18* | *This study* |

## Table 1.7: List of plasmids:

| Plasmid | Description | Reference |
|---|---|---|
| pCN366 | *2μ LEU2 ADH*-promoter $GAL4_{AD}$ *STN1* | Petreaca et al, 2007 |
| pCN367 | 2μ *LEU2 ADH*-promoter $GAL4_{DBD}$ *DBF4* | |
| pVL705 | 2μ *LEU2 ADH*-promoter $GAL4_{DBD}$ *CDC13*ΔBstBI | Petreaca et al, 2007 |

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