Role of the fission yeast Taz1p in promoting genomic stability

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Abstract

Telomeres define the ends of eukaryotic chromosomes and comprise multiprotein complexes bound to terminal DNA sequence repeats. An intrinsic role of telomeres is to protect chromosomal termini from being processed as damaged-induced DNA breaks; this role is critical for maintaining genomic integrity. The fission yeast Taz1 protein regulates telomere functions throughout the mitotic and meiotic cell cycles.

In this study, we employed biochemical, molecular and genetic analysis to dissect the regulation and role that \textit{taz1} and other telomere proteins play in promoting genomic stability. We find during growth at low temperatures, loss of \textit{taz1} results in decreased viability, chromosome missegregation and DNA damage checkpoint activation. Strikingly, these cells exhibit entangled chromosomes and a pronounced \textit{de novo} accumulation of DNA double strand breaks (DSBs). These defects are suppressed by altered topoisomerase II function, implicating unprotected telomeres as substrates for Top2p. Furthermore, \textit{taz1} cells are sensitive to treatments that induce DSBs suggesting a role for Taz1p in general DSB repair. Recent data obtained from 2-D DNA gel electrophoresis suggests that Taz1 is specifically required for telomere replication and that loss of Taz1 results in perturbed fork progression through both telomere and telomere associated sequences. These data suggest a model whereby aberrant replication in \textit{taz1} cells results in entangled chromosomes, leading to a requirement for altered Top2 activity and homologous recombination. These phenotypes are specific to \textit{taz1} cells and not other telomere mutants suggesting that Taz1 plays a central role in promoting genomic stability that is separable from its interacting partners.
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Abbreviations

°C: degrees celcius
µl: microliter
µM: micromolar
µm: microns (or micrometeres)
1D: first dimension
2D GE: two-dimensional gel electrophoresis
2D: second dimension
a.a.: amino acids
ATP: adenosine 5'-triphosphate
BND: Benzoylated Naphthoylated DEAE Cellulose
bp: base pairs
BSA: bovine serum albumin
cm: centimeter
DAPI: 4'-6-diamidino-2-phenylindole
dATP: deoxyadenosine 5'-triphosphate
dCTP: deoxycytosine 5'-triphosphate
ddH₂O: double distilled water
DIC: differential interference contrast
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
dNTPs: deoxynucleotide 5'-triphosphate
DSBs: double strand breaks
DTT: dithiothreitol
ECL: Electrochemiluminescence
EDTA: ethylenediaminetetraacetic acid
EMM: Edinburgh minimal media
EtOH: ethanol
G1: gap phase one
G2: gap phase two
GFP: green fluorescent protein
G-G: guanine to guanine base pairing
G-rich: guanine rich
HA: Hemagglutin epitope
HB: Hepes Buffer
Hepes-KOH:
HR: homologous recombination
hrs: hours
HU: hydroxyurea
IP: immunoprecipitation
kan: kanamycin
kb: kilobases
kDa: kilodalton
LiOAc: lithium acetate
LMP: low melting point agarose
mg: milligram
min: minutes
ml: mililiter
MMS: methylmethane sulfonate
M-phase: mitosis
MRN: Mre11/Rad50/Nbs1
ng: nanogram
NHEJ: nonhomologous end-joining
O.D.: optical density
ORF: open reading frame
PARP: poly(adenine diphosphate) ribose polymerase
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PEG4000: polyethyleneglycol 4000
PFGE: pulsed field gel electrophoresis
PMSF: Phenylmethylsulphonyl fluoride
PVDF: Polyvinylidene fluoride membrane
rDNA: ribosomal deoxyribonucleic acid
RNA: ribonucleic acid
rt: room temp
SDS: sodium dodecyl sulfate
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP1: spheroblast buffer
S-phase: synthesis phase
SSC: Saline-sodium citrate buffer
SV40: simian virus 40
TAE: Tris-acetate + EDTA
Taq: Thermus aquaticus
TAS: telomere associated sequence
TBE: Tris-borate + EDTA
TBS: tris buffered saline
TBS-T: tris buffered saline with Tween
TBZ: thiabendazole
TCA: trichloroacetic acid
TE: Tris + EDTA
Tm: melting temperature
Top2: topoisomerase two
ts: temperature sensitive
wt: wild type
X: times
YES: yeast extract supplements media
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1 Introduction

1.1 Telomeres

In the 1930's, experiments in the fruit fly, *Drosophila melanogaster*, hinted that the ends of chromosomes represented a specialized structure that was different from other DNA ends. For this historical observation, the *Drosophila* geneticist Hermann J. Muller was studying the behaviour of chromosomes that were treated with radiation. He noticed that after radiation treatments, broken chromosomes were fused with other broken chromosomes but that this activity was prohibited from happening at the very terminus of the chromosome suggesting that the ends act like "protective caps" that distinguish chromosomal termini from radiation-induced double strand breaks (Muller, 1938). Around the same time, Barbara McClintock was studying meiotic chromosome behaviour in maize and found that newly formed breaks were able to fuse to other breaks whereas natural chromosome ends were not during meiosis (McClintock, 1941; McClintock, 1942). This again suggested that telomeres had a special property to protect the end from fusing reactions. Since these landmark observations were made, intensive attention has been given to chromosome ends, termed ‘telomeres’ from the two Greek roots telos (end) and meros (part), and it is becoming clear that these ends are important structures that are involved in both aging and cancer. Indeed, alterations in telomere functions have since been shown to promote the genomic instability associated with cancerous cells, as well as to limit the lifespan of both cancerous and normal cells. The connection between telomeres, cancer and aging in humans
emphasizes the importance of gaining a better understanding of this relationship in hopes of developing additional therapies for combating the deleterious effects of cancer and aging. For this thesis, we have utilized the model organism, *Schizosaccharomyces pombe*, to study the interplay between telomeres and genomic instability in anticipation that insights gained from these studies will be applicable to humans.

The ends of linear chromosomes, called telomeres, are found in all eukaryotes. Telomeres are composed of three main components that are important for their function. First, telomeres are comprised of double stranded DNA simple sequence repeats that are 5' TTAGGG 3' in vertebrates and related sequences in other organisms (5' TG_{1-3} 3' in budding yeast and 5'TTACAG_{1-3} 3' in fission yeast) (Figure 1.1). Telomeric double stranded tracks vary in length in different organisms but can vary between 5 and 15 kb in human cells, up to 60 kb in mice and as little as around 300 bp in both budding and fission yeast. Telomeres also have a 3' single stranded overhang that extends from the 5' TTAGGG 3' G-rich strand past the 5' C-rich strand. These overhangs are a conserved feature of telomeres and range in length from ~ 150 nt in human cells to no less than 12 nt in budding yeast (Larrivee et al., 2004; McElligott and Wellinger, 1997). Telomeres also consist of proteins that bind to both the single- and double-stranded telomeric DNA tracts (Figure 1.2, (Ferreira et al., 2004)). These proteins bind and protect the telomere as well as nucleate other telomere complexes that regulate the many functions of telomeres. These proteins and their functions will be discussed later in more detail.
Telomeres

Figure 1.1 Components of telomeres. Top panel depicts a metaphase spread of human chromosomes with telomeres being labeled in yellow. Telomeres consist of repetitive DNA sequences (TTAGGG in humans) that are mostly double stranded except for a single stranded 3' overhang. Telomere sequences are bound by both double stranded and single stranded telomere binding proteins that bind and regulate multiple protein complexes to nucleate a functional telomere complex (green oval).
Figure 1.2 Telomere components in fission yeast, humans and budding yeast
1.2 End replication problem and telomerase

In the 1970’s, it was proposed that linear DNA molecules posed a specific problem to conventional DNA replication that was predicted to be incapable of fully replicating a linear DNA molecule (Olovnikov, 1973; Watson, 1972). This hypothesis was coined ‘the end-replication problem’ and purported that lagging strand synthesis would leave a 3’ overhang because of RNA primer removal and therefore would not fully replicate the linear DNA molecule (Figure 1.3). Conventional semi-conservative DNA replication is accomplished by the coordination of two modes of DNA synthesis, leading and lagging strand synthesis. DNA polymerases can only synthesize in the 5’ to 3’ direction. Therefore, a replication fork moves in only one direction and the strand that is replicated in this direction is achieved by leading strand synthesis that replicates the 3’ strand of the original DNA template. Lagging strand synthesis replicates the 5’ strand and is achieved by the synthesis of short RNA primers by the DNA polymerase α-primase complex that are extended as short DNA Okazaki fragments that are eventually ligated together after RNA primer removal to create a continuous lagging strand. There are no known mechanisms for replacing the terminal RNA primer of the lagging strand and its removal therefore results in a 3’ overhang and loss of DNA sequence (Figure 1.3). Thus, this defect was believed to constitute the end replication problem. However, the discovery that linear chromosomes end in a 3’ overhang and are not blunt required a re-examination of the end replication problem (Lingner et al., 1995). Since chromosome termini end in 3’ overhangs, conventional DNA replication would have a problem not only on the lagging strand but also on the
Figure 1.3 The end-replication problem. (A) End-replication problem for a linear DNA molecule caused by lagging strand synthesis. (B) End-replication problem for a linear chromosome which contains 3’ single stranded overhangs. Incomplete replication occurs through a leading strand synthesis problem from the lack of 3’ overhang formation. Only one of the two chromosomes produced from replication are shown. (cen: centromere)
leading strand. Since leading strand synthesis is processive, it is believed to continue to the very end of the chromosome and would therefore end in a blunt ended molecule (Ohki et al., 2001). This reaction would fail to recapitulate the 3' overhangs found at chromosomal termini and consequentially, DNA sequences and structure would be lost (Figure 1.3). Thus, leading strand synthesis can also contribute to the end replication problem for eukaryotic DNA chromosomal replication.

To combat the end replication problem, most eukaryotes utilize the specialized ribonucleoprotein enzyme telomerase (Smogorzewska and De Lange, 2004). Telomerase consists of a protein catalytic subunit containing reverse transcriptase activity and an RNA component (Greider and Blackburn, 1987; Greider and Blackburn, 1989; Lingner et al., 1997; Nakamura et al., 1997; Wellinger et al., 1993b). Telomerase has the ability to extend the 3' end of DNA using its RNA component as a template. Interestingly, telomerase needs a 3' overhang as a substrate for its activity and cannot act on a blunt ended molecule (Lingner and Cech, 1996). This is surprising given that the end replication problem predicts a blunt end and an overhang end for a replicated DNA linear molecule. Therefore, nucleolytic processing of at least one end of a chromosome is required before it can be actively elongated by telomerase. 3' overhangs have been found on both ends of chromosomes in several organisms, arguing that this is a general feature of telomere ends (Makarov et al., 1997; McElligott and Wellinger, 1997; Wellinger et al., 1996). Thus, telomerase adds telomeric sequences onto the 3' ends of chromosomes by reverse transcribing off of its RNA template, with the other strand being synthesized presumably by the lagging strand DNA polymerases α and δ.
counteracting any telomere shortening due to either replication or nucleolytic processing (Diede and Gottschling, 1999). Additionally, it has been shown that the passage of a replication fork is required before telomerase can act, suggesting that telomerase acts on sister chromatids after replication and not on the parental chromatid before replication (Dionne and Wellinger, 1998; Wellinger et al., 1993a).

Telomeres must be maintained for genomic stability and this maintenance is normally accomplished through telomerase. Indeed, mutations that abolish telomerase activity lead to the shortening of telomeres. In budding yeast, telomere loss has been calculated to be around 3-5 bp/end/generation (Lundblad and Szostak, 1989; Singer and Gottschling, 1994). In human and mouse cells, it has been calculated that between 50 – 200 nt are lost per generation in telomerase null cells (Blasco et al., 1997; Dionne and Wellinger, 1996; Harley et al., 1990; Huffman et al., 2000; Niida et al., 1998). These numbers are similar to the size of the 3’ overhang and might represent the loss from the end replication problem from the leading strand. However, telomerase null cells still have 3’ overhangs suggesting that nucleolytic processing still occurs in the absence of telomerase and suggests that nucleases might also contribute to the shortening of telomeres in the absence of telomerase (Dionne and Wellinger, 1996). If telomere shortening is not counteracted by lengthening mechanisms, telomeres become short and dysfunctional, leading to genomic instability (see below).

Telomere length homeostasis is accomplished through the actions of both the lengthening and shortening of telomeric tracts, which is regulated by a host of factors (Askree et al., 2004). Analysis of these factors are beyond the scope
of this introduction and are reviewed extensively elsewhere (Smogorzewska and De Lange, 2004). Astonishingly, a recently published genomic screen in budding yeast revealed over 170 genes that affected telomere length (Askree et al., 2004). Clearly, the regulation of telomere length is a complicated process and requires further analysis for mechanistic details to be revealed. However, a remarkably insightful study has recently redefined the way that we think about telomerase and its elongatation of telomeres (Teixeira et al., 2004). In this study, elongation of telomeres by telomerase was analyzed in a single cell cycle, allowing accurate resolution down to a single nucleotide addition. Amazingly, it was found that not all telomeres are elongated during one cell cycle and that shorter telomeres are preferentially elongated. Telomerase added anywhere from a few nucleotides to more than 100 bp to a selection of telomeres during one round of DNA replication. These results suggest a model whereby telomere length is maintained by telomerase through the balancing of telomere states; one being competent for telomerase extension and the other being unsuited for telomerase extension (i.e. a nonextendible state) (Teixeira et al., 2004).

1.3 Functions of telomeres

Telomeres are required for a surprisingly diverse array of biological functions. As explained above, telomeres are required to counteract the end replication problem by engaging telomerase, which synthesizes additional telomere repeat sequences so that chromosomes are fully maintained. Telomere repeat sequences are required to target telomere binding proteins to telomeres where they perform their functions. These proteins are responsible
for stabilizing chromosome ends; a requirement for maintaining genomic stability (see next section). Telomeres also regulate telomerase and telomere length. Telomeres are heterochromatic regions and are organized in non-nucleosomal structures (Shore, 2001). Proper telomere function is required to repress the transcription of genes that are placed near a telomere; a process termed the telomere position effect (or silencing) (Gottschling et al., 1990). Although controversial, the telomere position effect might have evolved to inhibit transposition and propagation of viruses and transposable elements that could be detrimental to the cell. Additionally, some functional genes have been located near telomeres and it is possible that their regulation is influenced by telomeres (Halme et al., 2004). Telomeres also play a critical role in some organisms during meiosis where they orchestrate chromosomal movements that are required for proper meiotic recombination and segregation (Cooper et al., 1998; Maddar et al., 2001). Thus, telomeres are an integral component of many chromosomal and cellular processes. A complete review of all the functions of telomeres would be extraneous and beyond the scope of this thesis. Thus, it is my intention to introduce the relevant concepts and background for understanding and evaluating the work presented in this thesis. Therefore, attention is placed on introducing the crucial roles of telomeres in protecting chromosome ends and promoting genomic stability.

1.4 Role of telomeres in chromosome end-protection and genomic stability

1.4.1 Telomere binding proteins

Fundamentally, telomeres are required to “cap” chromosome ends and
protect them from a battery of activities that, if left unguarded, can lead to chromosomal mistreatments resulting in genomic instability (Ferreira et al., 2004). Telomeres are required to protect chromosome ends from fusion reactions that could lead to the inappropriate joining of telomeres to each other or to genomic double strand breaks (DSBs). Telomere-to-telomere fusions result in dicentric chromosomes that are unstable during mitosis. When dicentric chromosomes are segregated by the mitotic spindle to the opposite poles of the cell, a spindle pole body tug-of-war ensues resulting in either fragmented chromosomes or chromosome loss and aneuploidy in the two daughter cells. Both of these events bring on unequal segregation of genetic material, a catastrophic event for a cell. However, as an organism, things can worsen since fragmented chromosomes lead to additional DNA breaks that can then fuse to other breaks leading to a chain of events called the breakage-fusion-bridge cycle which has been linked to genomic instability and cancer formation (see below). Telomeres must also be protected from fusing to genomic breaks that can result from both natural events and damaged-induced DSBs that can lead to the same detrimental events as telomere-to-telomere fusions. Telomeres prevent excessive processing of the chromosome end by nucleases. Nucleases, if left unchecked, can lead to the inappropriate degradation of telomeric sequences resulting in terminal deletions and telomere loss. Telomeres are comprised of repetitive sequences that are potentially recombinogenic. Therefore, telomeres must inhibit inappropriate recombination processes from acting on telomeric sequences that, if allowed to occur, can lead to genetic rearrangements and loss of genetic information. Damaged-induced DSBs trigger cellular checkpoint surveillance systems that can induce a
cell cycle arrest, activate DNA repair pathways and cause cellular death through apoptosis in mammalian cells. Telomeres must prevent these activities by protecting chromosome ends from being recognized and treated as damaged-induced DSBs. It appears that human cells, as well as numerous other organisms, have developed an ingenious way to duck DNA damage checkpoints by tucking in the 3' single strand overhang into the duplex region of the telomere to form a T-loop (de Lange, 2004; Griffith et al., 1999), see Figure 1.3. By forming a T-loop, chromosome ends would no longer resemble DNA breaks, helping to explain the unique properties of telomeres that distinguish them from other genomic DNA breaks. Thus, telomeres are specialized structures whose maintenance are essential for distinguishing chromosome ends from DSBs which is an all-important function for promoting genomic stability (summarized in Figure 1.4, (Ferreira et al., 2004)).

The fission yeast, *Schizosaccharomyces pombe*, has been a useful organism for studying chromosome biology. *S. pombe* has similar centromere structures, cell cycle phases and checkpoints and chromosome segregation mechanisms as higher eukaryotes which suggests evolutionary conservation and makes fission yeast a valuable model organism (Forsburg, 1999; Zhao and Lieberman, 1995). Additionally, fission yeast is as distantly related to budding yeast as humans, creating a useful evolutionary counterpoint for research in hope of discovering mechanisms that are conserved in higher eukaryotes. As for telomeres, fission yeast have 3 chromosomes in a haploid cell which facilitates cytological and physical studies of telomeres. Fission yeast have many telomere proteins that are homologous to human proteins suggesting conserved function between the two organisms. Additionally, because of the
Figure 1.4 Outcomes of telomere dysfunction that can promote genomic instability. Telomeres are responsible for capping the chromosome termini which inhibits recombination, degradation and end-joining activities, processes that are required to repair double stand breaks. Dysfunctional telomeres can allow these processes to occur at telomere ends, leading to the inappropriate treatment of telomeres as DSBs. These treatments can lead to checkpoint activation, apoptosis and senescence in mammalian cells and genomic changes such as translocations, deletions and chromosome end fusions resulting in genomic instability. Thus, telomeres play an important part in maintaining genome stability.

(Figure taken from Ferreira, Miller and Cooper Mol. Cell. 13 2004)
low number of chromosomes, fission yeast can survive with circular chromosomes that lack telomeres, a feature unique among eukaryotes (Nakamura et al., 1998). Therefore, this extraordinary situation should reveal universal answers to the basic biological questions of why eukaryotes evolved to have linear chromosomes and the role of telomeres in this process.

In fission yeast, double-stranded telomere sequences are bound by Taz1, a member of the TRF family of telomere binding proteins that include human TRF1 and TRF2 (Cooper et al., 1997a; Li et al., 2000). Taz1 is essential for the protection of telomeres as its loss results in telomere uncapping. Loss of Taz1 results in the elongation of the double-stranded telomeric repeat tracts as well as a deregulation of the 3′ single stranded overhang (Cooper et al., 1997a; Tomita et al., 2003). Taz1 is required for telomere chromatin structure, repression of the telomere position effect and telomere clustering during meiosis as well as efficient meiotic recombination (Cooper et al., 1997a; Cooper et al., 1998). Loss of Taz1 also results in end-to-end telomere fusions in the G1 phase of the cell cycle, a situation encountered during meiosis and upon nitrogen starvation (Ferreira and Cooper, 2001a; Tuzon et al., 2004). During normal growth, 70% of a cell cycle for a fission yeast cell is G2 and normally cells do not experience a detectable G1 phase. This is important because taz1′ telomere fusions are dependent on ku and lig4′, two components of the NHEJ (non homologous end-joining) pathway. DNA repair pathways are cell cycle regulated in fission yeast with HR (homologous recombination) being prevalent in G2 when a homologous sister chromatid is present that can be used for homology dependent DNA repair and NHEJ being favoured during G1 when no homologs, and only single chromatids, are present (Ferreira and Cooper, 2004).
Therefore, this unique cell cycle property of fission yeast allows taz1Δ cells to dodge DNA repair pathways that would otherwise fuse taz1' telomeres. However, if the HR pathway is compromised in taz1Δ cells by the deletion of rad22Δ or rhp51Δ (the budding yeast Rad52 and Rad51 homologs), telomere fusions are readily detectable throughout the cell cycle suggesting that HR protects taz1' telomeres from NHEJ reactions although the exact relationship between HR proteins and taz1' telomeres is still unclear (Ferreira and Cooper, 2001a). In human cells, there are two TRF members, TRF1 and TRF2. They both share similar sequence homology with Taz1 so it appears that two separate telomere binding proteins have evolved in human cells to encompass the functions of Taz1 (Li et al., 2000). TRF2 is required to prevent NHEJ-dependent telomere fusions (Smogorzewska et al., 2002). Knockout mice lacking TRF1, a protein usually linked to telomere length regulation, is embryonic lethal suggesting an involvement in some aspect of telomere end-protection (Karlseder et al., 2003). In budding yeast, Rap1, the major double stranded binding protein is both an essential transcriptional activator and a telomere binding protein (Shore, 1994). While homologs of Rap1 have been described in fission yeast and human, these homologs do not bind telomeres directly but are recruited to telomeres via Taz1/TRF2 (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001; Li et al., 2000). Rap1 is essential and has dual biological functions which complicates its analysis for telomere capping. However, mutations that affect its telomere function are required for chromosomal stability suggesting that Rap1 also is essential for telomere capping (Conrad et al., 1990; Kyrion et al., 1992). Thus, double stranded telomere binding proteins appear to play a universal role in protecting
chromosome ends from end-joining activities.

Taz1 is also involved in protecting the 3’ single-strand overhang. In wt cells, 3’ overhangs are not detectable in cycling cells, most likely due to the limitations of the assay. However, in taz1Δ cells, an intense signal is readily detected in cycling cells showing that the regulation of 3’ overhang formation requires Taz1 (Tomita et al., 2003). Interestingly, the formation of detectable 3’ overhangs in taz1Δ cells is dependent on the MRN complex. The MRN complex consists of Rad32 (MRE11 in humans and budding yeast), Rad50 and Nbs1 (Xrs2 in budding yeast) (van den Bosch et al., 2003). The MRN complex is required for DNA repair and the intra-S-phase checkpoint in fission yeast (Chahwan et al., 2003; Ueno et al., 2003). Therefore, it has been proposed that MRN is responsible for 3’ overhang resectioning in fission yeast. In budding yeast, MRN has also been shown to interfere, but not abolish, 3’ overhang formation (Larrivee et al., 2004). Disruption of TRF2 in mammalian cells results in the loss of the 3’ overhang which is dependent on the XRCC1/XPF nucleotide excision repair (NER) endonuclease (Zhu et al., 2003). This protein was also found to be part of the TRF2 complex in human cells and inhibition of XRCC1/XPF results in the production of telomeric DNA-containing double minute chromosomes (TDMs) suggesting that this protein is required to protect telomeres, perhaps from intrachromosomal recombination that could result in TDMs (Zhu et al., 2003). TRF2 also interacts with the MRN complex suggesting that this complex could also be involved in 3’ end formation in human cells (Zhu et al., 2000).

Telomeres also have single-stranded binding proteins that are required to cap chromosome ends. In budding yeast, the 3’ telomeric overhang is bound
by the essential protein Cdc13, which is required both for telomere capping functions and for in vivo telomerase activity (Evans and Lundblad, 1999; Garvik et al., 1995). Disruption of Cdc13 results in the formation of a dramatically extended 3' overhang due to 5' degradation and increased telomeric recombination, suggesting that long, unprotected 3' overhangs are recombinogenic (DuBois et al., 2002; Garvik et al., 1995; Grandin et al., 2001a). The role of Cdc13 in protecting telomeres from 5' degradation is exerted through interactions with Ten1 and Stn1, as ectopic delivery of Stn1 to the telomere suppresses a cdc13** allele, and mutations in these genes lead to similar phenotypes as CDC13 mutants (Chandra et al., 2001; Grandin et al., 2000; Grandin et al., 2001b; Pennock et al., 2001). A single-stranded binding protein has also been described in both fission yeast and humans. First found in fission yeast due to its homology with single-stranded telomere proteins in ciliates, Pot1 was cloned in both fission yeast and humans and was found to bind to the single-stranded overhang in both organisms (Baumann and Cech, 2001). Deletion of pot1' results in the rapid degradation of both the single- and double-stranded regions of the telomere in fission yeast. This phenotype is different from the loss of telomerase which leads to a gradual loss of telomeric sequences (Nakamura et al., 1997). Therefore, Pot1 has an additional role in protecting telomeres from degradative processes in fission yeast. In humans, the role of Pot1 in telomere capping has not been directly tested. However, Pot1 was shown to be a negative regulator of telomerase through an interaction with TRF1 (Loayza and De Lange, 2003). By analogy, Taz1 might also interact with Pot1 in fission yeast. Recently, a Pot1-interacting protein, PIP1/PTOP has been described in humans (Liu et al., 2004b; Ye et al., 2004). PIP1 appears to
recruit a TRF1-containing complex, possibly through TIN2, another protein involved in telomere length regulation (Kim et al., 1999). Taken together, the phenotypes elicited from the loss of Pot1 in humans and fission yeast suggests that Pot1 is required to both regulate telomerase action and cap telomeres. These functions are shared with Cdc13, suggesting that Pot1 and Cdc13 are functional homologs. Collectively, these data show that the telomeric 3' overhang must also be protected to maintain functional telomeres.

1.4.2 Pathways required to prevent telomere loss and maintain genomic stability

Telomere uncapping can also occur in situations where telomeric sequences are lost, thus diminishing the binding capacity of protective telomere binding proteins and resulting in telomere dysfunction. Telomere sequences must be maintained to preserve proper telomere end-protection. The main mechanism for maintaining telomere sequences is telomerase. In both budding and fission yeast, loss of the telomerase catalytic subunit (EST2 in budding yeast and trtV in fission yeast) or components of telomerase that are required for its in vivo activity (for example, estV), result in the gradual loss of telomeric sequences (Beernink et al., 2003; Lundblad and Szostak, 1989; Nakamura et al., 1997). After sufficient generations occur to deplete the telomere reserves, cells go through a period of senescence and crisis resulting in death for most of the cells. However, survivors do appear in both yeasts. In budding yeast, telomerase null cells survive through the maintenance of telomeres through two different recombination pathways resulting in type I and type II recombination-dependent survivors (Le et al., 1999; Lundblad and Blackburn, 1993; Teng et
al., 2000; Teng and Zakian, 1999). Type I survivors amplify the Y' elements that are found at budding yeast telomeres and end with a short stretch of telomeric DNA that are maintained by a Rad51, Rad52-dependent pathway. Telomeres in type II survivors end with long stretches of telomeric sequences that are maintained in a Rad50, Rad52-dependent pathway. In fission yeast, most telomerase minus survivors arise through the intra-chromosomal fusion of the three chromosomes to create circular chromosomes (Nakamura et al., 1998). Since fission yeast only have 3 chromosomes, the probability that 3 chromosomes will self-circularize instead of fuse with other chromosomes to create dicentric chromosomes is high enough that survivors are generated. However, recombination directed survivors that have linear chromosomes do arise in fission yeast lacking telomerase (Nakamura et al., 1998). Interestingly, deletion of Taz1 preceding loss of telomerase switches the mode of survival from circular chromosome formation to survival by recombination to form linear chromosomes containing long telomeric repeats (Nakamura et al., 1998). This is most likely due to the massively elongated telomeres and long 3' telomeric overhangs found in taz1Δ cells that could potentially promote recombination. Interestingly, deleting taz1Δ from trt1Δ cells or vice versa accelerated telomere loss and senescence 100-fold (Nakamura et al., 1998). This is very interesting because if telomere loss was solely due to the end replication problem, then elongating telomeres by deleting taz1Δ should suppress senescence which it does not. This data suggests that Taz1 inhibits recombination at telomeres and is involved in maintaining the stability of telomere tracts; perhaps through inhibiting nucleases or telomere rapid deletion, a mechanism that causes end deletions through intrachromosomal recombination and resolution (Lustig,
Therefore, telomerase minus survivors in budding yeast utilize recombination pathways while fission yeast survivors circularize their genomes. This might suggest that the pathways that generate telomerase minus survivors are different between the two yeasts. However, using a PCR assay that does not rely on survival of cells, several groups have shown that the loss of telomerase results in end-to-end fusion in budding yeast (Chan and Blackburn, 2003; Mieczkowski et al., 2003). These results show that telomere uncapping followed by fusion also occurs in budding yeast following telomere erosion caused by the loss of telomerase. The large number of chromosomes in budding yeast compared to fission yeast (16 versus 3 respectively) make it statistically improbable that circular survivors can exist in budding yeast which probably explains the mechanistic differences in survivor formation.

Budding yeast has been a useful organism for studying the genomic instability that accompanies telomere dysfunction. An informative assay utilizing the $CAN1$ gene has been developed that analyzes the loss or mutation of genetic material that arise in telomere mutants. The $CAN1$ gene encodes an arginine permease whose loss can be selected for on plates lacking arginine and containing the lethal arginine analog canavanine so that loss of $CAN1$ allows survival on this media (Whelan et al., 1979). In cells that have lost telomerase, genetic instability can be seen after telomeres become critically short (Hackett et al., 2001). Interestingly, analysis of the type of chromosomal rearrangements that occurred in these cells showed that larger terminal deletions, telomere fusions and nonreciprocal translocations had occurred, perhaps through breakage-fusion-bridge cycles. This data suggests that dysfunctional telomeres can result in genomic instability by allowing telomere
fusions and terminal deletions. Importantly, a later study showed that deletion of the exonuclease EXO1 largely suppressed the genomic instability observed in telomerase minus cells (Hackett and Greider, 2003). These results suggest that degradation of chromosome ends by unchecked nucleases cause terminal deletions that can recombine with other chromosomes leading to nonreciprocal translocations, two outcomes that can initiate genomic instability.

Telomerase has been implicated in pathways that promote genomic stability, cancer and aging in mammalian cells (Artandi and DePinho, 2000; Hackett and Greider, 2002). In mice and humans, telomerase activity is limited to germ cells and highly proliferating cells. Therefore, most cells in humans do not have detectable levels of telomerase activity. Primary human cells that do not contain telomerase activity display telomere attrition followed by increased levels of telomere fusions and apoptosis as their population doublings increase (i.e. as they age) (Harley et al., 1990; Hastie et al., 1990). Immortalization of primary cells as well as a suppression of telomere dysfunction can be achieved by the overexpression of telomerase, linking telomeres as a prerequisite for maintained cellular lifespan and chromosome integrity (Bodnar et al., 1998; Counter et al., 1992). The mechanism for telomerase reactivation leading to malignant transformation is depicted in more detail in Figure 1.5. Indeed, 90% of human tumors overexpress telomerase (Kim et al., 1994). Like in primary cells, telomerase inhibition in cancer cells also leads to telomere dysfunction and cellular death (Damm et al., 2001; Zhang et al., 1999). Telomerase negative cancer cells have been found which maintain their telomeres presumably through a HR pathway called ALT (alternative lengthening of telomeres, (Reddel, 2003)). Telomerase negative mice exhibit cancers in late
Figure 1.5 Relationship between telomeres and cancer in telomerase minus cells. Most human cells do not express telomerase. As these cells proliferate, telomeres gradually shorten and eventually become critically short. At this point, cells go through apoptosis, replicative senescence and genomic instability. Most cells go through crisis resulting in cellular death. However, some cells reactivate telomerase and begin to establish telomere reserves that were previously depleted. These cells have survived a period of genomic instability which results in chromosomal rearrangements and genetic changes that have increased their proliferative lifespan which can lead to malignant transformation and eventually to cancer.
generations when telomere reserves have been depleted (Blasco et al., 1997; Rudolph et al., 1999). These cancers show telomere fusions and chromosomal rearrangements, signs of genomic instability that are similar to human cancers (Artandi et al., 2000). Thus, telomeres must be maintained, either through recombination or telomerase, for unlimited proliferation, which underscores the importance of understanding telomeres and their multiple roles in tumorigenesis and aging (Maser and DePinho, 2002).

Telomerase activity is not the only prerequisite for maintaining telomeric DNA. Surprisingly, DNA damage checkpoints are required to maintain telomeric DNA. In fission yeast and budding yeast, the two ataxia telangiectasia related proteins (ATR/ATM, Rad3/Tel1 in fission yeast and Mec1/Tel1 in budding yeast) are required to maintain telomeres and simultaneous deletion of these pathways results in accelerated senescence (Craven et al., 2002; Naito et al., 1998). Telomerase activity is still active in these cells so telomerase recruitment and/or substrate preparation might be the function of the ATM and ATR proteins (Chan et al., 2001). Loss of telomeres and senescence is also accelerated in these cells suggesting that ATM and ATR proteins are involved in two pathways that inhibit degradation at chromosome ends. The MRN complex is thought to function in the ATM pathway as loss of Rad3/MRN or Mec1/MRX also results in telomere loss and senescence (Mieczkowski et al., 2003; Nakamura et al., 2002). In telomerase negative budding yeast, Tel1 loss also causes a synergistic increase in genomic instability, suggesting that Tel1 is required to cap telomeres in the absence of telomerase (Chan and Blackburn, 2003; Myung et al., 2001). These results evince a requirement for DNA checkpoint proteins for telomere maintenance and end-protection. Although
telomeres need to inhibit the inappropriate activation of checkpoint and repair pathways, components of these pathways are integral parts of normal telomeres (Ferreira et al., 2004) and reviewed in Chapter 3. Recent data suggests that telomere binding proteins actively inhibit DNA checkpoints at the telomere which could help explain how telomeres hide from checkpoints (Karlseder et al., 2004).

In budding yeast, Cdc13 and Ku, a protein required for NHEJ, represent another pathway that is required to maintain telomeres. Simultaneous deletion of CDC13 and KU results in senescence and survivor formation through the type II recombination pathway (DuBois et al., 2002; Grandin and Charbonneau, 2003; Nugent et al., 1998; Polotnianka et al., 1998). Therefore, Ku represents one of several examples of a DNA repair protein that is required to cap telomeres. Indeed, loss of Ku results in telomere dysfunction in many organisms (Downs and Jackson, 2004). Insights into the function of Ku at telomeres came from the finding that Ku binds to the RNA template of telomerase (Peterson et al., 2001). Separation of function mutations in budding yeast Ku have allowed for the dissection of its multiple interactions with telomeres. These studies have revealed that Ku is involved in telomere length regulation through its interaction with telomerase, which is separable from its role in protecting and regulating 3' overhang formation and DNA repair (Bertuch and Lundblad, 2003; Stellwagen et al., 2003). Thus, telomeres require a battery of DNA repair and checkpoint genes for their protection whose activities must by highly regulated to avoid inappropriate activation, which could lead to catastrophic consequences for the cell. Important questions remain about the symbiotic nature of repair/checkpoint proteins and telomeres and elucidating
these questions should reveal insights into the nature of telomere dysfunction and the cellular mechanisms that work to maintain these essential caps.

1.4.3 Cellular responses to uncapped telomeres

Telomeres must protect chromosome ends from being sensed as a DSB that would elicit checkpoint and repair responses. Little is known about the cellular responses to dysfunctional telomeres in fission yeast. However, in several cases, mutations that un-cap telomeres have been shown to activate DNA damage checkpoint pathway. In human cells, uncapping telomeres through the removal of TRF2 results in activation of an ATM-dependent DNA damage checkpoint and apoptosis through a p53-dependent pathway (Karlseder et al., 1999). Although undemonstrated, it has been suggested that the activation of ATM might be through the dismantling of the T-loop, a structure that could be required to avoid the DNA damage response. Uncapping telomeres, either through removal of TRF2 or in senescing cells results in DNA damage foci (d'Adda di Fagagna et al., 2003; Takai et al., 2003). These foci consist of several components including 53BP1, γ-H2AX, MRN (MRE11 and NBS1), RAD17, MDC1 and ATM, all proteins involved in either DNA repair or DNA damage checkpoints. This data shows that dysfunctional telomeres are sensed and processed as genomic DSBs in mammalian cells. This appears to be a general feature of dysfunction telomeres as defects in telomere maintenance also elicit checkpoint responses in budding yeast. Inhibition of CDC13 results in a DNA damage checkpoint arrest (Garvik et al., 1995). Interestingly, loss of Ku results in the activation of a subset of proteins within both the DNA damage and spindle assembly checkpoints (Maringele and
Lydall, 2002). Therefore, dysfunctional telomeres can be sensed as damage in various organisms suggesting that a major role of telomeres is to preserve proper end-structure and to inhibit checkpoint and repair activation at the telomere; activities which left unchecked, can result in genomic instability and cellular death.

1.4.4 Human diseases and telomeres

It is well established that telomere dysfunction is associated with cancer and aging. However, mutations in genes that affect telomere maintenance are also associated with genetic human diseases. Some forms of the rare human disease, dyskeratosis congenita (DKC) are associated with mutations in either the telomerase accessory factor, Dyskerin or in the RNA template of telomerase (hTERC) (Mitchell et al., 1999). Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia-related (ATR) disorders are caused by mutations in the checkpoint kinase genes ATM and ATR respectively. Patients with these disorders have shortened telomeres and these proteins have been shown to function at telomeres (Wong and Collins, 2003). Mutations in MRE11 are believed to lead to an AT-like disorder (AT-LD) and mutations in NBS1 lead to Nijmegen breakage syndrome (Michelson and Weinert, 2000; Stewart et al., 1999). Both of these proteins are part of the MRN complex that is involved in telomere maintenance. Fanconia anemia, an autosomal recessive disorder, is characterized with cancer predisposition and some data suggests that telomere dysfunction could be involved although the details are still unknown (Leteurtre et al., 1999). The REC-Q helicases, WRN and BLM, are mutated in the accelerated aging and cancer diseases, WRN syndrome and BLM syndrome.
(Franchitto and Pichierri, 2002). These helicases have been linked with telomere maintenance as well. Therefore, telomere dysfunction appears to be involved in not only cancer and aging in humans but also several human genetic diseases as well (Wong and Collins, 2003). Thus, defining the mechanisms that are required for proper telomere maintenance should lead to a better understanding of these disorders and cancer, hopefully leading to improved treatments for these diseases.

1.5 Aims of thesis

For these studies, we set out to use the fission yeast, *Schizosaccharomyces pombe*, to the study the role of telomeres in promoting genomic stability and to gain insights into the mechanisms that cap telomeres. This yeast has been a very useful model organism in studying many different aspects of chromosomal biology as well as cell cycle control, DNA repair, DNA replication and DNA checkpoints. Therefore, we wanted to study conditions where telomere dysfunction impinged upon these processes to gain a better understanding of the ways that cells respond to dysfunctional telomeres, how telomeres become uncapped and to use the genetic tools of this yeast to try to dissect the cellular pathways that are involved in coping with dysfunctional telomeres to preserve genomic integrity. The following thesis describes the results that were obtained from studies that were initiated in hopes of achieving these aims.
2 Material and Methods

2.1 Yeast Strains and Media

All fission yeast strains used in this study are listed in table I. Some strains were constructed by mating with another mutant and selecting for the appropriate markers on selective media to create the desired strain. Other strains were constructed in this study by one-step gene replacement of the entire ORF with a \textit{kanMX6} or \textit{ura4} cassette (Bahler et al., 1998). Many strains were kindly provided by various labs that are noted in table 1. Epitope tagging of the Taz1 protein with HA and GFP was performed using the Bahler one-step gene replacement method. All media and growth conditions were as previously described (Moreno et al., 1991). Cultures were grown at either 32°C or 20°C in rich medium (YES) with noted exceptions. All temperature sensitive strains were grown at 25°C unless otherwise stated. For nitrogen starvation experiments, cultures were grown in EMM media without \textit{NH}_4\textit{Cl} as previously described (Godinho Ferreira and Promisel Cooper, 2001).
Table 1. *Schizosaccharomyces pombe* strains used in this study.

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This study refers to the reference (Nakamura et al., 1998).
top1-710 L top1-710 h⁻ Yanagida Lab
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top2-191 (D191)
417 top2-191 top2⁻ U,L ura4::top2⁻ h⁻ This study
top2-191 taz1Δ L top2-191taz1::kan⁹ h⁻ This study
top2-3423 taz1Δ L top2-3423 taz1::kan⁹ h⁻ This study
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top2-191taz1Δ
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top2-191taz1Δ
421 top1-710 taz1Δ L top1-710 taz1::kan⁹ h⁻ This study
top2-191 trt1Δ H top2-191trt1::his3⁺ h⁻ #2 Clone 1 This study
top2-191 trt1Δ H top2-191 trt1::his3⁺ h⁻ #12 Clone 1 This study
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top2-191 rap1Δ L top2-191 rap1::kan⁹ taz1::kan⁹ h⁻ This study

taz1Δ
1033 taz1Δ+Top2 U,L,A1,H taz1::kan⁹+ 81X-Top2 h⁻ This study
1034 top2-191 taz1Δ L top2-191taz1::kan⁹ + 81X-Top2 h⁻ This study
+Top2
200 Taz1-HA taz1::3XHA-kan⁹ h⁺ This study
201 Taz1-HA taz1::3XHA-kan⁹ h⁻ This study
202 Taz1-HA taz1::3XHA-kan⁹ h⁹⁰ This study
203 Taz1-HA U,L,A1,H telo::his3 taz1::3XHA-kan⁹ h⁹⁰ This study
205 Taz1-GFP taz1::GFP-kan⁹ h⁺ This study
206 Taz1-GFP taz1::GFP-kan⁹ h⁻ This study
207 Taz1-GFP taz1::GFP-kan⁹ h⁹⁰ This study
208 Taz1-GFP U,L,A1,H telo::his3 taz1::GFP-kan⁹ h⁹⁰ This study
445 Rap1-Taz1myb U,L,rap1::ura4::taz1::ura4 lys1::Rap1-Taz1myb h⁹⁰ (Chikashige and Hiraoka, 2001)
451 Taz1myb U,L,rap1::ura4::taz1::ura4 lys1::Taz1myb h⁹⁰ (Chikashige and Hiraoka, 2001)
JCF.C  Taz1-HA, Swi6-GFP  L, taz1::3XHA-kan^{r} + 81X-Swi6-GFP^{*}  This study
495  internal telo  U,L ura4::LEU2-telo  This study
496  internal telo  U,L ura4::LEU2-telo taz1::kan^{r}  This study
taz1^{A}
2.2 Yeast Transformations

All yeast transformations were performed as previously described (Hill et al., 1991). Cells were grown to log phase in YES media. Ten mls of cells were pelleted and washed 1X in 10 mls of LiOAc solution (0.1 M LiOAc, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Cells were resuspended in 500 μl of LiOAc solution and 100 μl was used per transformation. 100 μl of cells were added to a tube containing the transformation DNA and 5 μl of previously boiled and iced salmon sperm DNA (10 mg/ml Stratagene). For the transformation DNA, several μg of gel purified PCR produced integration fragments were used or ~1 μg of prepped supercoiled plasmid depending on application. Cells were incubated at rt with the DNA for 5 min. 280 μl of PEG4000 (50% in LiOAc solution) was added, mixed by inversion and incubated at the optimal growing temperature for the strain used for 45 – 60 min. DMSO was added (43 μl) to a final concentration of 10% (v/v) and the tube was incubated at 42°C for 5 min. Cells were pelleted, washed in 1 ml ddH₂O and resuspended in 100 μl of ddH₂O. Cells were plated on the appropriate media and incubated at the appropriate temperature for selection.

2.3 Cytological Analysis

Cellular morphology was analyzed by collecting log phase cultures grown at the indicated temperatures. Cell were visualized by light microscopy or by differential interference microscopy. Analysis of nuclear morphology was achieved by fixing cells with ice-cold methanol, followed by rehydration in PBS, staining with DAPI (4',6-diamidino-2-phenylindole, Vectashield, Vector
Laboratories), and visualization on a Nikon Eclipse E600 fluorescence microscope. All images were captured on a MTI 300T-RC CCD camera using Scion Images software.

2.4 Viability and Sensitivity Assays

Cultures were grown to log phase (optical density at 595nm, 0.1-1) at the indicated temperature and counted on a hemacytometer. To score cold sensitivity, cells were logarithmically grown for at least 4 days at 20°C before plating. Cultures were concentrated to 10^7 cells/ml in water and 300 cells were plated on YES plates in triplicate. Colonies were counted after 4 days at 32°C or 7-10 days for 20°C. Cell viability was calculated as the ratio of colonies formed to cells plated and was compared to the growth of wild type cells under the same condition. For dilution assays, five fold serial dilutions of 2 X10^7 cells/ml cultures were prepared in 96 well microtiter plates and stamped onto YES plates and grown as described above. For drug sensitivity assays, cultures were prepared in the same manner and plated onto YES containing hydroxyurea (1 M stock in DMSO), methylmethane sulfonate (MMS, added as a percentage directly to media, for example, 1 µl/100mls gives .001%) or thiabendazole (TBZ, 10 mg/ml stock made fresh in DMSO).

2.5 Cell Synchronization using \textit{cdc25^{gs}} and \textit{cdc10^{gs}} strains

Asynchronous cultures were grown at the permissive temperature of 25°C to mid-log phase. To block cells in G2 (using the \textit{cdc25^{gs}} mutation) or G1 (using the \textit{cdc10^{gs}} mutation), cells were shifted to 37°C for 3 and 3.5 hrs respectively. To release from the block, cells were shifted to either 25°C or 20°C. Samples
were then taken at the indicated time intervals and fixed for cytological analysis. Mitotic index was calculated by either counting septum or binucleated cells.

2.6 Molecular biology techniques

All molecular biology was performed as described (Sambrook et al., 1989). Basic techniques using commercially available kits were used for some techniques and these were performed according to the manufacture's suggested protocols and so are not described in detail.

2.6.1 PCR

PCR was performed using standard reagents and conditions. Typically, high fidelity long template Expand Taq (Roche) was used when PCR was used to amplify a DNA fragment for cloning, gene knockouts or epitope tagging. Normal Taq supplied by Cancer Research U.K. stores facility was used for non-proofreading, verification reactions. The following reaction conditions were normally used: 1 μl template (14 ng for Bahler method plasmids, 1 μl genomic prep or 1 μl plasmid DNA from miniprep), 0.4 μM primers, Expand long template buffer 3, 0.5 mM dNTPs, 2.5 mM MgCl₂, and 1 μl Taq and ddH₂O until 50 μl.

Standard running programs used in this study are described below.

2.6.1.1 Programs

For short primers with 100% homology:

Step 1. 95°C 5 min
Step 2. 95°C 0.5 min
Step 3. 45-55°C 1 min (depending on Tm of primers)
Step 4. 68°C 1 min/kb of target fragment length
Repeat steps 2-4 30X

Step 5. 68°C 5 min
Step 6. 4°C ∞

For long primers with less than 100% homology:

Step 1. 95°C 5 min
Step 2. 95°C 1 min
Step 3. 50°C 2 min
Step 4. 72°C 2 min

Repeat steps 2-4 5X

Step 5. 95°C 1 min
Step 6. 56°C 2 min
Step 7. 72°C 2 min

Repeat steps 5-7 35X

Step 8. 72°C 5 min
Step 6. 4°C ∞
Table II. PCR primers used in this study.

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DNA sequences are provided as examples only and may not represent actual sequences.
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<td>upoftaz</td>
<td>53.90, 24</td>
<td>GATCATAACTGACCAAGCAGAATA</td>
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2.7 Isolation of genomic DNA for PCR and Southern applications (smash preps)

Genomic DNA isolations were done based on a previously described method (Hoffman and Winston, 1987). 10 mls of cells were grown to saturation or 50 mls of cells were grown to log phase depending on the application. Cells were washed in 10 mls of ddH₂O and resuspended in 350 μl of smash prep buffer (2% triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.), 1 mM EDTA). 400 μl of phenol:chloroform:isoamyl alcohol (25:24:1) was added along with 300 μl of acid washed beads in a 1.5 ml screw capped eppendorf. Tubes were vortexed vigorously for 3-5 min and then centrifuged for 10 min at 13,000 rpm. The supernatant was removed and ice-cold 100% EtOH was added up to 1.5 ml. The DNA was precipitated by incubation at ≤ -20°C for up to 12 hours. Samples were centrifuged for 10 min at 13,000 rpm, washed in 500 μl ice-cold 70% EtOH and re-centrifuged. All EtOH was removed and the pellet was allowed to air dry before being resuspended in 50 μl TE plus RNase (15 μg/ml).

2.8 Protein protocols

2.8.1 Extract preparations for Western blotting and immunoprecipitations

Cells were grown logarithmically and 20 O.D.s worth of cells were pelleted per IP. Pellets were washed in water and resuspend in 1 ml HB buffer. Lyse cells by adding glass beads and vortexing for 5 min (30 seconds then 30
seconds on ice 10X). Add SDS to a final concentration of 1%, heat cells at 100°C and clear lysate by centrifuging 13,000Xg for 15 min at 4°C. Determine protein concentration by Bradford assay (BioRad) and use extract for desired protocol.

**HB Protein Extraction Buffer Recipe**

<table>
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<th>Stocks</th>
<th>Volume for 10 ml</th>
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<td>600 mM</td>
</tr>
<tr>
<td>15 mM p-nitrophenylphosphate</td>
<td>150 mM</td>
</tr>
<tr>
<td>25 mM MOPS (pH 7.2)</td>
<td>250 mM</td>
</tr>
<tr>
<td>15 mM MgCl₂</td>
<td>1 M</td>
</tr>
<tr>
<td>15 mM EGTA(.3 M (pH 8.0))</td>
<td>500 μl</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>1 M</td>
</tr>
<tr>
<td>.1 mM Sodium Orthovanadate</td>
<td>.5 M</td>
</tr>
<tr>
<td>1 mM PMSF</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>20 ug/mL leupeptin</td>
<td>2.5 mg/ml</td>
</tr>
<tr>
<td>40 ug/mL aprotinin</td>
<td>5 mg/ml</td>
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<tr>
<td>pepstatin A</td>
<td>1 mg/ml</td>
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<tr>
<td>H₂O</td>
<td>5.218 ml</td>
</tr>
</tbody>
</table>

Additional info.

EGTA- 100 mls- 19.02g, pH 8.0 with NaOH

MOPS- 100 mls- 5.23 g pH 7.2 with HCL, **LIGHT SENSITIVE**

p-nitrophenylphosphate- 20 mls- 1.38 g- store in fridge, **LIGHT SENSITIVE**

B-glycerophosphate- 20mLs- 2.6g- store in fridge.

Luepeptin and aprotinin- stocks for −80°C freezer

PMSF- made in isopropanol, Pepstatin A- made in ethanol, stored at −20°C
2.8.2 Immunoprecipitation protocol

Extracts were prepared using the above protocol. For co-IPs, the SDS step was omitted to preserve protein complexes and the following protocol was used. Add HB buffer until the volume reaches 1 ml. Add primary antibody (approximately 1-5 μg/IP depending on antibody) and allow to rotate overnight at 4°C. Add 50 μl of a pre-equilibrated slurry of either Protein A or Protein G coupled to sephrose (depending on primary antibody). Incubate secondary antibody greater than 1 hr at 4°C. After incubation, centrifuge the tubes at 3,000Xg and wash 2X in ice-cold HB buffer. Wash 2X in 10 mM Tris-HCl (pH 7.5). Add 25 μl 5X sample loading buffer and boil for 5 min. Analyze using standard Western blotting protocol.

2.8.3 Western blotting protocol

Samples were resolved on SDS-Page gels (percentage depending on size of the protein being analyzed). Gels were transferred onto PVDF membrane (pre-charged in MeOH (Stratagene)). Blots were washed with TBS-TWEEN (0.1 %). Blots were then blocked for 1 hr in 1% low-fat dried milk in TBS-T. Blots were next incubated in primary antibody in 1% milk TBS-T overnight at 4°C, (α-HA 12CA5 (Roche), α-GFP (Babco)), using manufactures recommended dilutions. Blots were then washed 3X 10 min in TBS-T and incubated with secondary antibody (either anti-mouse or anti-rabbit HRP) in TBS-T for 1 hr at 4°C. Blots were washed 3X in TBS-T at rt. Finally, the blots were treated with ECL solution for 1 min and visualized by film.
2.9 Protein purification protocols

2.9.1 Extract preparations for Tazi-HA purification

Protein extracts were made based on a previously published protocol (Mortensen et al., 2002). Protein extracts were prepared from cell pellets frozen in liquid nitrogen. Pellets were first ground with dry ice in a coffee grinder (pre-chill with ground dry-ice), and the resulting material was homogenized with a mortar and pestle under liquid nitrogen until 70-80% lysis (ground extracts can be stored in -80°C). Ice-cold extraction buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 10% (vol/vol) glycerol, 300 mM NaCl, 0.1 mM DTT, 1 mM EDTA plus protease inhibitors (0.5 mM PMSF, 1 Complete-mini, EDTA-free protease inhibitor tablet (Roche)/10 ml extraction buffer) was added, and the extract was stirred at 4°C for 15 min (add 25 ml extraction buffer per 10-15 g extract) and was cleared by centrifugation (once for 10 min at 5,600Xg and twice for 10 min at 16,000Xg). Protein concentrations in cell extracts were determined by Bradford assay and were typically 10-20 mg/ml.

2.9.2 Immunoaffinity purification of Tazi-HA

This technique was developed in Doug Kellogg’s lab in Santa Cruz, CA and has been previously described (Mortensen et al., 2002). We followed this technique with minor modifications. Tazi-HA cells and an untagged strain (for the control) were grown logarithmically to a density of 1 O.D. Eight litres of cells were used per experiment. Cells were pelleted and washed with 50 mM Hepes-KOH (pH 7.6). Cells were either frozen in liquid nitrogen as pellets in the bottom of 50 ml conicals or as cellular “spaghetti”. To make “spaghetti”,
cells were pelleted in a 60 ml syringe tube. Using the plunger, cells were pushed through the syringe and into a 50 ml conical tube containing 30 ml of liquid nitrogen. This method will produce strings of frozen cells that facilitate their grinding by mortar and pestle. Next, extracts were prepared as explained in section 2.9.1 except that the following extraction buffer was used: 50 mM Hepes-KOH (pH 7.6), 100 mM \(\beta\)-glycerolphosphate, 25 mM NaF, 25 mM KCl, 1 mM MgCl\(_2\), 1 mM EGTA, 5% Glycerol, 0.25% Tween-20, and 2 mM PMSF (note: the PMSF is added from a 100 mM stock made in 100% EtOH and is added immediately before cells are resuspended). Additionally, the cell extract was cleared at 40k for 1 hr. Be sure to avoid particulates that are found at the bottom of the tube after clearing the extract. Extracts were placed in a 15 ml conical and .45 ml of Protein A beads (Affi-gel, BioRad) containing 0.45 mg of polyclonal antibody bound were added and mixed for 2-3 hr at 4°C. To prepare beads, pre-bind the antibody to the Protein A beads for at least 1 hr at rt in 1.5 ml PBS containing 0.1% Tween-20 (kindly provided by Kellogg’s lab). Wash 2X with 15 ml ice-cold extract buffer containing 25 mM NaF and no PMSF (spin 3.8K for 1 min to pellet beads). The solution was transferred to a column (Biorad 1.5 ml spin columns) and washed with 5 ml (1 ml at a time) of cold extract buffer (containing Tween-20). Columns were washed with 1 ml of extract buffer that does not contain Tween-20. Be sure to wear gloves to inhibit contamination in the protein sample. Move columns to rt and add 0.25 ml elution buffer and collect flow-through in a 2 ml tube. Elution buffer consists of 50 mM Hepes-KOH (pH 7.6), 100 mM \(\beta\)-glycerolphosphate, 1 mM MgCl\(_2\), 1 mM EGTA, 5% Glycerol, 0.35 mg/ml HA dipeptide (kindly provided by Kellogg’s lab). Allow column to incubate for 30 min at rt. Add another 0.25 ml elution buffer and
collect flow-through in a 2 ml tube. Columns were placed in cold room O/N.

Two more fractions were collected, waiting 30 min between each collection.

The last aliquot was taken by washing the column with .25 ml of elution buffer
that lacks HA peptide. The five fractions were stored on ice. Ten μl of each
fraction were taken and diluted in 5X sample loading buffer and analyze by
Western blotting with an anti-HA antibody to detect the purification of Taz1-HA.

Crude extract and supernatant fractions were analyzed to allow for the estimate
of the efficiency of purification. These fractions were diluted 1/10 or 1/100
depending on their concentrations. Fractions 2-5 were pooled (if purification
looks good by Western blotting) and samples were precipitated by adding
Tricholoroacetic Acid (TCA) to 10%. To precipitate, add TCA and place tubes
on ice for 5 min. Spin 14K at rt for 5 min. Remove supernatant, spin again and
be sure to remove all liquid. Resuspend the precipitated samples in 50 μl of 1X
sample loading buffer and analyze on Coomassie-blue stained gels. Note:
before loading sample, neutralize TCA sample with Ammonium OH which will
change the bromophenol blue from yellow (caused by TCA) back to blue. Add
crude extract and supernatants for comparisons.

2.10 Pulsed Field Gel Electrophoresis (PFGE)

2.10.1 Whole chromosome and double strand breaks

PFGE

For analysis of whole chromosome integrity and detection of DSBs, a
previously reported protocol (Cervantes et al., 2000a) was used with the
following modifications. Agarose plugs containing ~8 X 10^7 cells were incubated
for 60 min at 50°C in 1% SDS, 0.25 M EDTA, 50 mM Tris-HCl (pH 7.5). Plugs
were next treated with proteinase K (1 mg/ml) in 1% sarkosyl, 0.5 M EDTA, 10 mM Tris-HCl (pH 9.5) for 2 days at 50°C, and then washed twice in this buffer and stored at 4°C. Before electrophoresis, plugs were equilibrated in 1X TAE running buffer for at least 1 hr. Plugs were loaded in 0.8% pulsed-field certified grade agarose gels (Biorad) and electrophoresed at 14°C in a CHEF-DR III pulsed-field gel apparatus (Biorad) at 2V/cm, 30 min switch time, 100° included angle for 48 hr. Ethidium bromide stained gels were visualized using a Fluor-S Multilmager system (Biorad). Southern blotting analysis of these gels was performed using the protocol for telomere length analysis.

2.10.2 Not1 restriction fragment analysis

Protocol was performed as previously described (Ferreira and Cooper, 2001a). Cultures of cells were washed twice in SP1 buffer (1.2 M sorbitol, 50 mM citrate/phosphate [pH 5.6], 40 mM EDTA), resuspended at a density of 0.5 – 1 X 10^8 cells/ml, and treated SP1 buffer containing 0.6 mg/ml Zymolyase-T100 (ICN) for 1 hr at 37°C. Cells were pelleted and gently resuspended in 100 μl TSE buffer (0.9 M sorbitol, 10 mM Tris-HCl pH 7.5, 45 mM EDTA) plus one volume of 1% LMP agarose (GIBCO-BRL) in TSE. The cell suspension was placed in 2 100 μl plug molds (Biorad) and allowed to solidify. Agarose plugs were first incubated for 90 min at 50°C in 1% SDS, 0.25 M EDTA, 50 mM Tris-HCl (pH 7.5) and then for 48 hr at 50°C in 1% lauryl sarcosine, 0.5 M EDTA, 10 mM Tris-HCl (pH 9.5) containing 1mg/ml proteinase K (GIBCO–BRL). Plugs were then washed in TE10X (10 mM Tris-HCl pH 7.5, 10 mM EDTA) at rt, and proteinase K was inactivated by incubating with 0.04 mg/ml PMSF in TE10X for 4X 30 min at 50°C. The plugs were finally washed in 3X 30 min in 1X TAE and
stored at 4°C. For NotI restriction digest, plugs were pre-equilibrated overnight in NotI buffer (10 mM NaCl, 5 mM Tris-HCl pH 7.9, 1 mM MgCl₂, 0.1 mM DTT, 100 µg/ml BSA). NotI buffer was then replaced, and 100 units of NotI (NEB) were added and plugs were incubated for 8 hr at 37°C. An additional 100 units of NotI (NEB) was added and the plugs were incubated overnight at 37°C. The digested agarose plugs were then pre-equilibrated in 0.25X TBE and loaded onto 1% agarose gels in 0.25X TBE. PFGE was performed on a Beckman GeneLinerII system in 0.25X TBE at 14°C using the following program: step 1, current 350 mA, switch 1 min, time 12 hr 30 min; step 2, current 370 mA, switch 2 min, time 12 hr 30 min; and step 3, current 390 mA, switch 3 min, time 12 hr 30 min. After electrophoresis, DNA was visualized by ethidium bromide staining, and gels were processed for Southern blotting, which was performed using telomeric oligonucleotide probes as described (see below).

2.11 Telomere length Southern blotting analysis

Telomere Southern blotting were carried out as previously described (Cooper et al., 1997a). DNA samples were isolated and cut with the stated restriction enzyme using the optimal buffer and incubation condition unless otherwise stated. Normally, DNA samples were either cut with EcoRI or ApaI and incubated overnight at 37°C and 25°C respectively. Samples were diluted in 6X DNA loading dye and were typically run on 1X TAE, 1% agarose gels containing ethidium bromide (0.03 mg/ml). Gels were run at 5V/cm in 1X TAE buffer until the bromophenal blue band was 1/4 from the bottom of the gel. Gels were photographed for loading and quality control and treated for transfer. The gels were first incubated in 0.25N HCl for 15 min. Gels were then incubated in
Blot #1 solution (20g NaOH, 87.6g NaCl in 1L H2O) for 30 min and Blot #2 solution (77g NH4Ac, 0.8g NaOH in 1L H2O) for 60 min. During this time, the membrane was prepared for transfer by incubating in Blot #2 solution for 5 min. To set up the dry transfer, a stack of dry paper towels about 3 inches thick were placed on the bench. On top of these, 3 pieces of 3MM Whatman paper cut to the same size as the gel were placed before placing the gel on top of the Whatman paper. The gel was placed on top of the membrane, wells up. The stack was then covered with saran wrap and weighted down with two 500 ml bottles on top of a glass plate to ensure equal distribution of the weight over the transfer stack. The gel was allowed to transfer overnight before being removed from the stack and being air-dried. The membrane was then crosslinked using a Stratagene crosslinker and pre-hybridized for 30 min in Church-Gilbert buffer (1% BSA, 1 mM EDTA, 7% SDS, 0.5 M NaHPO4 (1 L of 1 M, 134g Na2HPO4 - 7H2O plus 4 ml 85% H3PO4) pH 7.2) at the appropriate temperature. For telomere Southern's, oligo probes were hybridized overnight at 45°C while random primed probes were hybridized overnight at 65°C. For preparation of probes, telomere oligos were labelled using the following reaction: 2 μL (50 μM stock) of each oligo (ABT and BCT, or ACT), 1 μl 10X kinase buffer, 1 μl T4 Polynucleotide Kinase (PNK), 1 μl H2O, 3 μL of [γ-32P] ATP. Probe mixture was in incubated for 1 hr at 37°C. 90 μl of TE (Tris-EDTA pH 8.0) was added and the solution was placed over a Sepharose G-50 or G-25 spin column (Amersham). For random primed probes, a cloned telomere fragment pSNU70 was used as a probe and was prepared using a random prime labelling kit (Stratagene). Briefly, 25 ng of purified telomere fragments (per probe) were labelled with α-32PdCTP and purified using G-25 spin columns. Probes were
incubated for 5 min at 100°C before being added to the hybridization buffer. After hybridization, blots were pre-rinsed in 100 ml of wash solution (2X SSC, 0.1% SDS) and then washed in 100 ml wash solution for 30 min at either rt or 50°C (oligo versus random primed probe). The blot was placed in saran wrap and the signal was detected using a Molecular Imaging phosphoimager system.

2.12 In-gel hybridization procedure for the detection of the telomere 3’ overhangs

In-gel hybridization analysis was performed according to previously published protocols (Dionne and Wellinger, 1996; Tomita et al., 2003). Smash prep purified DNA (approximately half of a smash prep from 10 ml overnight culture (1 O.D. (600nM)) was digested with EcoRI overnight at 37°C and electrophoresed on a 0.5% agarose gel in 0.5X TAE buffer containing 0.03 mg/ml ethidium bromide. The gel was vacuum dried at 45°C (45-60 min or until visibly dried). Single-stranded telomeric DNA probe was labelled with \([\gamma-^{32}\text{P}]\) ATP using T4 polynucleotide kinase. Hybridization buffers and blocking solutions were obtained from a commercially available kit (AlkPhos DirectTM, Amersham Pharmacia Biotech). The gel was pre-hybridized in hybridization buffer at 37°C for 15 min, and then the probe was added and the incubation was continued overnight at 37°C. The gel was washed with primary wash buffer at 37°C for 2X 10 min and then washed with secondary wash buffer at rt for 3X 5 min. The gel was placed on two layers of Whatman 3mm paper and overlaid with Saran Wrap. Detection of the signal was obtained by using a Molecular Imaging phosphoimager system. To detect total telomere signal (both double-stranded and single-stranded telomeric DNA), gels were treated
with denaturing solution (0.5 M NaOH, 150 mM NaCl) for 30 min at rt, and then treated with neutralizing solution (0.5 M Tris-HCl pH 8.0 150 mM NaCl) for 45 min (rt). The gel was then re-probed with both the C and G-strand probes using the same protocol as stated above.

2.13 Two-dimensional (2D) gel electrophoresis

2.13.1 Cell preparation

Cells were grown in YES media to reach a density $5 \times 10^6$ to $1 \times 10^7$ at either 32°C, 25°C or 20°C. Approximately 300 ml of cells were used per sample preparation. Sodium azide was added to a final concentration of 0.1% and mixed well. 40 ml of cell culture was added to 10 ml of frozen 0.2 M EDTA (pH 8.0) in a 50 ml conical and shaked vigorously until the mixture was thoroughly melted. Tubes were centrifuged for 3 min at 3,000 rpm at rt. The cells were next washed in 20 ml of ice-cold 25X TE (10 mM Tris-HCl pH 7.5, 25 mM EDTA), re-spun and then washed in 20 ml of ice-cold spheroplast buffer (SP1 buffer, 1.2 M D-Sorbitol, 50 mM Citrate Phosphate, 40 mM EDTA pH 8.0). The supernatant was removed and the pellet resuspended in 4 ml of ice-cold SP1 buffer. The cultures were aliquoted in 2 2 ml Eppendorf tubes and spun at 13,000 rpm for 10 seconds. The supernatant was removed and the cells resuspended in 1 ml of freshly prepared zymolyase buffer (0.6 mg/ml in SP1 buffer) and incubated at 37°C for approximately 30 min or until >90% of the cells had been spheroplasted. Percentage of spheroplasted cells was determined by adding 2 μl of 20% SDS to 2 μl of cells and counting cell shadows on a slide under a light microscope which is indicative of spheroplasted cells. The cells were centrifuged 10 sec at 13,000 rpm and resuspended gently in 0.4 ml/tube SP1 buffer.
buffer. To make cell agarose plugs, 0.4 ml of 1% low melting point agarose (LMP, in SP1 buffer) was added to each tube (0.5% LMP final concentration), gently mixed well with a p1000 tip which had been cut at the end to avoid mechanical shearing and placed in 100 μl plug molds (Biorad). The molds were placed at 4°C for at least 30 min to allow for complete agarose polymerization before proceeding. The plugs were placed in 10 ml digestion buffer (1% Lauroyl Sarcosine, 1 mg/ml Proteinase K, 25 mM EDTA pH 8) and incubated at 50°C for 30 min. This step was repeated twice with the final incubation going overnight at 50°C. The plugs were then washed in 50 ml 50X TE (10 mM Tris-HCl pH 7.5, 50 mM EDTA) for 3 hrs at 4°C and again overnight at 4°C with gentle rocking. The plugs were normally processed immediately after this step but could be stored at 4°C in 50X TE for several weeks without deleterious effects.

2.13.2 DNA digestion

Plugs were washed 2X in 1X TE (10 mM Tris-HCl pH=7.5, 1 mM EDTA) for 30 min at 4°C with agitation. Plugs were then washed in 2X restriction enzyme buffer and then 1X restriction enzyme buffer for 30 min each at 4°C with agitation. Plugs were incubated overnight in the appropriate restriction enzyme reaction buffer containing 40U of enzyme per 100 μl buffer per plug at the optimal temperature for the restriction enzyme. Plugs were pre-incubated at 70°C for 5 min to partially dissolve the agarose. β Agarase I (NEB) and RNAse (DNAse free enzyme, Sigma 50 mg/ml) was added to each tube (2 μL/plug for each) and incubated 60 min (or until agarose was completely digested) at 37°C. After digestion, plugs were incubated at 70°C for 10 min and centrifuged 13,000
rpm for 1 min. The supernatant was collected with a cut p1000 tip and salt concentration was increased to 300 mM by adding NaCl.

2.13.3 DNA purification

The solution was slowly added to a 1 ml Benzoylated Naphthoylated DEAE Cellulose (BND-cellulose, Sigma) column that had been pre-equilibrated with 3 ml equilibration buffer (10 mM Tris-HCl pH=7.5, 1 mM EDTA pH 8.0, 300 mM NaCl). Non-replicating DNA was first eluted from the column by the addition of 3 ml of wash buffer (10 mM Tris-HCl pH=7.5, 1 mM EDTA pH 8.0, 800 mM NaCl). 500 μL fractions were collected and put immediately in ice. Next, replicating DNA was eluted from the column by the addition of 3 ml of elution buffer (10 mM Tris-HCl pH=7.5, 1 mM EDTA pH 8.0, 1 M NaCl, 1.8% Caffeine). Again, 500 μL fractions were collected and put immediately in ice. Fractions were analyzed on a 1% agarose gel to determine the elution profile of the column. Peak fractions for the replicating and non-replicating fractions were pooled separately, diluted 3X in 1XTE and precipitated with 100% EtOH overnight at -20°C. The sample was centrifuged 12,500 rpm in a J13.1 Beckman rotor for 15 min to pellet the DNA. The pellet was washed in 70% EtOH, air dried and resuspended in 60-120 μL TEN (10 mM Tris-HCl pH=7.5, 0.1 mM EDTA pH 8.0, 10 mM NaCl). Samples were diluted in DNA loading dye and analyzed immediately or stored at 4°C.

2.13.4 Two-dimensional agarose gel electrophoresis

Samples were separated by electrophoresis in 1X TBE buffer on a 25 cm, 0.4% 1X TBE agarose gel (without ethidium bromide) for 48 hrs at 1V/cm. For loading, a 15 well comb was used with samples loaded every other space to
allow for second dimension analysis. A 1 Kb DNA ladder (NEB) was used for DNA migration standards. After completion of the run, a strip containing the DNA ladder was excised and visualized by staining with ethidium bromide (0.03 μg/ml). The lane containing the sample was excised with a clean razor blade and placed on a clean gel tray, 90° counter clockwise to the direction of migration in the first dimension. Gel trays with a width of either 15 cm or 25 cm were used depending on the length of the fragment that was analyzed. The second dimension 1X TBE gel containing 1.1% agarose and ethidium bromide (0.3 μg/ml) was poured into the tray containing the first dimension gel slice until the slice was completely submerged. The second dimension gel was ran at 180V at 4°C for 5 or 7 hrs (short and long trays respectively). The gel was photographed to determine proper second dimension migration and then transferred and probed using the telomere Southern blotting analysis protocol.
3 Telomeres containing Taz1p are required to prevent and repair genomic DNA breaks

3.1 Introduction

An intrinsic role of telomeres is to protect chromosomal termini from being sensed and processed as damaged-induced DNA breaks; this role is critical for maintaining genomic integrity (Blackburn, 1991; Lundblad, 2000). DNA damage generally activates checkpoint and repair pathways that are highly conserved from yeast to humans. In fission yeast, the intra-S-phase checkpoint arrests cells in S-phase in response to problems during DNA replication, and the G2/M checkpoint responds to DNA damage following S-phase (O'Connell et al., 2000; Rhind and Russell, 1998; Rhind and Russell, 2000). Both checkpoint pathways are initiated through activation of Rad3p, the fission yeast homolog of the human ataxia telangiectasia related (ATR) protein (Bentley et al., 1996; Cimprich et al., 1996). Specificity between the intra-S-phase and G2/M checkpoint pathways is found in the downstream protein kinases, Cds1 and Chk1 respectively (al-Khodairy and Carr, 1992; Boddy et al., 1998; Lindsay et al., 1998; Murakami and Okayama, 1995; Walworth et al., 1993). A third checkpoint system, the spindle checkpoint, delays anaphase onset until all chromosomes have been correctly captured by the spindle and thus ensures proper chromosome segregation (Burke, 2000; Hardwick, 1998). Although not traditionally considered a DNA damage checkpoint, recent reports (Su and Jaklevic, 2001) and our current study suggest a connection between the two. Additionally, DNA damage activates repair processes that include both
homology directed repair pathways (HR) and nonhomologous end joining (NHEJ).

Telomere dysfunction can lead to the inappropriate use of chromosome ends as triggers for both end-joining reactions and checkpoint responses. A prime illustration of this is provided by the genomic instability and irreversible cell cycle arrest that follow telomere attrition in human cells lacking telomerase, the ribonucleoprotein enzyme that synthesizes telomere repeats (de Lange and DePinho, 1999; Harley et al., 1990; Hastie et al., 1990). Likewise, inhibition of the human telomere binding protein TRF2 induces an ATM-dependent DNA damage checkpoint and end-to-end chromosome fusions. (Karlseder et al., 1999; van Steensel et al., 1998). In budding yeast, disruption of Cdc13 results in an extended single stranded telomeric region and G2/M arrest via the RAD9-dependent checkpoint pathway (Garvik et al., 1995). Interestingly, the loss of a single telomere in budding yeast also elicits a RAD9-dependent cell cycle arrest (Sandell and Zakian, 1993). Cells recover from this arrest, but subsequent propagation of the unrepaired chromosome leads to cell death after several generations. Therefore, mutations affecting telomeric function can lead not only to dicentric chromosome formation, but also to other types of damage, e.g. accumulation of single stranded DNA or loss of a protective telomere-specific chromatin structure, that are sensed by cellular damage surveillance systems (Collins, 2000; Godinho Ferreira and Promisel Cooper, 2001; Nakamura et al., 1998; van Steensel et al., 1998).

Despite the fact that DNA repair processes are normally prohibited from acting on telomeres, a growing body of evidence has led to the initially counterintuitive notion that repair and checkpoint proteins function in normal
telomere maintenance. Examples of checkpoint proteins that function at telomeres include fission yeast Rad3; deletion of rad3 \(^{+}\) results in short telomeres, although deletions of the downstream kinases \(chk1^{+}\) or \(cds1^{+}\) do not (Dahlen et al., 1998; Matsuura et al., 1999). Strikingly, deletion of both fission yeast ATM homologs, \(rad3^{+}\) and \(tel1^{+}\), results in the complete loss of telomeric DNA accompanied by cell death (Naito et al., 1998), mirroring the effects of telomerase loss (Nakamura et al., 1998), with survival occurring only via intramolecular end-to-end fusion of each of the three chromosomes. \(rad32^{+}\), an ortholog of budding yeast and human Mre11, was found to localize to fission yeast telomeres and to function in the \(tel1^{+}\) pathway that protects telomere attrition in the absence of \(rad3^{+}\) (Nakamura et al., 2002). These observations evince an important, though as yet ill-defined, relationship between repair and checkpoint proteins and telomeres.

In this chapter, we describe a growth defect in cycling \(taz1\Delta\) cells that reveals a novel mode by which telomere dysfunction can elicit checkpoint activation and chromosomal damage. We find that at 20°C, loss of \(taz1^{+}\) results in decreased viability, chromosome segregation defects, and activation of the G2/M DNA damage checkpoint. Strikingly, these cells also exhibit entangled chromosomes and a pronounced \(de\ novo\) accumulation of DSBs. These defects are suppressed by altered topoisomerase II function, implicating unprotected telomeres as substrates for Top2. Furthermore, \(taz1\Delta\) cells are sensitive to treatments that induce DNA double strand breaks (DSBs) at optimal growing temperature (32°C). Our data suggest a scenario in which \(taz1\Delta\) telomeres become entangled in the cold, leading to DNA breaks and checkpoint activation. In addition, Taz1 appears to play a role in general DSB repair.
throughout the genome.

3.2 Results

3.2.1 \textit{taz1Δ} cells exhibit a growth defect at 20°C

Although \textit{taz1Δ} cells exhibit deregulation of telomere length and structure as well as vulnerability to NHEJ-induced telomere fusions, their growth in rich medium at optimal temperature (32°C) appears normal. However, we observe several intriguing phenotypes when \textit{taz1Δ} cells are grown at 20°C. They become highly elongated (Figure 3.1A) and show aberrant chromosomal morphologies (Figure 3.1B) that indicate defects in chromosome segregation and are reminiscent of the phenotypes that accompany end-to-end chromosomal fusions in G1-arrested \textit{taz1Δ} cells (Godinho Ferreira and Promisel Cooper, 2001). While these defects are not observed in \textit{taz1Δ} cells at 32°C or wt cells at any temperature, \textit{taz1Δ} cells exhibit these problems at a frequency of \(-16\%\) of the population at 20°C. Three main classes are apparent: 56\% show anaphase bridging, 31\% have fragmented DNA and 11\% exhibit asymmetrical nuclear positioning. Furthermore, \textit{taz1Δ} cells show a \(-60\%\) reduction in viability at 20°C compared to \textit{taz1Δ} cells at 32°C or wt cells at either temperature (Figure 3.1C). Thus, \textit{taz1Δ} is important for viability at 20°C and its loss results in cellular elongation and chromosome missegregation.

3.2.2 The appearance of DNA segregation defects in \textit{taz1Δ} cells requires passage through S-phase in the cold

The chromosome missegregation in \textit{taz1Δ} cells at 20°C suggests that some process involved in duplicating and partitioning chromosomes requires
Figure 3.1 *taz1Δ* cells are cold sensitive. (A) *taz1Δ* cells elongate at 20°C. Logarithmically growing cells at 32°C and 20°C are compared. (B) Loss of *taz1Δ* results in chromosomal missegregation defects at 20°C. Cells from (A) were fixed and stained with DAPI. Wt cells at 20°C are shown in the grey box, and *taz1Δ* cells at 20°C in the right-hand box. The left-hand black boxes show magnified examples *taz1Δ* cells at 20°C. (C) *taz1Δ* cells exhibit decreased viability at 20°C. Cells were maintained in log phase by daily dilution at 32°C or 20°C for 3 or 5 days, respectively, and plated on rich medium at 32°C. Error bars indicate standard deviations.
taz1Δ in the cold. To determine the cell cycle stage during which these problems originate, we utilized a temperature sensitive mutant of Cdc25 that blocks cells at the G2/M transition. Asynchronous cultures of cdc25Δtaz1Δ and cdc25Δtaz1Δ cells were blocked at the restrictive temperature and then released at 20°C (Figure 3.2A). Within 3.5 hrs, both cultures go through a synchronous cell cycle and no apparent defects in chromosome segregation are indicated by DAPI staining. After the first cell cycle, the cdc25Δtaz1Δ strain loses synchrony. However, the cdc25Δtaz1Δ culture amasses in the second mitosis, indicating a mitotic delay relative to cdc25Δ cells containing taz1Δ, and goes through another semi-synchronous round of cell division. DAPI staining of these cells shows an approximately 5-fold increase in chromosomal abnormalities compared to growth at 32°C (Figure 3.2A). Thus, while the cdc25Δtaz1Δ culture is indistinguishable from the cdc25Δtaz1Δ culture in the first cell cycle following release from a G2/M block, it displays a mitotic delay and aberrant chromosome segregation patterns in the second cell cycle.

A salient difference between these two cell cycles is that the first proceeds through S-phase at 32°C while the second undergoes S-phase at 20°C. To determine whether passage through S-phase at 20°C elicits the defects in taz1Δ cells, we utilized a cdc10-V50 strain that arrests in G1 before the initiation of DNA replication at the restrictive temperature (Moreno et al., 1989). cdc10Δtaz1Δ and cdc10Δtaz1Δ strains were arrested in G1 and released at 20°C. Following release from the G1 block, both strains go through cell division at 20°C with identical kinetics (Figure 3.2B). However, the cdc10Δtaz1Δ cultures accumulate aberrant DAPI staining patterns upon passage through the first mitosis (asterisks, Figure 3.2B). Thus, unlike taz1Δ cells blocked at the
Figure 3.2  

**taz1Δ cells accumulate defects during S-phase in the cold.** (A)  
cdc25-ts and cdc25-ts taz1Δ cells were grown to early log phase at 25°C, transferred to 37°C for 4hrs to induce a G2/M block, released to 20°C, and taken at various time points for DAPI staining. The zero time point corresponds to release from the G2/M block. The inset picture shows an example of the chromosomal abnormalities that were scored, and asterisks signify time points at which such abnormalities were observed. (B) cdc10-ts and cdc10-ts taz1Δ strains were grown at 25°C, shifted to 37°C for 4 hours to initiate the G1 block and then released at 20°C. As in (A), asterisks signify time points at which chromosomal abnormalities were observed.
G2/M transition (Figure 3.2A), cells blocked just prior to S-phase sustain chromosomal abnormalities during the first cold cell cycle following release. Therefore, taz1Δ cells must undergo S-phase at 20°C to accumulate chromosome segregation defects in the subsequent mitosis.

To further analyze S-phase in taz1Δ cells, we treated taz1Δ cells with hydroxyurea (HU), a drug that inhibits ribonucleotide reductase and causes cells to arrest in S-phase. At 32°C, taz1Δ cells are indeed 5-fold more sensitive to HU than are wt cells (see Chapter 5, Figure 5.9). Thus, taz1Δ cells appear to be defective in some aspect of S-phase, and this defect is revealed by growth at 20°C or in the presence of HU.

3.2.3 taz1Δ cells activate the DNA damage checkpoint pathway and require rad3+ for viability at 20°C

The mitotic delay and cell elongation suggest that loss of taz1+ leads to a checkpoint-mediated cell cycle arrest in the cold. To explore the relationship between the loss of taz1+ and checkpoint pathways, we constructed a series of double mutants in which both taz1+ and a checkpoint gene were deleted. The rad3+, rad1+, rad9+, rad26+ components of the intra-S-phase and G2/M checkpoint pathways were tested, as were cds1+ (specific for the intra-S phase checkpoint) and rhp9+ and chk1+ (specific for the G2/M checkpoint). Although not strictly required for the G2/M DNA damage checkpoint pathway, the fission yeast ATM homolog tel1+ was also tested. Three categories of genetic interactions with taz1Δ are observed (Figure 3.3A and B). First, there are two genes, cds1+ and tel1+, whose deletion has no impact on the phenotype of taz1Δ cells at 20°C (Figure 3.3B). Second, there are several genes whose
Figure 3.3 Analysis of genetic requirements for elongation and survival of \textit{taz1\Delta} cells at 20\degree C. (A) Five fold serial dilutions of log phase cultures were stamped onto rich medium and incubated at the indicated temperature. Representative photographs are shown, and the data are fully summarized in panel B. (B) Table of genetic requirements for viability and elongation of \textit{taz1\Delta} cells at 20\degree C. Strains were cultured at 20\degree C for five days in rich medium, then scored for elongation. Viability data were obtained from dilution assays as in (A). Results are represented as +++ (wt growth), ++ (growth equivalent to that of \textit{taz1\Delta}, i.e. $\geq$ 5-fold decrease in viability relative to wt), + (significantly less than \textit{taz1\Delta} growth, i.e. $\geq$ 25-fold decrease in viability relative to wt) or +/- (nearly synthetic lethal). (c.s.) denotes reduced viability of the parental strain at 20\degree C. (C) Telomere length is reduced in \textit{taz1\Delta} cells at 20\degree C. Genomic DNA was digested with EcoRI, electrophoresed on 1\% agarose, blotted and probed with a telomeric oligonucleotide.
deletion suppresses the elongation of \textit{taz1Δ} cells in the cold; this category includes \textit{rad3Δ}, \textit{rad26Δ}, \textit{rad1Δ}, \textit{rad9Δ}, \textit{rhp9Δ}, and \textit{chk1Δ} (Figure 3.3B), establishing that \textit{taz1Δ} cells arrest in the cold through activation of the G2/M checkpoint and independently of the intra-S-phase checkpoint. The third category comprises the phenotype elicited by deleting \textit{rad3Δ}; in addition to suppressing the elongation of \textit{taz1Δ} cells in the cold, \textit{rad3Δ} deletion increases the lethality of \textit{taz1Δ} cells at 20°C (Figure 3.3A and B). This type of synthetic interaction is specific to \textit{rad3Δ} and does not occur upon deletion of the other G2/M checkpoint genes. To clarify whether this was indeed a checkpoint independent function of Rad3p, we analyzed a \textit{cds1Δchk1Δ} double mutant, defective in both the intra-S-phase and G2/M checkpoint pathways. Interestingly, the \textit{cds1Δchk1Δtaz1Δ} triple mutant shows the same viability at 20°C as \textit{taz1Δ} cells alone, although these cells do not elongate. Therefore, \textit{rad3Δ} acts in some checkpoint-independent capacity to promote survival of \textit{taz1Δ} cells.

As \textit{rad3Δ} deletion in wt cells (Dahlen et al., 1998; Matsuura et al., 1999) or \textit{taz1Δ} cells (Nakamura et al., 2002) confers a slight decrease in the telomere length of either strain, we addressed the possibility that the synthetic interaction between \textit{rad3Δ} and \textit{taz1Δ} stems from drastic telomere attrition. Growth at 20°C had no effect on telomere length in wt or \textit{rad3Δ} cells (Figure 3.3C), but did result in a modest reduction in \textit{taz1Δ} and \textit{rad3Δtaz1Δ} cells (compare 32°C to 20°C, Figure 3.3C). Thus, while reduced temperatures do result in slightly shorter \textit{taz1Δ} telomeres, they are still unusually long (~10-fold longer than wt telomeres), and this minor temperature effect seems unlikely to provide the basis for the cold sensitivities of \textit{taz1Δ} or \textit{rad3Δtaz1Δ} cells.
3.2.4 MRN complex is required for growth in the cold

Since \textit{taz1}\(\Delta\) cells require a subset of DNA repair and checkpoint proteins, we wanted to further our analysis with other genes that are known to be involved in these pathways. The MRN complex consists of Rad32/Rad50/Nbs1 in fission yeast. Rad32 is the Mre11 ortholog from both budding yeast and humans. This complex has been shown to be important in the intra-S-phase checkpoint and DNA repair (Chahwan et al., 2003; Ueno et al., 2003). Additionally, this complex has been found at telomeres in most organisms (D'Amours and Jackson, 2002). Therefore, we asked if MRN was required for survival of \textit{taz1}\(\Delta\) cells in the cold. Surprisingly, single mutants of the MRN complex were extremely cold sensitive and were essential for viability in the cold, irrespective of \textit{taz1}\(^{+}\) (Figure 3.4). A Rad32 nuclease dead mutant \((\text{rad32D25A})\) exhibited better growth in the cold but was still extremely cold sensitive (Figure 3.4)(Tomita et al., 2003). Additionally, deletion of \textit{taz1}\(\Delta\) in any of the MRN gene deletions did not increase the inviability of the single MRN mutant. This epistasis analysis suggests that the defect resulting in the cold sensitivity of \textit{taz1}\(\Delta\) cells is dependent on MRN, which places Taz1 and MRN in the same genetic pathway in regards to survival in the cold. Importantly, the importance of MRN in the survival of cells in the cold suggests that cold treatments induce stress in cells that require MRN. This severe cold sensitivity was not observed in other checkpoint or repair mutants (Figure 3.3A and B). Thus, MRN most likely functions in multiple pathways that are required to survive cold-induced stresses.
Figure 3.4 MRN complex is required for survival at 20°C. (A)

Loss of Rad32 (or Rad50, Nbs1), irrespective of taz1 status, results in a severe growth defect in the cold. Experiments were performed as in Figure 3.3.
3.2.5 A subset of spindle assembly checkpoint components are required for the survival of \textit{taz1}\textDelta cells at 20°C

To address why the foregoing phenotypes of \textit{taz1}\textDelta cells appear only at cold temperatures, we considered that cold sensitivity is often associated with mutations that affect spindle assembly checkpoints, microtubules, and centromeric functions (Bernard et al., 1998; Ekwall et al., 1996; Javerzat et al., 1999). To evaluate the relationship between the cold sensitivity of \textit{taz1}\textDelta cells and the spindle assembly checkpoint, double mutants were constructed in which \textit{taz1}\textDelta was combined with deletion of \textit{mph1}\textsuperscript{+}, \textit{bub1}\textsuperscript{+} or \textit{mad2}\textsuperscript{+}. Double mutants between \textit{taz1}\textDelta and \textit{mph1}\textDelta or \textit{bub1}\textDelta show greater lethality at 20°C than do either single mutant alone, while \textit{mad2}\textsuperscript{+} deletion has no effect (Figure 3.3A). Recent reports suggest that Bubi p functions independently of Mad2p in sensing chromosomal tension during mitosis (Skoufias et al., 2001). Therefore, our data suggest that the \textit{mph1}\textsuperscript{+}/\textit{bub1}\textsuperscript{+} dependent, tension-sensing branch of the spindle assembly checkpoint is required for the survival of \textit{taz1}\textDelta cells at 20°C.

To address the possibility that \textit{taz1}\textsuperscript{+} loss affects microtubule structure or function, we transformed \textit{taz1}\textDelta cells with a plasmid encoding α-tubulin tagged with GFP (Ding et al., 1998). Cytological analysis of these cells shows no obvious defect in microtubule morphology at 20°C (data not shown). However, the frequency of cells containing mitotic spindles is three times greater in \textit{taz1}\textDelta cultures at 20°C than wt cultures, consistent with a mitotic delay in \textit{taz1}\textDelta cells in the cold. We also assessed microtubule-associated functions by exposing the cells to the microtubule destabilizing agent thiobendazole (TBZ) and found that
taz1Δ cells are no more sensitive than wt cells at 32°C or 20°C (Figure 3.5).

Thus, survival of taz1Δ cells at 20°C depends on two proteins associated with the spindle assembly checkpoint, Mph1 and Bub1. However, since taz1Δ cells do not exhibit TBZ sensitivity or defective centromeric silencing (Nimmo et al., 1998b), this dependency is unlikely to stem from direct effects of Taz1p on microtubules or centromere function. Rather, we hypothesize that loss of taz1Δ leads to some other type of defect, e.g. chromosomal damage, that necessitates a functional spindle assembly checkpoint for detection and resolution.

3.2.6 Cold temperatures do not induce telomere fusions in taz1Δ cells

What might be the nature of the chromosomal damage incurred by taz1Δ cells in the cold? A reasonable candidate might be chromosome end fusions, as we have shown that Taz1 is required to protect telomeres from NHEJ-dependent fusion under conditions of nitrogen starvation or impaired homologous recombination (Godinho Ferreira and Promisel Cooper, 2001). Furthermore, the nuclear morphologies discussed above are virtually identical to those of taz1Δ cells that have fused chromosomes. Thus, we used pulsed field gel electrophoresis (PFGE) to determine whether taz1Δ cells grown at 20°C accumulate end fusions. Nitrogen starved taz1Δ cells yield bands that represent inter- and intra-chromosomal telomere fusions ((Godinho Ferreira and Promisel Cooper, 2001) Figure 3.6, arrows), but these are not observed in taz1Δ cultures grown at 20°C (Figure 3.6). We also examined rad3Δtaz1Δ and mph1Δtaz1Δ strains, since both yield high levels of inviability and should display
Figure 3.5 Loss of Taz1 does not affect microtubule or centromeric function. (A) Defects in microtubules and/or centromeres often result in sensitivity to the microtubule poison thiobendazole (TBZ). \textit{bub1}Δ cells, which lack a spindle assembly checkpoint, are shown as a control for TBZ sensitivity. The lack of sensitivity to TBZ suggests that loss of Taz1 does not greatly affect either centromere or microtubule function. Five fold serial dilutions of log-phase cultures were plated on YES without or with the indicated concentrations of TBZ and grown at 32°C.
Figure 3.6 PFGE analysis of \textit{taz1Δ} cells at 20°C. (A) Growth of \textit{taz1Δ} cells at 20°C does not induce telomeric fusions. \textit{NotI}-digestion of genomic DNA releases telomeres from chromosomes I and II (C, L, I and M, gray boxes in schematic), visible by PFGE and Southern blotting with a telomere probe. Telomere fusions are present only in nitrogen starved \textit{taz1Δ} cells (L+I, M+I, M+L).
fusions if any link exists between telomere fusions and cold sensitivity. However, no fusion bands were seen for these strains (Figure 3.6). Furthermore, loss of pu70 or lig4*, both required for the formation of taz1Δ telomeric fusions, did not rescue the inviability of taz1Δ cells at 20°C (Figure 3.3B), again ruling out that covalent telomeric fusions are the basis for their cold sensitivity.

3.2.7 taz1Δ cells accumulate double strand breaks at 20°C

The shared phenotypes of taz1Δ cells harboring fused chromosomes and taz1Δ cells at 20°C prompted us to consider that non-covalent intertwining of the telomeres, which might elicit chromosome missegregation and breakage in a manner similar to that seen for dicentric chromosomes, could lead to the phenotypes we observe. To explore this possibility, we utilized well-established PFGE assays to assess chromosomal integrity (Cervantes et al., 2000b). Mutations that result in chromosomal entanglement, e.g. those that cause incomplete DNA replication and the accumulation of unresolved replication forks, yield DNA samples that cannot enter pulsed field gels (Nishitani and Nurse, 1995). If taz1Δ cells were incurring intertwined chromosomes in the cold, we would expect to see a reduction in the amount of chromosomal DNA that enters the gel. Indeed, we consistently observe that the total amount of DNA entering the pulsed field gels is lower for taz1Δ strains grown at 20°C than for wt strains (e.g. Fig 3.7). To ensure that equal quantities of DNA were loaded in each lane, equal cell numbers were processed and half of each sample was subjected to digestion with NotI restriction endonuclease. The NotI-digested taz1Δ samples entered the gels to the same extent as the wt samples (Fig 3.7),
Figure 3.7  *taz1Δ* cells have entangled chromosomes at 20°C.

Undigested chromosomes were resolved by PFGE. DNA from *taz1Δ* cells grown at 20°C enters the gel less efficiently than DNA from *taz1Δ* cells at 32°C or wt cells at either temperature. *NotI* digestion of the same samples allows equal quantities of DNA to be released into the gel.
notwithstanding the fact that 30-70% less DNA from the corresponding undigested \textit{taz1A} samples entered the gels (Fig 3.7-3.9). This suggests that the \textit{taz1A} samples contain entangled DNA that can be resolved by NotI digestion.

Entangled DNA might lead to chromosome breakage when the intertwined chromosomes are pulled apart during mitosis. To detect DNA breaks, we again utilized PFGE. Homologous recombination is the primary mode of DSB repair in fission yeast (Manolis et al., 2001) and requires \textit{rad22^+}, the fission yeast \textit{RAD52} homolog (Ostermann et al., 1993; Schlake et al., 1993; Schmidt et al., 1989). If growth at 20°C were inducing DSBs, loss of Rad22p should cause these breaks to accumulate. When undigested chromosomes are separated on a pulsed field gel, DSBs are seen as a diffuse zone of faster migrating DNA fragments running below the 3 intact chromosomes ((Cervantes et al., 2000b), Figure 3.8). We find no detectable accumulation of DSBs in \textit{rad22A} cells at 20°C, indicating that growth in the cold does not induce DSBs (Figure 3.8). However, \textit{rad22Ataz1A} cells exhibit high levels of breakage at 20°C, as these cells yield a low ratio of intact chromosomes to DSBs (Figure 3.8). The \textit{rad22Ataz1A} double mutant also exhibits severely reduced viability (Figure 3.3A). In control experiments, we treated wt cells with sub-lethal concentrations of the alkylating agent methyl methanesulfonate (MMS) to induce DSBs. Increasing concentrations of MMS result in a progressive reduction in the ratio of intact to broken chromosomes (Figure 3.8), yielding a pattern identical to that observed for \textit{rad22Ataz1A} cells grown at 20°C. Wt cells show no decrease of viability following these MMS treatments (data not shown). Therefore, cells can survive this pattern of chromosomal breakage if they have normal repair processes, and this PFGE pattern is not simply an indication of
Figure 3.8 rad22Δtaz1Δ cells accumulate DSBs at 20°C. (A) Undigested chromosomes were resolved by PFGE and stained with ethidium bromide. DSBs are seen as a diffuse zone of faster migrating DNA fragments running below the three intact chromosomes (5.7, 4.6 and 3.5 Mbps). Growth at 20°C does not induce DSBs if Taz1p is present. (B) Wt cells were treated with 0.001% to 0.006% MMS for 1 hour before processing for PFGE.
dying cells.

Deletion of taz1Δ alone also leads to a modest accumulation of DSBs at 20°C, but not at 32°C (Figure 3.7 and 3.9). Thus, an inability to repair these breaks may underlie the taz1Δ cold sensitivity. If this were true, we might expect the double mutants with greater inviability at 20°C to display increased levels of DSBs; as shown above, this expectation is fulfilled for the rad22Δtaz1Δ double mutant. Another double mutant exhibiting drastically reduced viability was rad3Δtaz1Δ, and this double mutant also results in a pronounced accumulation of DSBs at 20°C (Figure 3.9), though rad3Δ deletion alone does not promote DSBs at 20°C. Thus, Taz1 is required to prevent DSB accumulation at 20°C, and these DSBs may arise as a consequence of telomeric entanglement.

3.2.8 taz1Δ cells are sensitive to treatments that cause double strand breaks

The persistence of DSBs in taz1Δ cells at 20°C prompted us to ask whether taz1Δ might be involved in general DSB repair. When growth of taz1Δ and wt cells are compared on plates containing MMS (Figure 3.10A) or bleomycin (data not shown), it is evident that the taz1Δ cells are hypersensitive at 32°C. Loss of rad3Δ leads to severe MMS sensitivity even at low drug concentrations (al-Khodairy and Carr, 1992; O’Connell et al., 2000), while double mutants of taz1Δ with the checkpoint kinases chk1Δ and cds1Δ yield enhanced MMS sensitivity. The synthetic lethality between cds1Δ and taz1Δ at high levels of MMS was unanticipated based on their lack of genetic interaction in the cold and on previous studies showing that cds1Δ deletion alone does not yield MMS sensitivity (Figure 3.10A). Surprisingly, loss of bub1Δ or mph1Δ also
Figure 3.9 *taz1Δ* cells accumulate DSBs at 20°C. Undigested chromosomes were resolved by PFGE at both 32°C and 20°C. Chromosomal integrity in *taz1Δ* cells was identical to wt cells at 32°C. At 20°C, *taz1Δ* cells displayed a marked accumulation of DSBs and this was exacerbated by deletion of *rad3*⁺.
enhanced the sensitivity of $taz1\Delta$ cells to MMS (Figure 3.10A), indicating a requirement for components of the spindle assembly checkpoint in recovery from drug induced DNA damage. Therefore, loss of $taz1^+$ confers extreme drug sensitivity on checkpoint defective strains that are not themselves drug sensitive.

To address the possibility that $taz1\Delta$ cells are specifically sensitive to alkylation damage, we subjected the cells to $\gamma$-irradiation, a treatment that induces immediate DSBs in $taz1\Delta$ cells. Indeed, $taz1\Delta$ cells are hypersensitive to gamma irradiation compared to wt cells, though they are not as sensitive as $rad3\Delta$ cells (Figure 3.10B). These results demonstrate that Tazi is involved in general DNA repair.

3.2.9 Loss of telomeres results in cold and drug sensitivity

Since $taz1\Delta$ cells accumulate DSBs throughout their genomes at 20°C, we wondered if the basis for cold sensitivity originates at telomeres or instead reflects a telomere-independent role of Taz1. To clarify this issue, we utilized $taz1\Delta$ strains lacking telomeres entirely. When telomerase ($ttr1^+$) is deleted, cells gradually lose telomeres and most eventually die. However, two different types of survivors can be isolated. Most have lost all telomeric sequences and have circularized each of their three chromosomes. A second class containing linear telomeres, maintained via recombination, can be isolated from cultures in which $taz1$ deletion preceded $ttr1$ deletion (Nakamura et al., 1998). We compared these two types of survivors for their ability to grow in the cold. The $ttr1\Delta taz1\Delta$ linear strain showed the same cold sensitivity as the $taz1\Delta$ single mutant, indicating that $ttr1^+$ is itself not required for growth in the cold.
Figure 3.10 Taz1 is required for survival of DNA damage. (A) Loss of *taz1*+ confers sensitivity to MMS. Five fold serial dilutions of log-phase cultures were plated on YES without or with the indicated concentrations of MMS and grown at 32°C. (B) Loss of *taz1*+ confers sensitivity to γ-irradiation. Plated cells were irradiated and grown at 32°C.
However, trt1Δ cells containing circular chromosomes exhibit extremely low viability at 20°C (Figure 3.11), revealing a requirement for telomeres and/or chromosome linearity in coping with growth in the cold. Furthermore, deletion of taz1+ from the circular strain does not exacerbate its inviability, indicating that the effects of taz1+ loss at 20°C are exerted specifically through telomeres (Figure 3.11).

We found a similar pattern of responses to MMS. Strikingly, trt1Δ circular cells exhibit extreme sensitivity to MMS, irrespective of their taz1+ status (Figure 3.11). This sensitivity is revealed on plates containing only 0.0005% MMS, a concentration 16 times lower than that needed to observe drug sensitivity in taz1Δ cells (Figure 3.11, compare last two panels). The drug sensitivity of these circular strains does not stem from a defect in the DNA damage checkpoint, as all of the strains shown in Figure 3.11 elongate upon MMS treatment (data not shown). Hence, the drug sensitivity most likely reflects a defect in recovery and repair. These data reveal a requirement for telomeres in coping with both growth in the cold and survival of DNA breakage. The fact that loss of taz1+ has no further effect on these parameters in cells that lack telomeres implies that Taz1 acts via its association with telomeres.

3.2.10 Topoisomerase II activity promotes chromosomal defects in taz1Δ cells at 20°C

The fission yeast type II topoisomerase, Top2, is required for many aspects of chromosome organization, including resolution of intertwined sister chromatids following replication (Uemura et al., 1987). We hypothesized that if taz1Δ cells accumulate intertwined chromosomes in the cold, alterations in
Figure 3.11 Loss of telomeres results in both cold- and drug- sensitivity.

Five fold serial dilutions of log-phase cultures were plated onto YES plates without or with the indicated concentrations of MMS and grown at 32°C. For analysis of cold sensitivity, plates were incubated at 20°C. Loss of telomeres, irrespective of \textit{taz1} status, results in enhanced cold sensitivity suggesting that the involvement of Taz1 in surviving growth in the cold and treatments that induce DSBs is via telomeres.
Top2 activity might affect their cold sensitivity. As top2\(^{-}\) is an essential gene, we utilized the temperature sensitive allele top2-191 to explore this possibility. top2-191 contains a single amino acid substitution (A801V) in the C-terminus which is reported to be involved in the DNA breakage/joining and DNA recognition domain of Top2 (Shiozaki and Yanagida, 1991; Uemura et al., 1986). Surprisingly, top2-191 suppressed the growth defect of \(taz1\Delta\) cells at 20°C (Figure 3.12A) as well as the cell elongation. The inability of \(taz1\Delta\) chromosomes to efficiently enter pulsed field gels was also suppressed (Figure 3.12B), as was the accumulation of DSBs. These data suggest that Top2 activity is responsible for generating the defects seen in \(taz1\Delta\) cells at 20°C.

Since top2-191 suppressed the cold sensitivity of \(taz1\Delta\) cells, we wondered if it might also suppress the cold and drug sensitivities of circular chromosome containing strains. To address this possibility, \(trt1\Delta\) was deleted from a top2-191 strain and circular survivors were isolated (data not shown). Analysis of two independent top2-191\(trt1\Delta\) circular survivor strains showed that these strains had identical cold and drug sensitivity profiles as \(trt1\Delta\) or \(trt1\Delta taz1\Delta\) cells (Figure 3.13). Therefore, we conclude that the mechanism for top2-191 suppression of \(taz1\Delta\) cells is specific for these cells and not all cold sensitive cells. For drug sensitivity, we have also found that top2-191 does not suppress the drug sensitivity of \(taz1\Delta\) cells suggesting that drug and cold sensitivity of \(taz1\Delta\) cells do not share a common mechanism (Chapter 5). Since circular strains do not contain telomeres, it is conceivable that top2-191 acts specifically on telomeres explaining why it suppresses the cold sensitivity of \(taz1\Delta\) cells and not any circular survivor strain.

*top2-191* was isolated as a temperature sensitive topoisomerase II mutant
Figure 3.12 Altered topoisoamerase function suppresses \textit{taz1}\textDelta cold sensitivity phenotypes. (A) \textit{top2-191} suppresses the loss of viability of \textit{taz1}\textDelta cells at 20°C. Analysis was performed as in Figure 3.3A. (B) \textit{top2-191} suppresses the inability of genomic DNA from \textit{taz1}\textDelta cells to enter PFGE at 20°C. Experiments were performed as in Figure 4C except that gels were processed for Southern blotting with a telomeric probe. Again, identical plugs were digested with \textit{Not1} to confirm approximately equal quantities of DNA in each sample.
Figure 3.13  Altered topoisomerase function does not suppress drug or cold sensitivity of circular strains. Analysis was performed as in Figure 3.3A.
(Uemura and Yanagida, 1984). Therefore, any altered activity of Top2 might suppress the cold sensitivity of \textit{taz1\Delta} cells. To explore this possibility, we analyzed additional \textit{top2}\textsuperscript{ts} alleles that were isolated in the original screen. We also analyzed a topoisomerase I mutant that was isolated in the same screen. We found that only \textit{top2-191} had an effect on the cold sensitivity of \textit{taz1\Delta} cells (Figure 3.14). We confirmed that both isolates of \textit{top2-191} (the original from Yanagida's lab and an additional isolate from Nurse's lab) suppressed the cold sensitivity of \textit{taz1\Delta} cells. Altered Top1 activity or loss of endonuclease I had no effect on \textit{taz1\Delta} cells growth in the cold. Taken together, these data show that \textit{top2-191} specifically suppresses the cold sensitivity of \textit{taz1\Delta} cells.

To gain further insights into the nature of the \textit{top2-191} mutation, we wanted to determine if this mutation was dominant or recessive with wt \textit{top2}\textsuperscript{ts}. For this aim, we created a strain containing both \textit{top2}\textsuperscript{ts} and \textit{top2-191} and tested its ability to grow at 37°C and suppress the cold sensitivity of \textit{taz1\Delta} cells. Concerning the temperature sensitivity, we found that \textit{top2-191} was recessive with \textit{top2}\textsuperscript{ts} as this strain was able to grow at 37°C (data not shown). This suggested that \textit{top2-191} was a loss of function mutation with respect to temperature sensitivity. Surprisingly, we found the opposite scenario for the cold sensitivity. \textit{top2-191} was proficient in suppressing the cold sensitivity of \textit{taz1\Delta} cells even when wt \textit{top2}\textsuperscript{ts} was present (Figure 3.14). To further this analysis, we cloned \textit{top2}\textsuperscript{ts} into an overexpression vector (pRep81X-Top2) and expressed it in both \textit{taz1\Delta} and \textit{top2-191taz1\Delta} cells. Overexpression of Top2 in either \textit{taz1\Delta} or \textit{top2-191taz1\Delta} cells had no effect on the cold sensitivity of the strain (Figure 3.15). Taken together, our results suggest that \textit{top2-191} is dominant over wt \textit{top2}\textsuperscript{ts} and indicates a gain of function mutation in \textit{top2}\textsuperscript{ts} with
Figure 3.14  Suppression of taz1Δ cold sensitivity by altered topoisomerase function is specific to top2-191 which acts like a dominant mutation. top2-191 suppresses the loss of viability of taz1Δ cells at 20°C. Additional topoisomerase mutant alleles were analyzed for genetic interactions with taz1Δ cells at 20°C. All mutant strains that carried the top2-191 ts allele suppressed the cold sensitivity of taz1Δ cells, regardless of their endonuclease or topoisomerase 1 (Top1) status. This suppression was specific to the top2-191 allele since another top2 ts allele did not suppress the cold sensitivity of taz1Δ cells. D191 is a strain that lacks endonuclease activity, contains a ts allele of topoisomerase I and the top2-191 ts allele of top2. top2-1913 is a different isolate of the top2-191 strain obtained by Yanagida’s lab where it was originally isolated. top2-3421 is a different top2 ts allele isolated in the lab of Yanagida. Dual expression of top2-191 and the wt allele of top2 still suppressed the cold sensitivity of taz1Δ cells, suggesting top2-191 is a dominant mutation. For this experiment, top2 was cloned into the ura4 locus so that is was expressed from the ura4 promoter. Activity was verified by its ability to suppress the temperature sensitivity of top2-191 (data not shown). Analysis was performed as in Figure 3.3A.
Figure 3.15  *top2-191* acts like a dominant mutation at 20°C. Elongation of *taz1Δ* cells is not suppressed by the overexpression of topoisomerase II (81X-Top2) at 20°C (top panel). Additionally, overexpression of topoisomerase II does not revert the suppression of *top2-191* of the cold sensitivity in *taz1Δ* cells (bottom panel). Failure of overexpressed Top2 to alter the cold sensitivity of *taz1Δ* cells suggests that *top2-191* is dominant over wt Top2 in respect to the cold sensitivity of *taz1Δ* mutants.
respect to the cold sensitivity of \( taz1\Delta \) cells (see discussion and Figure 3.16).

3.3 Discussion

3.3.1 Taz1p loss triggers activation of the G2/M DNA damage checkpoint and chromosome missegregation at 20°C

While \( taz1\Delta \) cells have an aberrant telomeric chromatin structure and dramatically elongated telomeres under all conditions, they exhibit no detectable growth defects during normal vegetative growth. Here, we show that this apparent normality disintegrates at cold temperatures, revealing previously unknown roles for Taz1 in regulating telomeric functions that are required for normal cell cycle progression, DNA repair and genomic integrity. \( taz1\Delta \) cells lose viability at 20°C and become highly elongated, suggesting a cell cycle delay. Indeed, cell elongation depends on an intact G2/M DNA damage checkpoint pathway. These cells also exhibit excess mitotic spindles at 20°C and depend on components of the spindle assembly checkpoint for survival, despite showing no evidence of gross microtubular or centromeric defects. Accompanying the aberrations in growth and cell size, \( taz1\Delta \) cells show cold-specific chromosome segregation defects. These phenotypes are exacerbated by deletion of the fission yeast ATR homolog, \( rad3^+ \), which appears to act in a checkpoint-independent capacity to promote survival of \( taz1\Delta \) cells at 20°C. The chromosomes of \( taz1\Delta \) cells appear to be entangled and show high levels of DNA breakage at 20°C. Strikingly, the cold sensitivity
and chromosome breakage phenotypes are alleviated by altered topoisomerase II function, suggesting that these problems stem from the inappropriate use of topoisomerase action in \textit{taz1} cells.

3.3.2 Roles for Taz1p in both preventing and repairing DNA damage

The checkpoint-dependent elongation of \textit{taz1A} cells at 20°C indicates that the checkpoint surveillance system is functional and activated. What is the nature of the damage signal generated when Taz1 is lost, and why is it only detected in the cold? We have ruled out the possibility that this damage signal stems from covalent telomere-telomere fusions, as such fusions, while seen in \textit{taz1A} cells arrested in early G1 by nitrogen starvation, are not detected in cycling \textit{taz1A} cells at 20°C (Figure 3.6). \textit{taz1} telomeres have an aberrant chromatin structure which could, in principle, be detected as damage by checkpoint systems, although it is not obvious why this would occur exclusively at cold temperatures. However, we find that another type of damage, DSBs, does occur only at cold temperatures in \textit{taz1A} cells. Our data suggest that these DSBs reflect two aspects of Taz1 function, a role in preventing DSBs and a role in repairing them. Here we consider the evidence and potential bases for each of these roles in turn.

3.3.2.1 Potential bases for the accumulation of DSBs in \textit{taz1A} cells at 20°C

The accumulation of DSBs is accompanied by nuclear morphological defects that evince chromosome missegregation. The appearance of these
defects requires passage through S-phase at 20°C. Thus, the event that initiates the cascade of cold-specific defects must occur during S-phase. Importantly, this cascade is curtailed in cells with compromised topoisomerase II function. \textit{taz1A} cells display extremely long telomeres at all temperatures ((Cooper et al., 1997b), and Figure 3.3C), which do not themselves appear to pose a problem at 32°C, as \textit{taz1A} cells grow normally. However, these long G-rich repetitive tracts could conceivably pose a problem during replication in the cold, e.g. by invading stalled replication forks or by engaging in excessive levels of recombination. Such problems could lead to intertwining of DNA strands from different chromosomes or different regions within a chromosome. In wt cells, intertwined sister chromatids are resolved by Top2 (Spell and Holm, 1994; Uemura et al., 1987). Therefore, intertwined chromosomes in \textit{taz1A} cells may inappropriately provide a stimulus for Top2, which might cleave and re-join DNA strands inaccurately, leading to entangled chromosomes that become broken as they are sheared during cell division. Alternatively, Top2 may normally facilitate resolution of intertwined \textit{taz1A} chromosomes at 32°C. However, Top2 function may be compromised at 20°C such that it can cleave DNA but cannot re-join it efficiently. In support of this latter possibility, \textit{top2} mutants at restrictive temperatures exhibit similar chromosomal missegregation defects to \textit{taz1A} cells at 20°C (Krien et al., 1998; Uemura et al., 1987). However, \textit{top2-191} appears to act in a dominant fashion with respect to the cold sensitivity of \textit{taz1A} cells. We suggest a model whereby \textit{top2-191} is a gain of function mutation which allows it to act on \textit{taz1} telomeres while wt Top2 cannot (Figure 3.16). Since the mutation in \textit{top2-191} maps to the DNA recognition domain, this mutation might allow Top2-191 to recognize telomeric sequences while normal
A

possible scenarios:

1. **Gain of function** - top2-191 protein is able to resolve chromosomes at 20°C

   - disentanglement and suppression of cold sensitivity

2. **Loss of function** - Top2 entangles chromosomes in taz1Δ cells in the cold

   - potentially lethal event

   Top2-191 doesn’t act on DNA: no entanglement

   Suppresses cold sensitivity

B

viable, suppresses temperature sensitivity
(recessive: loss of function for temp. sensitivity)

\[ taz1Δtop2-191^{ts} + top2^+ \]

viable, suppresses cold sensitivity
(dominant: gain of function for cold sensitivity of taz1Δ cells)

37°C

20°C
Figure 3.16 Possible scenarios to explain Top2-191 suppression of the cold sensitivity of \textit{taz1Δ} cells. (A) This figure shows an explanation of how either a gain of function (part 1) or loss of function (part 2) mutation could suppress the cold sensitivity of \textit{taz1Δ} cells. (B) Experimental data (from Figure 3.14 and 3.15) suggests that \textit{top2-191} can act as both a dominant and recessive mutation with wt top2\textsuperscript{+}. Since \textit{top2-191} acts like a dominant mutation with respect to the cold sensitivity of \textit{taz1Δ} cells, we favor the gain of function model in part 1A.
Top2 does not. In support of this, telomere sequences have been shown to be substrates for drug-inhibited Top2 in vitro which could be analogous to the gain of function mutation of top2-191 (Lee and Huang, 2001; Yoon et al., 1998). Topoisomerase II enzymes bind to preferred DNA sequences that are not telomeric (Spitzner et al., 1990). Additionally, normal telomeres in yeast are only 300 bp which limits the binding capacity of Top2. Therefore, in wt cells, Top2 might not be required to act on telomeres. Telomeres are normally attached to the nuclear matrix (Chikashige et al., 1997; Gasser, 2002; Luderus et al., 1996). However, telomeres may become unbound from the nuclear membrane when replicated which would expose a free DNA end allowing rotation to alleviate any positive supercoiling that would occur ahead of the fork alleviating the need for Top2. In \( \text{taz1} \Delta \) cells, telomeres are ten times longer than in wt cells. Conceivably, loss of Taz1 could create a situation requiring Top2 to act on telomeres in the cold. Replication fork progression/resolution and recombination are activities that are known to require Top2 (Wang, 2002). Why would \( \text{taz1} \Delta \) cells require these activities specifically at 20°C? Growth at cold temperatures require the action of the MRN complex since mutations in these genes render cells extremely cold sensitive (Figure 3.4) The MRN complex is required for recombination, intra-S-phase checkpoint and telomere maintenance in fission yeast. Therefore, cold temperatures appear to stress cells in a manner that creates the need for these activities. Since MRN is involved in so many biological functions, it is difficult to attribute the cold sensitivity to a specific pathway or process. However, the deletion of Taz1 did not increase the cold sensitivity of any of the MRN mutants (Figure 3.4). This data suggests that MRN and \( \text{taz1} \Delta \) cells share common cold-specific defects,
which places them in the same genetic pathway for surviving temperatures in
the cold. Thus, cold sensitivity of \textit{taz1A} cells could be explained if MRN was
compromised in these cells. MRN proteins localize to wt telomeres and to \textit{taz1-}
dysfunctional telomeres, a process that could compromise the activities of MRN
((Tomita et al., 2003), see below). Since MRN is required for both
recombination and the intra-S-phase checkpoint, it has been proposed that it
might process stalled replication forks or replication DNA damage bypass
mechanisms (Kowalczykowski, 2000; McGlynn and Lloyd, 2002; Paques and
Haber, 1999). MRN localizes to stalled replication forks in various organisms,
validating its role in processing replicative DNA damage (Mirzoeva and Petrini,
2003; Robison et al., 2004). Therefore, it seems plausible that growth in the
cold results in replicative stresses that require MRN. Since MRN and \textit{taz1A}
mutants appear to share the same genetic pathway leading to their cold-specific
demise, replication defects caused by the loss of Taz1 might promote the cold
sensitivity of these cells. This hypothesis is supported by the S-phase specific
initiation of the cold sensitivity in \textit{taz1A} cells. A detailed examination of
replication in \textit{taz1A} mutants should give insights into the possible defects that
could contribute to the observed phenotypes of \textit{taz1A} cells in the cold (see
Chapter 4)

Interestingly, cells containing circular genomes that might be expected to
mimic entangled chromosomes display similar nuclear morphologies to those
seen in \textit{taz1A} cells at 20°C. Furthermore, these circular chromosome-
containing cells are extremely cold sensitive, suggesting that any situation that
engenders chromosome entanglement is detrimental at 20°C.
3.3.2.2 Potential roles for Taz1 in DNA repair

A role in the repair of DSBs is indicated by both the failure of taz1Δ cells to efficiently repair those breaks that arise in the cold, and by their sensitivity to treatments that induce DSBs at optimal growing temperature. Furthermore, taz1Δ mutants become hypersensitive to DSBs when combined with other mutations that affect checkpoint signaling pathways but do not normally confer drug sensitivity. In particular, single mutations that affect the intra-S-phase checkpoint or the spindle assembly checkpoint pathways do not confer drug sensitivity, but loss of taz1+ in these backgrounds (cds1Δ, mph1Δ and bub1Δ) results in a >25 fold increase in sensitivity to DSBs. Such genetic interactions could indicate that Taz1p and the respective checkpoint associated proteins play redundant roles in promoting DSB repair. Alternatively, Taz1 could participate in a DSB repair process that normally obviates the need for the intra-S and spindle assembly checkpoint pathways. In this scenario, loss of Taz1 would compromise the DSB repair process, prolonging the presence of DSBs in the cell and creating a requirement for the checkpoint pathways for cellular survival. As taz1Δ cells have functional DNA damage checkpoint systems, their sensitivity to DSBs is likely to stem from a defect in DNA repair. A role for telomere proteins in DSB repair has been suggested by studies of protein translocations upon DNA damage. In budding yeast, the Sir and Ku proteins are normally located at telomeres, but upon induction of DSBs, a sub-population of these proteins relocates to sites of DNA damage (Martin et al., 1999; McAinsh et al., 1999; Mills et al., 1999). It is well established that Ku is required for the NHEJ pathway of DSB repair, but whether the Sir proteins are actually involved in DSB repair is unclear (Astrom et al., 1999; Lee et al., 1999).
Conceivably, the telomere-specific chromatin structure nucleated by Taz1 might be required to mobilize the repair machinery or to inhibit competing deleterious processes. A good candidate requiring proper telomere function to act would be the MRN complex. Rad32 has been localized to telomeres and acts inappropriately on taz1 telomeres (Nakamura et al., 2002; Tomita et al., 2003). Therefore, the loss of Taz1 might disrupt the normal function of the MRN complex, either by inhibiting its action or by sequestering MRN repair activities by presenting telomeres as substrates rather than bona fide DSBs. An alternative explanation for the hypersensitivity of taz1Δ cells to DSBs would invoke elevated levels of NHEJ stimulated by DSB induction, leading in turn to NHEJ-mediated fusions between taz1Δ telomeres. However, this scenario is unlikely since inactivation of NHEJ via deletion of pku70 or lig4 does not suppress the sensitivity of taz1Δ cells to DSBs (data not shown).

### 3.3.3 Roles of checkpoint and repair proteins in the survival of taz1Δ cells

This study has revealed a complex network of genetic requirements for taz1Δ cells to survive cold temperatures or DNA damaging agents. While deletion of the G2/M DNA damage checkpoint pathway suppresses the cold-specific elongation of taz1Δ cells, only the Rad3 component appears critical for their survival. We suspect that this reflects a role for Rad3 in prompting the repair of DSBs that arise in taz1Δ cells at 20°C. Similarly, Rad22, a protein central to the HR repair pathway, is crucial for the survival of taz1Δ cells at 20°C, again suggesting that prevention of DSB accumulation is a major challenge for these cells.
In addition, survival of \textit{taz1}\textDelta cells in the cold requires the Mph1 and Bub1 components of the spindle assembly checkpoint. Entanglement between \textit{taz1}\textDelta telomeres at 20°C might alter the degree of tension exerted by the linked chromosomes upon the spindle and thereby activate the spindle checkpoint. Our data could also reflect a role for Bub1 and Mph1 in promoting DNA repair during mitosis, as discussed above. In Drosophila embryos, DNA damage causes a mitotic arrest, alleviation of which results in DNA breakage in the subsequent anaphase (Su and Jaklevic, 2001). Thus, our data is supportive of an important, though as yet incompletely defined, connection between the spindle assembly checkpoint and DNA repair.

This work demonstrates that Tazi-containing telomeres are critical for growth and the prevention of DSBs at 20°C. Understanding the cold-specificity of the \textit{taz1}\textDelta phenotypes should illuminate those processes that maintain the integrity of chromosome ends at all temperatures. Our data suggest a model in which defective telomeres become intertwined during S-phase, leading to DSB accumulation, checkpoint activation and chromosome missegregation (Figure 3.17). Our data further suggest that functional telomeres are required for general DSB repair. Deciphering the role of telomeres in recovery from DNA damage will be important for understanding the processes that preserve genomic integrity.
Figure 3.17 Model for the generation of cold sensitivity in \textit{taz1\Delta} cells. Upon passage through S-phase in the cold, \textit{taz1\Delta} telomeres become entangled, leading to DSB formation and G2/M DNA damage checkpoint and spindle assembly checkpoint activation. Genes indicated in grey type are required for elongation of \textit{taz1\Delta} cells at 20°C, and underlined genes are important for viability of \textit{taz1\Delta} cells at 20°C. All cold sensitivity phenotypes are alleviated by the \textit{top2-191} mutation. MRN complex is essential for viability in the cold independently from Taz1 and is epistatic with the loss of Taz1, suggesting that the role of Taz1 in surviving growth in the cold requires MRN.
4 Conventional DNA replication through telomeres

4.1 Introduction

Although roles for telomere binding proteins in regulating telomerase activity are well recognized (Smogorzewska and De Lange, 2004), little is known about their role in controlling telomere replication by the conventional replication machinery. Loss of Taz1 results in ca. 10-fold telomere elongation due to deregulation of telomerase (Cooper et al., 1997a). It also results in chromosome end-to-end fusions, but only when the level of nonhomologous end-joining is elevated via G1 arrest, a situation not normally experienced by fission yeast as the normal cell cycle lacks a discernible G1 phase (Ferreira and Cooper, 2001b). This unique property of fission yeast cells allows them to grow despite telomere dysfunction, and has therefore allowed us to address the involvement of telomere binding proteins in conventional telomere replication.

4.2 Telomere replication

To analyze telomeric replication intermediates, genomic DNA from asynchronous cultures was subjected to BND cellulose chromatography to enrich for replication intermediates. These partially purified replication intermediates were then analyzed by two-dimensional gel electrophoresis (2D GE) in which DNA fragments are separated by molecular weight in an agarose gel, then electrophoresed in a second dimension under conditions in which gel mobility is a function of shape. Branched replication structures lead to gel retardation resulting in unique patterns of gel mobility that have been definitively associated with specific replication states (Friedman and Brewer, 1995) (figure 113).
4.1B).

Of the 6 telomeres in fission yeast, four (at each end of Chromosomes I and II) are bordered by the semi-repetitive ‘telomere associated sequences (TAS)’, while the telomeres at either end of Chromosome III directly abut the rDNA repeats. While TAS sequences are not entirely identical from telomere to telomere, they share a common pattern in which the so-called TAS1 sequences are the most distal and the distinct TAS2 sequences are more centromere-proximal (figure 4.1A). We utilized a cloned telomeric restriction fragment to make specific probes to these regions (Nakamura et al., 1998) (figure 4.1A). Nsi1 digestion released 5 telomere-containing restriction fragments from wild type (wt) chromosomes, as seen by Southern blotting in the 1st dimension (1D) (Figure 4.1C). 2D GE revealed that each of these are replicated as simple Y-arcs, indicating that these telomeric fragments are replicated by an origin that is centromeric to the terminal Nsi1 fragment. This result is consistent with previous work on fission yeast replication origins, which showed that a region 10-20 kb centromeric to the TAS sequences contains multiple origins, all of which are markedly A/T-rich, in contrast with the G/C-rich telomeric region (Segurado et al., 2003). Interestingly, some of the wt telomere-containing fragments showed replication pause sights in the subtelomeric region (Figure 4.2), a phenomenon noted in budding yeast (see below).

1D Southern analysis of taz1Δ telomere-containing fragments revealed a heterogeneous smear of products, ranging from 3.5 – 9 kb, with the majority of hybridization between 5 and 7 kb. Hence, we expected that 2D GE of taz1Δ mutants would yield an amalgamation of superimposed Y-arcs ranging from 5
Figure 4.1. Analysis of conventional replication through telomeres using two-dimensional gel electrophoresis. (A) Map of the cloned telomeric fragment used to derive subtelomeric probes. (B) The Y-arc pattern seen by 2D GE analysis. For each restriction fragment, the unreplicated form runs in the 1N spot, while the 2N spot represents the theoretical duplication of that fragment. All unreplicated DNA fragments will run in the arc of linears. The simple Y-arc replication profile is generated by the unidirectional movement of a single replication fork across a DNA fragment. Unreplicated DNA: grey, replicated DNA: red. (C) 1D analysis of telomeric Nsi1 fragments.
Figure 4.2. Conventional replication through telomeres is inefficient and requires the telomere binding protein Taz1. 2D GE analysis of Nsi1 digested, telomere-containing fragments from wt and taz1Δ cells. Inset shows the expected area and shape of the signal expected from taz1Δ cells (yellow dashed line) if their telomeres, which are heterogeneous in length, were replicated as Y-arcs. The lower panel shows DNA from taz1Δ cells that were arrested in G1 by nitrogen starvation for 24 hours. DNA markers indicate molecular weights derived from 1D GE.
kb for the 1N spot to 18 kb for the 2N spot, with the vast majority of signal appearing as a semicircle encompassed by the dotted line in Figure 4.2 (inset). Surprisingly, this pattern was not observed. Instead, the telomere signal intensified at the top of the superimposed Y-arcs and then increased into higher molecular weight fragments, forming a plume that extended well beyond the predicted size for the replicating telomeres (Figure 4.2). 2D GE of DNA from G1-arrested taz1Δ cells yielded only the arc of linear fragments, excluding the possibility that the plume represents artifactual interactions between the long 3' G-strand overhangs that are present throughout the cell cycle at taz1Δ telomeres (Tomita et al., 2003) (Figure 4.2; Ferreira and Cooper 2004). We did not observe a signal descending from the top of the arcs to the 2N region, suggesting that forks do not progress to the ends of taz1Δ telomeres.

To verify that Y-arcs representing long DNA fragments would be detectable in our gel system, we analysed the replication profile of the rDNA locus, which has been studied extensively in fission yeast. Nsi1 digestion yielded 3 bands on the 1D Southern with the main ~ 8 kb fragment corresponding to the multiply reiterated rDNA repeat (Figure 4.3A). 2D GE showed that deletion of taz1Δ has no effect on rDNA replication, as the pattern obtained for DNA from both wt and taz1Δ cells is indistinguishable and identical to previously published replication patterns (Sanchez et al., 1998; Sanchez-Gorostiaga et al., 2004) (Figure 4.3B). Importantly, a larger molecular weight fragment (>12 kb), most likely corresponding to the most centromeric rDNA repeat, is also detected as a simple Y-arc (Figure 4.3B). Thus, the anomalous 2D GE behavior of taz1Δ telomeres does not stem from an inability of our gel system to detect the replication of large fragments. These results also indicate
Figure 4.3. rDNA replication is unaffected by the loss of Taz1. (A) Map of rDNA repeat region, found at either end of Chromosome III. A probe corresponding to the entire rDNA locus was used. (B) 2D GE of rDNA replication in wt and taz1Δ cells is identical, and the ~12 kb (F2) fragment yields a simple Y-arc.
that Taz1 loss affects telomere replication specifically, and has no effect on the replication of repeated DNA sequences in general.

4.3 Subtelomeric replication

To explore the basis for the anomalous replication of taz1Δ telomeres, we examined replication in subtelomeric regions. The TAS1 sequence lies just centromeric to the telomere tract and is separated from the telomere by Apa1 digestion (Sugawara, 1988). Analysis of the TAS1 region in wt cells revealed four fragments, also observed in taz1Δ mutants (Figure 4.4A), three of which could be analyzed under our 2D GE conditions. Notably, cells lacking Taz1 display hyper-recombination in the TAS regions as evidenced by the variability of the hybridization patterns. As expected, TAS1 fragments are replicated as simple Y-arcs (Figure 4.4B). Strikingly, taz1Δ mutants accumulated paused replication forks at the 2N spot, which lies adjacent to the telomeric tracts (Figure 4.4B). Therefore, Taz1 is required for efficient fork progression specifically through the telomeric end of TAS1. Simple Y-arcs corresponding to replication of the two major TAS2 fragments, which lie centromeric to TAS1, were unaffected by Taz1 loss, again pointing to the telomere itself as the site of elevated replication fork stalling in taz1Δ cells (Figure 4.4C and D). As unwinding activities associated with the replication fork occur ahead of the fork itself, we conclude that in the absence of Taz1, these activities are obstructed as they encounter the telomere.

Fission yeast Rap1 mediates some functions of Taz1 (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). Unlike budding yeast Rap1, fission yeast and human Rap1 do not bind telomeric DNA directly, but instead are
Apa1 digest, TAS1 probe

Apa1 digest, TAS2 probe

Nsi1 digest, TAS2 probe
Figure 4.4. Taz1 is required for replication through the TAS1, but not TAS2. (A) 1D analysis of Apa1 digested DNA. Additional bands in taz1Δ and rap1Δ cells are most likely due to hyperrecombination. (B) 2D GE showing replication of TAS1 fragments 1 and 2. The 1N spot of F3 overlaps with the end of F1, which obscures its analysis. Arrows denote replication pause sites. Both wt and rap1Δ mutants lack these replication pause sites. (C) 1D analysis of Nsi1 digested DNA, probed with TAS2. Loss of F3 in taz1Δ and rap1Δ mutants stems from hyperrecombination. (D) 2D GE of TAS2 fragments 1 and 2. Both are replicated as simple Y-arcs and are identical among the three strains.
recruited to telomeres by binding Taz1 or TRF2, respectively. Surprisingly, rap1Δ deletion did not elicit replication fork pausing at TAS1 (Figure 4.4B). Thus, Taz1 binding to telomeres is itself sufficient to prevent fork stalling at the TAS1/telomere boundary, and this protection does not require Rap1. As rap1Δ telomeres are at least as long as taz1Δ telomeres, this finding rules out the possibility that fork pausing stems from increased telomere length per se. Rather, it is the loss of Taz1 binding that renders these elongated telomeres difficult to maneuver by the conventional replication machinery.

4.4 Taz1 is required for efficient replication of internally placed telomeric tracts

To assess replication of telomere repeat DNA lacking Taz1 without the complication of heterogeneous telomere size, we analyzed the replication of an internally placed telomeric tract of 300 bp (Figure 4.5A). This internal telomere sequence was replicated as a simple Y-arc in wt cells (Figure 4.5B). However, taz1Δ deletion resulted in fork stalling specifically through the telomere repeat stretch (Figure 4.5B). Therefore, telomeric DNA lacking Taz1 yields fork pausing regardless of whether the telomere stretch terminates with a chromosome end.

4.5 Involvement of the RecQ helicase Rqh1 in cold sensitivity

If cold sensitivity of taz1Δ cells resulted from unresolved replication intermediates, we hypothesized that replication and recombination resolving enzymes would be required for the survival of taz1Δ cells in the cold. Rqh1 belongs to the RecQ helicase family whose members include both WRN and
Figure 4.5. Taz1 is required for efficient replication through internally placed telomere tracts. (A) Map of the genomic region containing an ectopically inserted telomere repeat tract. The ARS 2003-5 cluster is located 3 kb centromeric to the \textit{ura4} locus and has been shown to replicate this locus. The telomere tracts are positioned 2.6 kb from the beginning of replication of the Nsi1 fragment. Arrows denote direction of transcription. (B) Loss of Taz1 results in replication pausing through internal telomere tracts. Arrow indicates replication pausing.
BLM helicases, two genes mutated in the human diseases Werner’s syndrome and Bloom’s syndrome respectively (Khakhar et al., 2003). These diseases are associated with genomic instability and premature aging, which highlights the importance of RecQ helicases in promoting genomic stability (Hickson, 2003). Rqh1 is believed to play many important functions in the cell which include control of recombination, resolution of aberrant replication intermediates and telomere maintenance (Khakhar et al., 2003; Laursen et al., 2003; Stewart et al., 1997; Wilson et al., 1999). Loss of Rqh1 resulted in decreased viability when grown in the cold (Figure 4.6) Deletion of rqh1* did not enhance the loss of viability observed in taz1Δ cells in the cold (Figure 4.6). These results place rqh1* and taz1* in the same genetic pathway for cold sensitivity and suggest that the inability of Rqh1 to act at taz1 telomeres might be contributing to the cold sensitivity of these cells. Altered topoisomerase activity suppresses the cold sensitivity of taz1Δ cells (Chapter 3). RecQ helicases have been shown to interact with both type I and II topoisomerases (Laursen et al., 2003; Watt et al., 1995). Therefore, the suppression of taz1Δ cells cold sensitivity by top2-191 might require Rqh1. Indeed, loss of Rqh1 resulted in a decrease in viability in top2-191taz1Δ cells equivalent to that seen for taz1Δ cells (Figure 4.6).

Although difficult to quantitate by dilution assay, visual inspection of top2-191rqh1Δtaz1Δ cells grown in the cold showed many dead and highly elongated cells that were not visible in rqh1Δ cells (data not shown). Therefore, Rqh1 is required for the suppression of taz1Δ cells cold sensitivity by top2-191. Collectively, our data suggests that unresolved replication intermediates could be causing the cold-specific defects observed in taz1Δ cells.
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Figure 4.6. Involvement of Rqh1 in surviving growth in the cold. Like taz1Δ cells, rqh1 mutants are cold sensitive. Interestingly, the double mutant, taz1Δrqh1Δ, displays the same cold sensitivity as the loss of Taz1 alone, suggesting that Rqh1 is involved in the survival of taz1Δ cells in the cold. top2-191 suppresses the cold sensitivity of taz1Δ mutants and this suppression requires Rqh1. Five fold serial dilutions of log phase cultures were stamped onto rich medium and incubated at the indicated temperature.
4.6 Discussion

One of the outcomes of paused replication at \textit{taz1A} telomeres may be the presence of unwound, unreplicated strands of DNA, which may invade other chromosomes. The G-rich nature of telomere repeats and/or the long 3' single strand overhang in \textit{taz1A} cells might facilitate such interactions between strands of DNA from distinct duplexes (Tomita et al., 2003). In support of these hypotheses, the placement of \textit{rqh1} in the same pathway as \textit{taz1} for cold growth survival suggests that defective Rqh1 activity could explain the cold sensitivity of \textit{taz1A} cells. The budding yeast and human homologs of Rqh1, Sgs1 and BLM respectively, have been shown to unwind guanine-guanine (G-G) interactions (Sun et al., 1999; Sun et al., 1998). Unbound telomeric sequences could inappropriately base pair, a reaction normally prohibited by Watson-Crick base pairing. However, during replication, these base pairings are unpaired, exposing them to deleterious interactions. Taz1, in conjunction with Rqh1, might protect replicating telomeric sequences from G-G base pairing or inappropriate recombination. Thus, the ‘plumes’ of hybridization seen upon 2D GE of \textit{taz1A} telomere sequences may represent branched structures generated from telomeric strand invasion reactions or inappropriate G-G base pairing among telomeres. \textit{rqh1A} cells display shorter telomeres which suggests that Rqh1 functions at telomeres, even in wt cells (Wilson et al., 1999). The inability of Rqh1 to act at \textit{taz1} telomeres might not be too surprising, given the fact that TRF2, the human ortholog of Taz1, binds to and stimulates the helicase activity of WRN, the human homolog of Rqh1 (Opresko et al., 2002). Intriguingly, \textit{taz1A} mutants accumulate entangled chromosomes upon passage through S-phase.
at cold temperatures, and this can be alleviated by a specific mutation in
topoisoformsase II (Miller and Cooper, 2003) (Top2). Thus, it is tempting to
speculate that Top2 activity is critical for resolving aberrant structures that result
from replication fork stalling at \textit{taz1} \textit{telomeres}. Since Rqh1 is required for top2-191 suppression, altered Top2 activity might allow Rqh1 to function at \textit{taz1-}
telomeres while normal Top2 is unable to accommodate this activity in the cold.
Interestingly, a physical interaction between Top2 and Sgs1, the budding yeast
homolog of Rqh1, has been documented (Watt et al., 1995). Additionally, WRN
cells have been shown to have a defective decatenation checkpoint, a
checkpoint that monitors proper decatenation of chromatids by Top2 following
replication (Deming et al., 2001; Franchitto et al., 2003). Taken together, this
data suggests that aberrant replication intermediates arise in \textit{taz1} \Delta cells in the
cold that require Rqh1 and altered Top2 activity for their resolution. These
proteins might function together through the resolution of replication and/or
recombination intermediates, perhaps through the activation of a decatenation
checkpoint. This idea is further supported by the observed mitotic delay and
activation of mitotic checkpoints in \textit{taz1} \Delta cells when cultured in the cold
(Chapter 3). Our data also revealed a requirement for Rqh1 in surviving growth
in the cold. We previously found that the MRN complex is also essential for
viability in the cold (Chapter 3). Since mutations in either of these pathways
were epistatic with loss of \textit{taz1-}, it will be interesting to investigate if MRN and
Rqh1 physically interact in fission yeast. Indeed, these proteins have been
shown to localize to S-phase specific damage foci in various organisms and in
human cells, WRN was found to associate with the MRN complex (Cheng et al.,
2004). The cold sensitivity should provide a useful tool in further deciphering the
mechanistic interplay between MRN and Rqh1, answers of which should unveil the importance of these proteins in dealing with dysfunctional telomeres and their principal function in promoting genomic stability.

It has been proposed that replication fork pausing results from steric hindrance by stable DNA:protein complexes (Ivessa et al., 2003). Indeed, pausing has been observed in telomeric regions of budding yeast, and in both rDNA and heterochromatic regions of budding and fission yeast (Ivessa et al., 2003; Ivessa et al., 2002; Makovets et al., 2004). Fork stalling at these regions in budding yeast is alleviated by the activity of the PIF1-like helicase, Rrm3 (Ivessa et al., 2003). Deletion of a battery of genes encoding telomere-related factors or truncation of the Rap1 C-terminus had no effect on replication pausing through budding yeast telomeres, while shorter telomeres alleviated pausing. These results led to the conclusion that the Rap1 N-terminal DNA binding domain itself generated an obstacle to replication fork passage, with the larger number of bound Rap1 molecules at long telomeres exacerbating the effect (Makovets et al., 2004). The effects of losing the DNA binding domain of Rap1 would be more equivalent to taz1 deletion in representing the loss of direct contacts between the telomere and its major binding protein, and might reveal a role for Rap1 in promoting, rather than impeding, fork passage. However, such experiments are difficult as budding yeast Rap1 is not telomere specific and deletion of its DNA binding domain leads to a lethal loss of transcriptional regulation (Shore, 1994). In an in vitro SV40 replication system, creation of a telomeric DNA:protein complex via loading of human TRF1 and TRF2 onto telomeric tracts creates a replication block (Ohki and Ishikawa, 2004). Our data suggest that, by analogy with Taz1, physiological
concentrations of hTRFs may coordinate the passage of the replication fork in vivo.

Replication fork pausing can result in fork collapse and DNA breakage, which can trigger DNA recombination systems for repair and/or fork reactivation (Cox et al., 2000; Rothstein et al., 2000). Our results predict that stalled forks will result from the progressive loss of telomere binding proteins that accompanies telomere attrition in human telomerase-negative cells. Hence, replication pausing may be a trigger for the recombination reactions that constitute the 'alternative lengthening of telomeres' pathway seen in some cancer cells. Furthermore, if left unrepaired, stalled replication forks could create double strand breaks that would precipitate further telomere shortening.

If conventional replication forks are unable to effectively traverse taz1Δ telomeres, then the majority of the 3-5 kb taz1Δ telomeres must be synthesized by telomerase each cell cycle. Indeed, this prediction has been borne out, as loss of Taz1 in cells lacking telomerase accelerates telomere attrition and cellular senescence by 100-fold (Beernink et al., 2003; Nakamura et al., 1998). Hence, the 'end replication problem' begins before the replication fork reaches the chromosomal terminus, and necessitates not only telomerase but also telomere binding proteins.
5 Role of Rap1 and Rif1 in promoting genomic stability in Fission yeast

5.1 Introduction

Telomeres and the proteins that bind to them are important in regulating and executing a multitude of cellular processes. In fission yeast, many proteins have been discovered that are involved in telomere metabolism (Figure 5.1). In fission yeast, most efforts have been focused on understanding the function of Taz1 since it is the only protein that has been shown to bind directly to duplex telomere DNA. Because of this special property, it has been proposed that Taz1 acts as a platform that allows other telomere regulators to localize to the telomere where they perform their various essential functions including heterochromatin formation, checkpoint and DNA repair activities, telomere length regulation and DNA replication (Figure 5.1). Indeed, loss of Taz1 results in a diverse range of defects that encompass these activities supporting the idea that Taz1 recruits multiple protein complexes that then mediate their specific activities.

Recently, two Taz1-interacting proteins have been described. These proteins, Rap1 and Rif1, were found through their homology to the Rap1 and Rif1 proteins in budding yeast (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). In budding yeast, Rap1 is the major telomere regulator that binds directly to the double stranded region of the telomere where it regulates many telomere functions (Shore, 2001). Rif1 interacts with Rap1 and is involved primarily with telomere length regulation (Hardy et al., 1992). Rap1 is essential in budding yeast and has dual functions in both telomere maintenance
Figure 5.1  

Telosome components in fission yeast. Diagram showing the fission yeast telomere and some of the known components. For simplicity, many functional groups are depicted without specific proteins being named. Location of these groups is meant to show general telomere function and localization and not specific interaction sites within the telomere.
and transcriptional activation (Shore, 1994). These properties have made it difficult to assess the role of Rap1 specifically in telomere end-protection. The involvement of Rif1 in telomere end-protection is still unclear. In humans, a RAP1 ortholog has been described (Li et al., 2000). Human RAP1 does not bind directly to telomeric DNA but rather localizes to telomeres through its interactions with TRF2 (Li and de Lange, 2003). hRAP1 has been shown to primarily regulate telomere length and its role in telomere capping has not been fully addressed. A human RIF1 ortholog has also recently been described (Silverman et al., 2004). Remarkably, hRIF1 was shown to not be a constituent of normal telomeres but rather to be involved in the DNA-damage-response pathway, specifically in the intra-S-phase checkpoint. Therefore, telomere proteins between budding yeast and Humans have evolved to encompass different cellular functions.

A better understanding of the function of these proteins should be obtained by their analysis in fission yeast where their homologs have been described. In fission yeast, deletion of Rap1 was shown to be almost phenotypically identical to loss of Taz1 since rap1Δ mutants displayed elongated telomeres, de-repression of the telomere position effect and lethal meiosis (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). In another clever study, a fusion protein was made between the C-terminal fragment that contains the MYB DNA binding domain of Taz1 and Rap1 (Rap1-Taz1myb). In these experiments, Rap1-Taz1myb was able to confer meiotic telomere clustering and proper meiosis (Chikashige and Hiraoka, 2001). rif1Δ mutants displayed defects in telomere length and meiotic spore viability (Kanoh and Ishikawa, 2001). Rap1 and Rif1 are independently recruited to telomeres.
through Taz1. Fission yeast Rap1 appears to be more homologous to human RAP1 since these proteins are recruited to telomeres through other proteins while the budding yeast Rap1 binds directly to duplex telomeric DNA. Together, these data support a model whereby Taz1 acts as a binding platform for Rap1 and Rif1, which perform the essential functions of telomeres.

We have shown that Taz1 is important for capping telomeres (i.e. protecting from end-joining, degradative and recombinagenic activities), surviving growth in the cold, general DNA repair and telomere replication (Chapter 3 and 4). Previous studies of fission yeast Rap1 and Rif1 have not examined their involvement in these processes. To further elucidate the role of telomeres and their associated proteins in these processes, we analyzed the involvement of other telomere proteins for these functions. These analyses should confirm or dispel the model that Taz1 plays a structural role rather than an active role at the telomere. Additionally, determining the role of Rap1 and Rif1 in telomere end-protection should enhance our understanding of the function of these proteins in both humans and budding yeast. Finally, we have developed a protein purification protocol for Taz1 that should lead to the discovery of novel proteins involved in telomere biology. We hope that these studies will help illuminate the mechanisms and pathways that telomeres orchestrate for promoting genomic stability.

5.2 Results

5.2.1 Telomere length analysis of fission yeast mutants

Taz1 is a key regulator of telomere length in fission yeast as its loss leads to a massive elongation of telomeres resulting from the deregulation of
telomerase. Rap1 and Rif1, two proteins that interact with Taz1, have also been shown to be involved in telomere length maintenance. Deletion of rap1 results in very long telomeres while deletion of rif1 has a modest effect on increasing telomere length. To study the role of these genes in promoting genomic stability in fission yeast, we created deletions of these genes in our wt strain backgrounds (JCF1) by single-step gene replacement. To confirm that these mutants behaved like the previously reported strains, we analyzed telomere lengths in these mutants. Wt cells release a major 1 kb telomere containing fragment in EcoR1 digested DNA of which ~300 bp are telomeric and the other 700 bp are subtelomeric sequences. Deletion of taz1 results in a 5-10 fold increase in telomere length. As expected, deletion of rap1 in our strain background resulted in elongated telomeres comparable to taz1 mutants while loss of rif1 lead to a small increase in telomere length compared to wt cells (Figure 5.2A). These results are with agreement to previously published results for these strains. This result, along with confirmation by PCR of the gene knockouts, showed that the rap1Δ and rif1Δ mutants were correct and allowed us to proceed in analyzing these mutants. Previously, the C-terminal 146 a.a. of Taz1 was fused N-terminally with GFP (Taz1myb) and was shown to localize to telomeres (Chikashige and Hiraoka, 2001). Another construct was made which fused Rap1 to the N-terminus of this construct to create a Rap1-gfp-Taz1myb protein (referred to as Rap1-Taz1myb, Figure 5.2B). Rap1-Taz1myb was shown to be sufficient to cluster telomeres to the SPB during meiosis while Taz1myb was not. Although this result suggests that Taz1 is dispensable for telomere clustering when Rap1 is ectopically tethered to telomeres, we wanted to ask if Taz1 was dispensable for other telomere functions such as telomere
Figure 5.2 Telomere length analysis. (A) Loss of \textit{taz1} or \textit{rap1} or the substitution of these genes with Rap1 fused to the DNA binding domain of Taz1 (Rap1-Taz1myb) results in elongated telomeres. Loss of \textit{rif1} results in a small increase in telomere length. Cells were grown in rich medium and genomic DNA was isolated and digested with EcoR1, electrophoresed on 1% agarose, blotted and probed with a telomeric oligonucleotide. (B) Diagram of Rap1-Taz1myb construct which is inserted in the \textit{lys1} locus and expressed by the inducible promoter NMT1.
length. Interestingly, Rap1-Taz1myb and Taz1myb mutants (which are both in a taz1Δrap1Δ double mutant background) have severely elongated telomeres that are identical to both rap1 and taz1 mutants (Figure 5.2 and data not shown). These results suggest that these chimeric proteins do not fully complement a taz1Δ or rap1Δ mutant for telomere length.

5.2.2 Roles for Rap1 and Rif1 in surviving cold temperatures

taz1Δ is important for viability at 20°C and its loss results in cellular elongation and chromosome missegregation (Chapter 3). To test the Taz1 platform model and to further explore the role of telomere proteins in promoting genomic stability, we analyzed the growth of rap1Δ and rif1Δ mutants in the cold. Surprisingly, rap1Δ and rif1Δ single mutants (or rap1Δrif1Δ double mutants) showed no growth defect at 20°C (Figure 5.3A and B and data not shown). This result suggests that Taz1 can function independently from Rap1 or Rif1 to survive growth in the cold and that these two telomere proteins alone are not required for survival in the cold. To explore the relationship between the loss of Taz1 and other telomere proteins, double and triple mutants were created that both lacked taz1 and other telomere proteins. Surprisingly, rap1Δ and rif1Δ mutants that lacked taz1 showed genetic interactions in the cold even though the single mutants did not. rap1Δtaz1Δ mutants exhibited extreme cold sensitivity while loss of rif1 suppressed the cold sensitivity of taz1Δ cells (Figure 5.3A and B). If rap1Δtaz1Δ mutants were cold sensitive for the same reason as taz1Δ, then deletion of rif1 in this background should also suppress the cold sensitivity. Indeed, this was found to be the case as rap1Δtaz1Δrif1Δ cells grew much better than rap1Δtaz1Δ cells suggesting a common mechanism for the
Figure 5.3 Analysis of fission yeast telomere mutants in the cold. (A) Loss of taz1 but not rap1 or rif1 results in a loss of viability at 20°C. However, rap1Δtaz1Δ mutants display severe cold sensitivity while loss of rif1 suppresses the cold sensitivity of taz1Δ mutants. Five fold serial dilutions of log phase cultures were stamped onto rich medium and incubated at the indicated temperature. (B) Loss of rif1 suppresses the elongation of taz1Δ cells in the cold. Logarithmically growing cells in rich media at 20°C are compared.
demise of these cells in the cold. Based on these data suggests a new model for Taz1 in that it can function independently from Rap1 and Rif1 since only \( taz1 \Delta \) cells are cold sensitive and the others are not. Additionally, this data suggests that Rap1 and Rif1 can function independently from Taz1 in both promoting (i.e. Rap1) and preventing (i.e. Rif1) survival in the cold when Taz1 is absent.

### 5.2.3 \textit{top2-191} suppresses the severe cold sensitivity of \( rap1 \Delta taz1 \Delta \) cells

\( taz1 \Delta \) cells exhibit entangled chromosomes at 20°C which is suppressed by \textit{top2-191}, a temperature-sensitive allele of topoisomerase II (Chapter 3). If the severe cold sensitivity of \( rap1 \Delta taz1 \Delta \) cells was due to defective Top2 activity, like in \( taz1 \Delta \) cells, then \textit{top2-191} should also suppress the cold sensitivity of \( rap1 \Delta taz1 \Delta \) cells. Indeed, \textit{top2-191} suppressed the synthetic growth defect of \( rap1 \Delta taz1 \Delta \) cells at 20°C (Figure 5.4) as well as the cell elongation (data not shown). This data suggests that Rap1, like Taz1, protects cells from a cellular process that can be suppressed or resolved by altered topoisomerase II activity in \( taz1 \Delta \) cells at 20°C and that the basis for the extreme loss of viability of \( rap1 \Delta taz1 \Delta \) cells is identical to that of \( taz1 \Delta \) cells in the cold.

### 5.2.4 Cell length of \( taz1 \Delta \) cells increases at 20°C and is suppressed by \textit{top2-191} or deletion of \textit{rif1}\( \Delta \)

\( taz1 \Delta \) cells elongate in the cold due to the activation of the G2/M DNA damage checkpoint (Chapter 3). Cellular elongation is a difficult phenotype to
Figure 5.4 *top2-191* suppresses the severe cold sensitivity of *rap1Δtaz1Δ* mutants. Experiments were performed as in Figure 5.3.
quantitate visually and differences between mutants can be missed. Therefore, we wanted to quantitate cell length in various mutants in the cold to determine precisely the increase in length that is accompanied by their growth at 20°C. Since \textit{taz1Δ} cells and other telomere mutants did not show a decrease in viability or any gross change in cell length at optimal growing conditions (32°C), cell lengths were only determined at 20°C. \textit{wt} cells grew at an average length of around 10 \textmu m at 20°C which is in accordance with the normal average length of \textit{wt} cells grown at 32°C (reference, Figure 5.5). \textit{taz1Δ} and \textit{rap1Δtaz1Δ} cells showed a large increase in cell length (17 and 18 \textmu m respectively, Figure 6.4) compared to \textit{wt}. Surprisingly, \textit{rap1Δ} cells showed a small increase in cell length compared to \textit{wt} cells (Figure 5.5). Although within the error bars of \textit{wt} cells, this data might suggest that \textit{rap1Δ} cells might be slightly cold sensitive. \textit{top2-191} cells also exhibited a slight increase in cell length compared to \textit{wt} cells (Figure 5.5). This elongation phenotype is most likely due to the lack of full activity of topoisomerase II in this temperature sensitive mutant since these cells also elongate at the permissive temperature (25°C). \textit{top2-191} or deletion of \textit{rif1Δ} suppressed the cellular elongation of \textit{rap1Δtaz1Δ} cells in the cold (Figure 5.5). Therefore, \textit{top2-191} or deletion of \textit{rif1Δ} suppress both the elongation and inviability of \textit{taz1Δ} mutants in the cold suggesting that these mutants are full suppressors of the cold sensitivity of \textit{taz1Δ} and \textit{rap1Δtaz1Δ} cells.

\textbf{5.2.5 Requirements for Taz1 in surviving growth at 20°C}

To further examine the requirements of Taz1 in surviving growth in the cold, we analyzed the cold sensitivity of Rap1-Taz1myb and Taz1myb strains. Both of these fusion proteins localize to telomeres and can regulate some telomere
Figure 5.5 *top2-191* and loss of *rif1* suppress the elongation of *taz1* mutants in the cold. Cells were maintained logarithmically in rich medium at 20°C for several days and then cell length was determined. Over 100 cells were analyzed for each strain. Error bars represent the standard deviation.
functions but not others ((Chikashige and Hiraoka, 2001), Figure 5.2A).

Surprisingly, both Rap1-Taz1myb and Taz1myb strains did not display any defect in cellular growth compared to taz1A cells at 20°C (Figure 5.6). These data suggest the C-terminal 167 a.a. of Taz1 is sufficient to confer survival for taz1A cells at 20°C.

5.2.6 Swi6 is required for growth in the cold but acts independently from Taz1

Swi6 is a heterochromatin protein that localizes to telomeres and is required for telomeric silencing (Ekwall et al., 1995). To assess its role in surviving cold temperatures, we analyzed its growth in the cold. We found that swi6A mutants exhibited a loss of viability when cultured in the cold (Figure 5.6). However, we found that deletion of swi6A only had an additive effect on the growth defect of taz1A cells at 20°C (Figure 5.6). Therefore, we conclude that Swi6 is not involved in the same pathway as Taz1 in regards to cold sensitivity.

5.2.7 Telomere requirements for surviving double strand breaks

We have previously shown that Taz1 is required to survive treatments that induce double strand breaks (Chapter 3). This fact prompted us to determine the role of other telomere proteins in surviving DNA damage. For this examination, various telomere mutants were plated onto rich media plates with or without MMS, a drug that alkylates DNA resulting in DNA damage, including DSBs. Like taz1A cells, both rap1A and rif1A cells exhibited sensitivity to high concentrations of MMS (Figure 5.7). However, the sensitivity of both of these...
Figure 5.6 Requirements of Taz1 for survival at 20°C. Rap1-Taz1myb and Taz1myb are sufficient to suppress the cold sensitivity of taz1Δ mutants in the cold. Experiments were preformed as in Figure 5.3.
mutants was notably less than \textit{taz1A} cells. Epistasis analysis revealed that the sensitivity to MMS was additive in \textit{rap1Arif1A} cells since the sensitivity was more severe in the double mutant compared to either single mutant alone. However, we observed the drug sensitivity of \textit{rap1Ataz1A} and \textit{rif1Ataz1A} cells to be identical to \textit{taz1A} cells. The most sensitive strain examined was \textit{rap1Arif1Ataz1A}, which also showed an additive effect when compared to the double mutants (Figure 5.7). Therefore, we conclude that Rap1 and Rif1 play separate, minor roles in general DNA repair that define two independent pathways that are both dependent on Taz1. However, since the triple mutant is more sensitive than either double mutant, this suggests that both Rap1 and Rif1 can function independently from Taz1.

We have shown that \textit{trt1A} circular survivors that lack telomeres are very sensitive to DNA damage (Chapter 3). Additionally, loss of Taz1 in these cells did not exacerbate the sensitivity to DNA damage suggesting that Taz1 functions via telomeres to promote DNA repair. To ascertain if Rap1 and Rif1 functioned through telomeres, we created \textit{rap1A} and \textit{rif1A trt1A} circular survivors and compared their viability to MMS to both \textit{trt1A} circular survivors and \textit{trt1Ataz1A} circular survivors. Additionally, we created a new \textit{trt1Ataz1A} circular survivor by deleting \textit{taz1} with the \textit{kan} gene in an already circular \textit{trt1A} strain. Since \textit{trt1A} circular survivors are sick and display chromosome instability, we wanted to analyze the immediate loss of Taz1 in this strain rather than an older strain which might have been selected for, masking a potential role for Taz1 in the survival of circular strains to DNA damage. However, both \textit{trt1Ataz1A} circular strains showed identical sensitivity to MMS, suggested that Taz1 functions through telomeres to promote recovery from DNA damage.
Figure 5.7 Drug sensitivity of telomere mutants in fission yeast. Loss of \textit{taz1}, \textit{rap1} or \textit{rif1} confers sensitivity to MMS although \textit{taz1}\Delta mutants display the greatest sensitivity. Epistasis analysis suggests that the drug sensitivity of these mutants are independent from each other since the double and triple mutants show additive effects towards drug sensitivity. Five fold serial dilutions of log-phase cultures were plated on YES without or with the indicated concentration of MMS and grown at 32°C.
(Figure 5.8 A and B). Like deletion of Taz1 from either \textit{trt1}Δ circular survivor strains, deletion of Rap1 or Rif1 had no effect on the already severe drug sensitivity of \textit{trt1}Δ circular survivors that we had previously reported (Figure 5.8 A and B and Chapter 3). Wt cells did not show a significant drop in viability when subjected to these levels of MMS (Chapter 3 and data not shown). Therefore, like Taz1, these results suggest that both Rap1 and Rif1 act via their association with telomeres.

**5.2.8 Taz1 is specifically required for surviving Hydroxyurea**

We have previously showed a requirement for Taz1 is surviving hydroxyurea (HU), a drug that inhibits ribonucleotide reductase and causes cells to arrest in S-phase (Chapter 3). This data suggested that Taz1 was required specifically in S-phase. To assess the possible role of other telomere proteins in S-phase, we analyzed the sensitivity of various telomere mutants to HU. At 32°C, \textit{rap1}Δ cells did not exhibit sensitivity to HU when compared to wt cells (Figure 5.9). Additionally, loss of Rap1 or Rif1 had no effect on the sensitivity of \textit{taz1}Δ cells to HU. Thus, \textit{taz1}Δ cells appear to be uniquely defective in some aspect of S-phase compared to other telomere mutants suggesting Taz1 is specifically required to survive HU. These data might be explained by the central role that Taz1 plays in telomere replication, an event that occurs primarily in S-phase.

**5.2.9 Requirements for the survival of DNA damage in \textit{taz1}Δ cells**

To further examine the requirements of Taz1 in surviving DNA damage, we
Figure 5.8 The role of telomere proteins in surviving DNA damage requires telomeres. (A) Loss of *taz1*, *rap1* or *rif1* confers sensitivity to MMS in strains that contain telomeres. However, loss of these proteins in strains that do not contain telomeres has no effect. Five fold serial dilutions of log-phase cultures were plated onto YES plates without or with the indicated concentrations of MMS and grown at 32°C. (B) Quantification of the drug sensitivity of telomere mutants. Cells were grown logarithmically and 300 cells were plated on rich media that either contained various amounts of MMS or no drug. Plates were grown at 32°C for 3 days. Colonies were counted and the ratio between colonies formed on MMS versus no drug control plates is plotted. Together, these results suggest that Taz1, Rap1 and Rif1 function through telomeres to promote proper DNA repair and survival from DNA damage.
Figure 5.9 HU sensitivity of telomere mutants in fission yeast. Loss of \textit{taz1} specifically confers sensitivity to the replication inhibitor hydroxyurea (HU). Five fold serial dilutions of log-phase cultures were plated on YES without or with the indicated concentrations of HU and grown at 32°C.
analyzed the drug sensitivity of Rap1-Taz1myb and Taz1-myb strains. At low concentrations of MMS, both Rap1-Taz1myb and Taz1-myb were able to suppress the drug sensitivity of \textit{taz1A} cells (Figure 5.9). However, this suppression was not complete since at higher drug concentrations (i.e. .008% MMS), Taz1myb, and to a lesser extent, Rap1-Taz1myb strains lost viability compared to wt cells (Figure 5.9). Thus, Rap1-Taz1myb, and to a lesser extent Taz1-myb, are able to partially fulfil the requirements of Taz1 in surviving DNA damage.

Since altered topoisomerase II activity (i.e. \textit{top2-191}) suppresses the cold sensitivity of \textit{taz1A} cells, we asked if \textit{top2-191} could also suppress the drug sensitivity of \textit{taz1A} cells. \textit{top2-191} had no effect on the drug sensitivity of various \textit{taz1A} mutants (Figure 5.10). Therefore, these data suggests that altered topoisomerase II activity is not the basis for the drug sensitivity of \textit{taz1A} cells and that the cause of the cold and drug sensitivity of \textit{taz1A} cells is mechanistically different.

We found that the loss of Swi6 did not affect the cold sensitivity of \textit{taz1A} cells (Figure 5.6). However, we wondered if heterochromatin could play a role in survival of DNA damage in fission yeast. We found that \textit{swi6A} cells were more sensitive to MMS than wt cells (Figure 5.10). We also observed that \textit{swi6Ataz1A} mutants were more sensitive than \textit{swi6A} mutants alone (Figure 5.10). This result suggests that Swi6 plays some role in the survival of cells after DNA damage but this role appears to be independent from Taz1.
Figure 5.10 Genetic requirements for the drug sensitivity of \textit{taz1}Δ mutants. Rap1-myb and Taz1-myb are sufficient to suppress the drug sensitivity of \textit{taz1}Δ mutant at low MMS concentrations. Top2-191 had no effect on the drug sensitivity of \textit{taz1}Δ cells. Interestingly, loss of \textit{swi6} resulted in drug sensitivity. Experiments were performed as in Figure 5.7.
5.2.10 Taz1 does not require DNA binding to survive DNA damage or growth in the cold

A single amino-acid substitution in Taz1 that abolishes the ability of Taz1 to function has previously been described (Nimmo et al., 1998a). This Taz1 mutant, called A606V, has an alanine to valine substitution at amino acid 606, which lies in the DNA MYB domain of Taz1. Previous experiments in the lab have shown that this protein is unable to bind telomeric DNA in vitro, had elongated telomeres and did not localize to telomeres in vivo unless overexpressed (Atul Deshpande and Julia Promisel Cooper, unpublished results). It was also originally described to have a defect in telomere silencing and meiotic telomere clustering (Nimmo et al., 1998a). The availability of this mutant version of Taz1 prompted us to ask if this mutant was drug or cold sensitive. For analysis, we obtained the original strain where this allele was isolated that contained the A606V substitution in Taz1 (strain 2114). In this strain, we knocked out the entire \textit{taz1} gene so that we could compare isogenic strains. Surprisingly, the deletion of Taz1 in the A606V background affected both the drug and cold sensitivity of this strain. We found that loss of Taz1 resulted in both cold and drug sensitivity compared to the A606V strain (Figure 5.11). Compared to wt strains, A606V showed some sensitivity to both MMS and growth in the cold (data not shown). Additionally, A606V cells became elongated in the cold even though a major drop in viability was not observed (data not shown). Therefore, these results suggest the Taz1 protein containing the A606V mutation is able to partially suppress both the cold and drug sensitivity of \textit{taz1}\textDelta{} cells. This is a very interesting result since it suggests that
Figure 5.11 Taz1-A606V is sufficient to suppress the drug and cold sensitivity of taz1Δ cells. Strain 2114 is the original strain used in the mutagenesis screen to identify Taz1A606V. From this strain, the full length taz1 gene was deleted by single-step gene replacement (material and methods). The two, isogenic strains (one containing full length Taz1 with an A606V a.a. change and the other a complete knockout of taz1) were used for comparisons. Experiments were performed as in Figure 5.3 and 5.7.
Taz1 is able to partially function in both DNA repair and surviving the cold that is independent from its DNA binding, telomere length and telomere chromatin structure.

5.2.11 3’ single strand overhang formation in various telomere mutants in fission yeast

Normal telomeres end with a short single stranded 3’ overhang. The size of normal 3’ overhangs is unknown in fission yeast but in budding yeast, the size has been calculated to be around 12-14 bases in G1 and longer in S-phase (Larrivee et al., 2004). In fission yeast, loss of Taz1 results in the deregulation of 3’ end formation which results in the strong detection of single-stranded 3’ overhangs that are formed by the MRN complex (Tomita et al., 2003). The presence of unprotected, single stranded 3’ overhangs might contribute to the drug and cold sensitivity of \( \text{taz1} \Delta \) cells. Unprotected single-stranded telomeric DNA has been shown to activate DNA damage checkpoints and this could help explain the checkpoint activation that occurs in \( \text{taz1} \Delta \) cells when grown in the cold (Garvik et al., 1995; Maringele and Lydall, 2002). If 3’ overhangs were specifically contributing to the demise of \( \text{taz1} \Delta \) cells, we predicted that long single-stranded 3’ overhangs would not occur in \( \text{rap1} \Delta \) cells or other telomere mutants that are not cold sensitive. To test our hypothesis and to further elucidate the role of other telomere proteins in 3’ telomere end formation, we determined the presence of telomeric 3’ single-strand overhangs in various mutants at both 32°C and 20°C. As previously reported, G-strand overhangs were undetectable in wt cells while \( \text{taz1} \Delta \) cells displayed an intense, heterogeneous signal: indicating a deregulation of 3’ telomere end formation.
(Tomita et al., 2003) and Figure 5.12. Interestingly, loss of Rap1 but not Rif1 resulted in the detection of G-strand overhangs (Figure 5.12). Like \textit{taz1}\textDelta cells, Rap1-Taz1myb and Taz1myb cells have long G-strand overhangs (Figure 5.12). For all strains analyzed, temperature did not change the qualitative detection of G-strand overhangs as the signal detected was similar in all strains with detectable G-strand overhangs when compared to the total amount of telomeric DNA at both temperatures (Figure 5.12, compare top and bottom gel). Thus, deregulation of G-strand overhang formation does not correlate with cold sensitivity suggesting long single-stranded telomeric DNA is not the basis for the cold sensitivity of \textit{taz1}\textDelta cells. The fact that \textit{rif1}\textDelta\textit{taz1}\textDelta still has detectable G-strand overhangs at both temperatures suggests again that unprotected 3’ single-strand overhangs do not trigger the entangled chromosomes and cold sensitivity in \textit{taz1}\textDelta cells. Additionally, we find that Rap1, like Taz1, regulates G-strand overhang formation while Rif1 does not.

Taz1 is also important in protecting telomeres from NHEJ-dependent telomere fusions (Ferreira and Cooper, 2001a). To analyze the role of other telomere proteins in capping telomeres and preventing fusions, we subjected \textit{rap1}\textDelta and \textit{rif1}\textDelta mutants to nitrogen starvation, a condition resulting in telomere fusions in \textit{taz1}\textDelta cells. We find that deletion of Rap1 results in telomere fusions while deletion of Rif1 does not (Miguel Ferreira and Julia Promisel Cooper, unpublished results). We also found that Rap1-Taz1myb did not prevent telomeres from fusing under these conditions. Thus, we conclude that Rap1 is a major factor in both protecting telomeres from fusion and regulating end structure (i.e. 3’ G-strand), two important processes that cap telomeres and provide end-protection. Our data shows that Rif1 is not involved in these
Figure 5.12 3' overhang analysis of telomere mutants. Loss of *taz1* or *rap1* results in long 3' telomere overhangs. Rap1-myb and Taz1-myb strains also exhibit long 3' overhangs. Growing temperature does not appear to affect the formation of 3' overhangs in these mutants. Cells were grown at either 32°C or 20°C, DNA isolated and subjected to an in-gel telomere overhang protocol. Non-denatured gels were first probed with a c-strand specific probe (which detects the 3' G-strand overhang) and then denatured and probed with a total (C- and G-strand probes) telomere probe to detect total telomeric DNA.
processes and therefore is not a major protein involved in telomere capping.

5.2.12 Purification of Taz1-HA and analysis of Taz1-interacting proteins

We have studied the involvement in telomere capping of two Taz1-interacting proteins, Rap1 and Rif1. Our data suggests that there could be additional Taz1-interacting proteins that regulate the functions of Taz1 that are not dependent on Rap1 or Rif1 (i.e. telomere replication and cold sensitivity). To identify additional Taz1-interacting proteins, we set out to purify Taz1 using immunoaffinity purification techniques followed by mass spectrometry analysis to identify any Taz1 binding partners. To begin, we utilized a previously characterized c-terminally tagged Taz1 with 3XHA at its endogenous genomic location so that we could purify endogenously expressed Taz1-HA from fission yeast cells. Previous analysis of Taz1-HA showed that this protein was fully functional suggesting that any proteins identified using this construct would be bona fide Taz1-interacting partners (data not shown). First, we wanted to do a proof-of-principle experiment by seeing if we could co-IP known telomere proteins with Taz1-HA. A good candidate for this experiment was Swi6 since this protein has been localized to telomeres and plays a role in telomere heterochromatin formation, a function shared with Taz1 (Cooper et al., 1997a; Ekwall et al., 1995). To test if Taz1-HA can co-IP Swi6, we made a strain that contained both Taz1-HA and Swi6-GFP. Western blot analysis of whole cell extracts showed that both proteins were easily detectable using the appropriate antibodies (Figure 5.13). Immunoprecipitation analysis revealed that Taz1-HA can co-IP Swi6-GFP and vice versa (Figure 5.13A). This data suggests that
Figure 5.13 Purification of Taz1 from fission yeast. (A) Taz1-HA co-IPs Swi6-GFP. Arrows denote the two forms of Taz1 and the asterisks shows a HA immunoreactive background band. (B) Swi6-GFP co-IPs Taz1-HA. Arrows denote Swi6-GFP. A strain containing Taz1 c-terminally tagged with HA and Swi6 tagged with GFP was used. Cells were grown logarithmically and extracts were made and analyzed either by IP followed by western blotting or just western blotting for whole cell extracts using anti-HA (A) or anti-GFP. (C) Taz1-HA was purified using an HA immunoaffinity column. Both untagged (control strain) and Taz1-HA cells were subjected to the purification protocol and purified extracts were analysed by SDS-PAGE and visualized by coomassie blue staining.
Taz1 interacts with Swi6, a fact not surprising given their roles in telomere heterochromatin formation. This experiment shows that immunoprecipitation analysis can pull-down Taz1-interacting proteins which suggests that this technique could be successful in identifying novel telomere proteins that interact with Taz1.

To identify novel telomere proteins, we utilized a previously developed technique for single-step immunoaffinity purification of 3XHA tagged proteins that has been used to identify interacting proteins (see material and methods). Indeed, this technique was successful in purifying Taz1-HA that was clearly visible on a coomassie-blue stained gel when compared to an untagged strain control purification (Figure 5.13B). Unfortunately, no additional bands were visible in the Tazi-HA lane when compared to the control lane (Figure 5.13B). This data suggests that we were unable to purify any interacting proteins that were stoichiometric with Taz1. However, Taz1 is the only known binder of telomeres so other telomere proteins might not be expressed at the same levels as Taz1. Additionally, Taz1 exists in two forms with the full length larger form being expressed 3X more than the lower form which is produced through internal translation initiation (Kyle Miller and Julia Cooper, manuscript in preparation). In this experiment, only the upper, more highly expressed form, of Taz1 was visible, suggesting that any proteins that are less highly expressed than Taz1 might not be visible under these conditions. Western blotting of this extract did confirm that both forms of Taz1 were purified which supports the afore mentioned conclusion (data not shown). Regardless, we have developed a technique that is able to purify large quantities of endogenous levels of Taz1-HA that are visible on a coomassie-blue stained gel. With some optimization,
this technique should be useful in the identification of additional, novel Taz1-interacting proteins.

5.3 Discussion

5.3.1 Telomeres are required to promote genomic stability

5.3.1.1 Requirements of telomeres in surviving growth in the cold

While taz1A cells exhibit an activated DNA damage checkpoint, broken and entangled chromosomes in the cold, the requirement of other telomere proteins in inhibiting these defects was unknown. Here we show that the loss of Rap1 or Rif1 does not result in cold sensitivity, pointing to a central role for Taz1 in inhibiting the telomere dysfunction that causes the observed defects in taz1A cells grown in the cold. However, in cells that lack Taz1, loss of Rap1 exacerbates the cold-specific aberrations observed in taz1A cells while loss of Rif1 actually results in a diminution of these aberrations (Figure 5.3). Thus, Rap1 and Rif1 can function independently from Taz1 to both help and hinder, respectively, the survival of taz1A cells in the cold.

Our previous studies of the cold sensitivity of taz1A cells has been limited to the analysis of complete taz1+ knockout cells. To gain further insights into the specific requirements of Taz1 in promoting growth in the cold, we utilized a previously reported point mutant and truncations of Taz1 to see if we could better define the activity or region of Taz1 that was involved in cold sensitivity. Interestingly, the point mutant A606V of Taz1, which abolishes the DNA binding activity of the protein, can partially protect cells from cold
sensitivity (Figure 5.6 and 5.11). A N-terminal truncation of three-quarters of Tazi that preserves its DNA binding activity is sufficient to allow for normal growth of these cells in the cold. These data suggest that the C-terminal portion of Tazi is required for growth in the cold and DNA binding activity is not wholly necessary for this function.

### 5.3.1.2 Requirements for telomeres in DNA repair

Loss of Tazi results in the hypersensitivity of cells to treatments that induce DNA damage (Chapter 3). Thus, proper telomere function is required for the repair of damaged DNA in fission yeast. If Rap1 and Rif1 are delocalized in a \( taz1A \) mutant and function completely through Tazi, deletion of these genes should have no effect on drug sensitivity in a \( tazi' \) background. Interestingly, deletion of both Rap1 and Rif1 resulted in hypersensitivity to MMS in Tazi-containing cells and loss of these proteins in a \( taz1A \) mutant resulted in the same sensitivity as \( taz1A \) cells (Figure 5.7). These data suggest both Rap1 and Rif1 play minor roles in DNA repair and function with Tazi but in independent, separate pathways. However, we found that Tazi was specifically required for survival from HU, a drug that inhibits S-phase by nucleotide depletion resulting in stalled replication forks that halt S-phase and which can be processed into damaged DNA (Figure 5.9). Thus, proper telomere functions are necessary to survive DNA damage in fission yeast and Tazi might be uniquely required when DNA damage results in the perturbation of S-phase.

We wondered if, like cold sensitivity, mutations that partially disrupt the function of Tazi could still promote DNA repair. Again, like cold sensitivity, the drug sensitivity of \( taz1A \) cells was partially suppressed by Taz1-A606V and
Taz1myb (Figure 5.6 and 5.10). The tethering of Rap1 to Taz1myb (Rap1-myb) gave an even greater suppression of the drug sensitivity in \( taz1\Delta \) cells, again supporting the conclusion that Rap1 is involved in the survival from DNA damage. Again, these mutants of Taz1 are able to partially function with respect to surviving DNA damage, suggesting that the C-terminus of Taz1 is important in this function and that Taz1 can partially function independently of its DNA binding.

### 5.3.1.3 Requirements of telomere proteins for telomere length maintenance, end-protection and 3’ single strand overhang formation

Telomere length homeostasis is believed to be achieved through the action of multiple proteins that regulate telomere states that are either extendable or non-extendable by telomerase (Smogorzewska and De Lange, 2004). Taz1 is a key negative regulator of this process as its loss leads to the deregulation of telomerase and extremely long telomeres. Deletion of Rap1 also lead to the same extent of telomere lengthening seen in \( taz1\Delta \) cells suggesting a common pathway of telomere length regulation for these proteins. Surprisingly, we found that Rap1 tethered to the telomere via the DNA binding domain of Taz1 or the DNA binding domain of Taz1 alone was insufficient to regulate telomere length and resulted in the same long telomere phenotype seen in either \( taz1\Delta \) or \( rap1\Delta \) cells. Although the Rap1-Taz1myb construct recapitulates the requirements for telomeres for telomere clustering in meiosis, we conclude that additional parts of Taz1 (i.e. the N-terminal 493 a.a.) are required for telomere length homeostasis and that Rap1 is required but not
sufficient to regulate this process at the telomere. Additionally, the DNA binding of Taz1 is essential for telomere length regulation as the A606V mutation results in identical telomere lengthening as seen in taz1Δ cells (data not shown). As previously reported, we also found that Rif1 was required to regulate telomere length, although to a lesser extent than either Taz1 or Rap1.

An essential function of Taz1 is to protect telomeres from NHEJ reactions that can result in lethal telomere fusions (Ferreira and Cooper, 2001a; Tuzon et al., 2004). Our lab has found that Rap1, but not Rif1, is required to prevent telomere fusions in G1 arrested cells (Ferreira and Cooper, unpublished data). We also found that tethering Rap1 to the telomere via Rap1-Taz1myb was insufficient to prevent telomere end fusions. This result, like that for telomere length, might suggest that additional proteins that interact with the N-terminal region of Taz1 are also required to cap telomeres and that Rap1 is required but not sufficient to inhibit telomere end fusions.

Taz1 is required to regulate 3’ telomere overhang regulation as its loss leads to long, deregulated 3’ G-rich overhangs. We found the same genetic requirements for 3’ overhang formation as for telomere end-protection from fusions. Therefore, Rap1, along with Taz1, appears to play a critical role in orchestrating the degradative and replicative processes that accompany 3’ overhang formation. Since telomere length, telomere end-protection and 3’overhang formation share similar genetic requirements, it is tempting to speculate that the underlying mechanism for these defects are related. We propose that Taz1, along with Rap1, regulate and form a non-extendable telomere state that protects the chromosome ends from being treated as a DSB by the NHEJ machinery and nucleases which can lead to both telomere fusions
and deregulated, long, 3’ overhangs. Unprotected 3’ overhangs can also act as substrates for telomerase addition leading to deregulated telomere length homeostasis. Since Rap1-Taz1myb exhibit the same defects as either taz1Δ or rap1Δ cells, we speculate that additional proteins may be involved in regulating this closed telomere state. In human cells, Pot1 has been shown to regulate telomere length homeostasis through its interactions with TRF1 (Loayza and De Lange, 2003). Therefore, in fission yeast, Pot1 might ultimately be required to regulate telomere length and end protection through its interactions with both Taz1 and Rap1. Further analysis will be required to better understand the molecular basis for telomere length regulation and telomere end-protection in fission yeast.

5.3.2 Potential bases for the role of telomeres in surviving growth in the cold

We have previously shown that telomere dysfunction resulting from the loss of Taz1 causes entangled chromosomes, checkpoint activation and a unique requirement for altered Top2 activity to survive growth in the cold. What aspect then of telomere dysfunction results in these cold-specific defects? Surprisingly, loss of Rap1 does not result in cold sensitivity, even though taz1Δ or rap1Δ cells share many telomere defects. Specifically, loss of these proteins both lead to deregulated telomere lengthening, deregulated 3’ overhang formation and susceptibility to NHEJ dependent telomere fusions. Based on these results, the causative defect resulting in the observed cold sensitivity of taz1Δ cells appears to be independent from these aspects of telomere dysfunction. Thus, the observed phenotypes of taz1Δ cells are not a result of
long, unprotected telomeres, aberrant telomere chromatin structure, deregulated 3’ overhang formation or telomere fusions. These results point to a central role for Taz1 in inhibiting chromosomal entanglement and cold sensitivity that is independent from Rap1 or Rif1. What telomere deficiency then could lead to the observed cold sensitivity phenotypes specifically in \textit{taz1A} cells? Since the cold-specific defects originate in S-phase, replication defects are likely to be the \textit{raison d’etre} for the observed cold sensitivity when \textit{taz1} is deleted. We suggest that the underlying mechanism of telomere dysfunction that results in cold sensitivity is the aberrant telomere replication caused by the loss of Taz1 (Chapter 4). Interestingly, we found that the C-terminal 146 a.a. of Taz1 (Taz1myb) was able to partially suppress the cold sensitivity. These data suggest that the binding of Taz1 to telomeres is sufficient to allow both proper replication and/or resolution of replication structures that could be the cause of the cold sensitivity in \textit{taz1A} cells. Perhaps counterintuitively, we also found that a mutant form of Taz1 that does not bind telomeres can partially suppress the cold sensitivity of \textit{taz1A} cells. Taz1-A606V cells are still elongated in the cold and have a loss of viability but this is less than \textit{taz1A} cells. These results suggest that Taz1 is required at two steps, one dependent on DNA binding which suppresses the DNA damage and loss of viability and another step which does not require DNA binding that is involved perhaps in the resolution and survival of the DNA damage that occurs when Taz1 can not bind directly to telomeric DNA.

The cold sensitivity of \textit{taz1A} cells is exacerbated by the loss of Rap1. How might the absence of this protein affect the cold sensitivity when Taz1 is lost? It has been reported that the localization of both Rap1 and Rif1 were
dependent on Taz1 (Kanoh and Ishikawa, 2001). In another study, Rap1 was found to still localize to telomeres but inefficiently in a taz1Δ mutant (Chikashige and Hiraoka, 2001). Our results suggest that Rap1 is still able to function at telomeres independently from Taz1. Since the same altered Top2 activity can suppress the severe cold-induced lethality of rap1Δtaz1Δ cells, this data suggests that the mechanism of cold sensitivity in these cells is the same as in taz1Δ cells (Figure 5.4). Therefore, the reduced localization of Rap1 to telomeres in taz1Δ cells appears to be sufficient to partially protect cells from cold-specific defects and recovery from DNA damage. In the absence of Taz1, Rap1 might still be able to organize telomeric complexes that are required to survive these stresses. Since Rap1 mutants are required for survival of DNA damage independently from Taz1, Rap1 might function in the recovery pathway after chromosomal entanglement occurs which is more specifically caused by the loss of Taz1. We have shown that rap1 mutants do not display major replication defects in subtelomeric sequences whilst taz1 mutants do (Chapter 4). Therefore, the loss of Taz1 might lead to chromosomal entanglement caused by stalled replication forks independently of Rap1. Loss of Rap1 alone does not result in cold sensitivity or subtelomeric replication fork pausing supporting this hypothesis. Thus, Rap1 might facilitate resolution of entangled taz1Δ chromosomes, perhaps through its interactions with repair or resolving proteins (i.e. Rad22 or Rqh1).

The cold sensitivity of taz1Δ cells is suppressed by the loss of Rif1. How might Rif1 function in the demise of taz1Δ cells in the cold? Rif1 was shown to localize diffusely in wt or taz1Δ cells, although its localization to the telomere by CHIP analysis was dependent on Taz1 (Kanoh and Ishikawa, 2001).
Interestingly, Rif1 was shown to localize as foci to telomeres in rap1Δ cells. This result has been interpreted to mean that Rif1 might localize to dysfunctional telomeres, like human RIF1 (Silverman et al., 2004). However, our data suggest that taz1Δ telomeres are even more dysfunctional than rap1Δ telomeres and since Rif1 does not localize to taz1Δ telomeres, this strongly suggests that fission yeast Rif1 is not involved in the DNA damage response in the same manner as human RIF1. Unlike human RIF1, fission yeast Rif1 does not localize as foci in MMS treated cells supporting our conclusion that Rif1 is not involved in the cellular DNA damage response (data not shown). Although it has been shown that Rap1 and Rif1 bind to Taz1 independently from each other, the increased localization of Rif1 to rap1Δ telomeres might simply occur from a competition between Rap1 and Rif1 for binding to Taz1 rather than a localization to dysfunctional telomeres. Our data suggest that free Rif1 is detrimental to taz1Δ or rap1Δtaz1Δ cells when grown in the cold. Our results also indicate that Rif1 does not play a major role in the DNA damage response in fission yeast. Therefore, we propose that Rif1 has a novel function at telomeres that when delocalized from telomeres in the absence of Taz1, results in the cold sensitivity of taz1Δ cells. Genetic analysis has shown that both recombination and topoisomerase activity are essential for the survival of taz1Δ cells (Chapter 3). Therefore, the suppression of the cold sensitivity when Rif1 is deleted from taz1Δ cells can readably be understood if Rif1 was a negative regulator of either homologous recombination, resolution of intertwined chromosomes or fork resolution/restart activities. Interestingly, budding yeast Rif1 was shown to be associated with telomeres when Rap1, the protein that binds directly telomeric DNA, was not associated (Smith et al., 2003).
extension then, fission yeast Rif1 might interact with telomeres independently from Taz1. Since Rif1 is a negative regulator of telomere length, Rif1 is predicted to keep telomeres in a closed confirmation, inhibiting telomerase access to the telomere. This protective confirmation might also inhibit other activities from acting at the telomere. For \textit{taz1A} cells, Rif1 could inhibit homologous recombination and/or proper Top2 activity from gaining access to the telomere DNA which could lead to the observed cold-specific defects in these cells. Budding yeast Rif1 was also shown to be maximally associated with telomeres in G2/M, a time when Top2 is predicted to be most active and telomerase inactive (Smith et al., 2003). Perhaps in \textit{taz1A} cells, the loss of Rif1 suppresses the cold sensitivity through the action of normal Top2 activity that can access the telomere specifically when \textit{rif1} is deleted to disentangle the chromosomes. Rif1 has been shown to interact with the sequences of telomere related genes in budding yeast, raising the possibility that it might regulate their expression (Smith et al., 2003). Taken together, these studies suggest that Rif1 is involved in many complex biological functions that have not been fully realized. Additional studies on both the genetic interactions of Rif1 with other genes and the discovery of additional Rif1 interacting proteins should shed light on the function of this poorly understood telomere associated protein.

A N-terminal truncated version of Taz1, Taz1myb, was sufficient to allow efficient growth of \textit{taz1A} cells in the cold (Figure 5.6). This form of Taz1 retains its ability to bind to telomeric DNA although it has lost its ability to regulate telomere length, end-protection and 3’ end formation (Figure 5.12 and data not shown). Therefore, the binding of telomeric DNA appears to be an important requirement for Taz1 in promoting growth in the cold. However, we also found
that a version of Taz1 mutated in the MYB DNA binding domain that abolishes telomeric DNA binding, Taz1-A606V, is also sufficient to partially suppress the cold sensitivity of \( taz1\Delta \) cells (Figure 5.11). This data appears contradictory with the Tazi myb data that suggests DNA binding is essential for protection from growth in the cold. However, the Taz1-A606V mutation is a partial suppressor of the cold sensitivity and cellular elongation and cell death is still observed in these cells when grown in the cold. We propose that Taz1myb is sufficient to allow proper replication and resolution of telomeres in the cold and that Taz1-A606V is insufficient to allow proper replication of telomeres but can promote the resolution of aberrant replication intermediates which leads to a partial suppression of the cold sensitivity of \( taz1\Delta \) cells. Thus, this data supports our hypothesis that Taz1 is required to both promote replication fork progression through telomeres and to resolve any aberrant replication intermediates which if left unresolved, could lead to entangled chromosomes and the observed cold sensitivity of \( taz1\Delta \) cells. Since Taz1-A606V is only a point mutant, this form of Taz1 might still efficiently form telomeric complexes through protein-protein interactions with an as yet unidentified telomere associated protein that would help protect telomeres from cold-specific defects. Additionally, Taz1-A606V might sequester free Rif1 molecules away from the telomere, which would be equivalent to a \( rif1\Delta taz1\Delta \) cell, a condition we known experimentally that suppresses the cold sensitivity of \( taz1\Delta \) cells.

Heterochromatin is believed to be involved in many cellular processes including transcription, mating-type switching, centromere function and meiotic telomere clustering (Ekwall et al., 1995; Tuzon et al., 2004). The role of heterochromatin in telomere functions is not well understood. In human cells,
inhibition of Tankyrase, a poly (ADP-ribose) polymerase (PARP) involved in
telomere function, results in mitotic arrest and anaphase bridging, defects that
are very reminiscent of the events that occur in taz1Δ cells when grown in the
cold (Dynek and Smith, 2004). Telomere end-fusions are not the cause of these
defects in Tankyrase knockdown cells prompting the authors to propose the
involvement of a telomere-specific cohesion in the maturation of these mitotic
abnormalities. In their model, Tankyrase is required to cleave telomere
cohesion to allow proper mitosis. If Tankyrase is inhibited, telomeres remain
associated through uncleaved cohesion leading to the activation of a mitotic
checkpoint which eventually is overridden resulting in anaphase bridging and
chromosomes missegregation. Since taz1Δ cells grown in the cold and
Tankyrase inhibited cells share many phenotypic aberrations, we analyzed the
role of cohesion and heterochromatin in the cold sensitivity of taz1Δ cells. Swi6
is required for heterochromatin formation and silencing at telomeres, mating-
type loci and centromeres and when deleted, results in decreased cohesion
loading at centromeres and the mating-type loci (Ekwall et al., 1995; Nakayama
et al., 2000; Nonaka et al., 2002). Although it has not been analyzed, we
hypothesized that Swi6 might also be involved in cohesion loading at the
telomere. Therefore, if excessive cohesion resulted in the cold sensitivity of
taz1Δ cells, deletion of Swi6 should alleviate the cold-specific defects in taz1Δ
cells. swi6Δ cells did show a minor decrease in viability when cultured in the
cold (Figure 5.6). However, we found that loss of Swi6 had no additional effect
on the cold sensitivity of taz1Δ cells (Figure 5.6). Swi6 has been shown to bind
to subtelomeric regions independently from Taz1 and it has been hypothesized
that it could be involved in subtelomeric heterochromatin formation (Sadaie et
Therefore, we cannot rule out that taz1-independent subtelomeric heterochromatin plays a role in protecting cells from growth in the cold since we did observe a drop in viability in swi6Δ cells. However, the more likely explanation for the observed cold sensitivity in swi6Δ mutants is its role in centromere function, which is defective and has been linked to an increase in chromosome missegregation, a defect that is predicted to be exacerbated in the cold due to destabilized microtubules. Although not conclusive, our data suggests that excessive telomere cohesion does not contribute to the cold sensitivity of taz1Δ cells. In budding yeast, a telomere specific cohesion mechanism has been described (D'Amours et al., 2004). The involvement of cohesion and heterochromatin at telomeres in fission yeast requires further analysis to determine its role in various telomere functions and to completely rule out its involvement in the cold sensitivity of taz1Δ cells.

5.3.3 Potential role for telomeres in DNA repair

We have previously shown that Taz1 is involved in proper DNA repair and have discussed the possible significance of telomeres in the repair of DNA damage (see Chapter 3 discussion). Our finding that the loss of Rap1 and Rif1 confer sensitivity to MMS might not seem surprising since loss of these genes lead to telomere dysfunction. Since the localization of Rap1 and Rif1 was thought to be dependent on Taz1, loss of these genes should be epistatic with the loss of Taz1 for the DNA damage response. Indeed, our data suggests that Rap1 and Rif1 function in the survival of DNA damage in a Taz1-dependent manner. However, since the triple mutant is more sensitive than the taz1Δ mutant alone, this suggests that there are multiple pathways at work to survive
DNA damage. Since the loss of these genes from circular strains does not exacerbate their sensitivity to DNA damage, this data suggests the Rap1 and Rif1 function via telomeres to assist cells in the recovery from DNA damage. Since, like taz1Δ mutants, DNA damage checkpoints appear to be fully functional in these mutants (data not shown). In human cells, the MRN complex has been shown to localize to telomeres through TRF2, the homologue of Tazi (Zhu et al., 2000). In fission yeast, the MRN complex has also been localized to telomeres, even in taz1Δ cells (Nakamura et al., 2002). The MRN complex is essential for the intra S-phase checkpoint and proper DNA repair in fission yeast. Since this complex localizes to telomeres, the efficient utilization of this complex in DNA damage recovery might require fully functional telomeres. This could explain why telomere dysfunction resulting from the loss of various telomere proteins results in drug sensitivity. Additionally, the MRN complex acts on telomeres to create deregulated 3' overhangs in taz1Δ cells and presumably rap1Δ cells as well ((Tomita et al., 2003) and Figure 5.12). The additional employment of this complex to the telomeres in these mutants might perturb the efficiency of the DNA damage response in these cells. Like in fission yeast, loss of human RIF1 renders cells sensitive to DNA damage (Silverman et al., 2004). This defect has been attributed to a role of human RIF1 in the intra-S-phase checkpoint, a function that fission yeast Rif1 could potentially be involved in. Regardless, this work highlights an importance for proper telomere function in surviving DNA damage. As most human cells proliferate and divide, telomere reserves become depleted due to the lack of telomerlase activity. The inability to deal with genomic DNA damage can lead to genomic instability, which could be influenced by shortened, dysfunctional telomeres. Indeed, mice
lacking telomerase activity have been shown to be sensitive to DNA damage as telomeres shortened (Wong et al., 2000). Therefore, fission yeast could be a useful model in defining the role of telomeres in DNA repair, which could potentially lead to a better understanding of the involvement of telomeres in genomic instability and tumorigenesis in humans.

5.3.4 Fission yeast telosome

Taz1 plays a major role in organizing and regulating the proteins that constitute the fission yeast ‘telosome’. Indeed, loss of Taz1 results in a myriad array of cellular defects. Interestingly, only two proteins that interact with Taz1 have been reported. We have found that the loss of these proteins, Rap1 or Rif1, does not mirror all the effects of losing Taz1. This data suggested that there might be additional Taz1-interacting partners. To pursue this inquiry, we epitope tagged and immunoaffinity purified Taz1 in hopes that we could co-purify additional proteins that interact with Taz1. Our attempts to purify Taz1 were successful (Figure 5.13). However, we were unsuccessful in co-purifying any additional Taz1-interacting proteins. Time constraints and lack of reagents prohibited us from further developing this technique. Regardless, the successful purification of Taz1 suggests that this technique should be applicable to purifying additional telomere proteins with some optimizations. For example, we analyzed the Taz1 purified extract on a coomassie gel. These gels are far less sensitive than silver gels. The use of silver gels might have revealed some Taz1 specific bands that could have been analyzed by mass spectrometry for their identification. Purifying from a larger volume of cells could also have increased the sensitivity of the purification protocol. Taz1 is involved in many cell cycle
specific functions. Our extracts were taking from cycling cells, which are primarily in the G2 phase of the cell cycle. The use of drugs or cell cycle mutant alleles could synchronize cells in different parts of the cell cycle that could reveal the association of different proteins to Taz1. Taz1 is a telomere binding protein so much of the protein might be complexed with DNA, complicating its purification. The use of ethidium bromide or DNase might facilitate the purification procedure by releasing Taz1 from DNA. There are many additional conditions that could aid in the biochemical purification of additional telomere proteins. Our protocol is not limited to just Taz1 and could be used to purify other interacting proteins of additional telomere proteins, such as Rap1 or Rif1. Biochemical purification techniques have been successfully employed in human cells to identify telomere components (Liu et al., 2004a; Ye et al., 2004; Zhu et al., 2000). The successful identification of additional Taz1-interacting proteins would greatly facilitate our understanding of how Taz1 organizes the 'telosome' to regulate telomere functions and maintain genomic stability.
6 Conclusion and perspectives

These studies have revealed several interesting findings about telomeres and mechanisms that preserve their integrity, a function essential for ensuring genomic integrity and survival. The telomere binding protein Taz1 plays a pivotal role in this process in fission yeast. In this thesis, we present a situation whereby Taz1 becomes essential for capping telomeres. When taz1Δ cells are grown in cold temperatures, cells exhibit a high frequency of chromosome missegregation typified by anaphase bridging, fragmented chromosomes and defective nuclei. Cellular survival is compromised and both DNA damage and spindle assembly checkpoints are activated. Genetic analysis revealed that homologous recombination, the ATM homolog Rad3 and a subset of spindle assembly checkpoint proteins were required for survival. Analysis of chromosomal integrity in taz1Δ cells grown in the cold showed that chromosomes became broken and entangled. Importantly, these defects were separable from covalent end-to-end fusions, a defect most commonly associated with the phenotypes exhibited by taz1Δ cells in the cold. Additionally, the entanglement of chromosomes and all phenotypes were suppressed in taz1Δ cells that carried an altered topoisomerase II gene. These data suggest a link between dysfunctional telomeres and topoisomerase II and suggest that DNA topology is also an important factor that must be coordinated at telomeres. Thus, telomere covalent fusions are not the only defect that can result in anaphase bridging and genomic instability, an important consideration when analysing telomere mutant phenotypes. Based on these findings, we propose that uncapped $\text{taz}^+$ telomeres become entangled in the cold leading to
damaged chromosomes that activate checkpoints and repair pathways for cellular survival. The involvement of topoisomerase II at telomeres is novel and warrants further investigations. It will be very interesting to analyse Top2 and see if it acts at telomeres. Since top2-191 appears to act like a dominant mutation, it will be important to see if this protein has gained the function of binding to and resolving telomeres. Answers to these questions should give mechanistic insights into the possible involvement of topoisomerase II with telomeres, a potentially important subject since both topoisomerases and telomeres are linked with genomic stability and cancer.

Based on our analysis of taz1Δ cells in the cold, we wondered if unresolved replication intermediates could be the molecular explanation for the defects seen in these cells. Therefore, we set out to determine if Taz1 was required for proper replication at the telomere. We found that Taz1 was required for efficient replication of telomere and subtelomeric sequences. These data are in contrast to the dogma that states that protein-DNA complexes act as replication blocks. Our results suggest that Taz1 is required to orchestrate replication fork progression through telomeric DNA. Thus, telomere binding proteins might actively be involved in organizing the replication of chromosomal termini. It is tempting to speculate that telomeres require special mechanisms for their replication and resolution since telomeres are repetitive in sequence and are an end, a feature that is unique to telomeres throughout the genome. Thus, we have defined a new function for telomere binding proteins. It will be very interesting to delve further into this topic to understand their role in replication. It seems likely that telomere proteins must be removed before the replisome transverses the telomere and our data suggests that this step must be
regulated. We speculate that the inability to resolve telomeric replication intermediates accounts for the cold sensitivity of \textit{taz1Δ} cells in the cold. In support of this hypothesis, we found that MRN and Rqh1, two pathways involved in replication repair, were in the same genetic pathway as \textit{taz1} for cold sensitivity.

Taz1 binds to the duplex DNA region of telomeres and interacts with several other protein complexes to nucleate a functional telomere. Loss of Taz1 results in a variety of defects suggesting that it interacts with a host of other telomere factors that regulate telomere functions more directly. To dissect the genetic requirements for various telomere functions, we characterized the defects associated with the loss of Rap1 and Rif1, two known Taz1-interacting factors. Interestingly, we found that these factors were involved in a subset of telomere functions that were distinct from Taz1. Thus, Rap1 and Rif1 do not exert all of the activities of Taz1, a model that has been proposed in the literature. Instead, we propose that Taz1 can function independently, or through an as yet unidentified interacting factor, for some telomere functions. Conversely, we also suggest that Rap1 and Rif1 are functional for some telomere activities independently from Taz1. We hope that this work will present a genetic blueprint for the various functions of telomeres and help place specific telomere proteins into distinct pathways.

Although we are a long way from understanding telomeres, we hope that the work presented in this thesis will aid researchers in putting together a more detailed molecular model of a capped telomere and defining the cellular responses to dysfunctional telomeres. Answers to these questions are important given the essential role of telomeres in preserving genomic stability
and their association with human cancer and aging.
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