TELOMERE SPECIFIC HOMOLOGOUS RECOMBINATION IN THE ALTERNATIVE LENGTHENING OF TELOMERES

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DEDICATION

To my mother, for always believing in me and for teaching me to do the same.

TELOMERE SPECIFIC HOMOLOGOUS RECOMBINATION IN THE ALTERNATIVE LENGTHENING OF TELOMERES

by

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Over twenty years have passed since the discovery of telomerase-independent telomere maintenance, yet the precise details of the ALT mechanism remain a mystery. A growing body of evidence suggests that ALT cells maintain telomeres by homologous recombination (Reddel 2003 for Review). Groundbreaking work by Oliver Bechter demonstrated that ALT cells and telomerase-positive cells show no difference in the rate of general HR. This study fundamentally shaped our current concept of the ALT mechanism, implying that it involves preferential recombination of telomeric repeats. Since ALT seems to require proteins involved in normal HR, it follows that this telomeric recombination must be suppressed in telomerase positive or normal cells. However, to date there has been no direct evidence to support this hypothesis.

Seeking to investigate the rates of telomere specific recombination, previous work in the Shay and Wright lab utilized the Tel-Tel vector. However,

due to the method of integration only a limited number of clones could be analyzed and no statistically significant conclusions could be made. My work has focused on remedying this limitation. I have developed a strategy for integrating the Tel-Tel vector into a variety of host cell lines and generating a large number of distinct clones for each line. Using this strategy I was able to measure the average rates of telomeric HR for each cell line and provide the first direct evidence that ALT cells show distinctly elevated levels of increased telomere-specific HR. Additionally, I have constructed a control vector which functions in the same manner as Tel-Tel, differing only in that the telomeric repeats are replaced by non-telomeric repeat sequence. Using this vector (referred to as Mut-Mut) and the same incorporation method, I have demonstrated that there is no significant difference in the rates of general HR in ALT and telomerase positive cells. Finally, I used this novel ALT reporter as well as previously established methods to investigate some proteins that may play a central role in the ALT mechanism.

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ALT – Alternative Lengthening of Telomeres

APB – ALT Associated PML Body

bp – Base Pair

DNA – Deoxyribonucleic Acid

FACS – Fluorescence-Activated Cell Sorting

GFP – Green Fluorescent Protein

HDR – Homology Directed Repair

HR – Homologous Recombination

loxP – Locus of X over P1

MCS – Multiple Cloning Site

NHEJ – Non-Homologous End Joining

PCR – Polymerase Chain Reaction

PML – Promyelocytic Leukemia

RCA – Rolling Circle Amplification

SCE – Sister Chromatid Exchange

TRAP – Telomere Repeat Amplification Protocol

T-SCE – Telomere Sister Chromatid Exchange

Chapter One: Introduction and Literature Review

A History of Telomeres and Telomerase

Telomeres, the long stretches of repetitive DNA that cap mammalian chromosomes, play crucial roles in a number of biological processes. For nearly forty years scientists have been focusing their energy on understanding this highly specialized structure. Explorations in telomere biology have shed light on the mechanism by which cells divide and eventually become senescent, a process fundamental to aging and certain age related diseases. Additionally, the cellular immortalization required for all types of cancer requires certain alterations involving telomere maintenance. The fundamental role telomeres play in human aging and disease has attracted extraordinary interest in this field of study and indeed the efforts of a great number of scientists have opened the door for promising therapeutic opportunities. Many questions remain however, and this dissertation seeks to further our understanding of telomere biology and its role in certain types of cancer.

In the early 1960's, Leonard Hayflick published the first conclusive evidence that normal human cells have only a limited replicative potential. His work showed that human fibroblasts cultured in the laboratory undergo a finite number of divisions before becoming senescent (Hayflick 1961). This

phenomenon, referred to as the Hayflick limit, requires the existence of some counting mechanism within dividing cells. A decade later, Alexy Olovnikov implicated the chromosome ends as this molecular "clock". He theorized that DNA-replication machinery cannot fully replicate the ends of linear chromosomes and thus each round of cell division would lead to progressive loss of DNA (Olovnikov 1971). Although experimental evidence was hindered by the technology of the day, this end-replication problem and consequent telomere shortening would later prove to be at the heart of replicative aging and cellular senescence.

In 1978, Elizbeth Blackburn provided some of the first biochemical characterization of telomeres. Her work found that extrachromasomal genes coding for ribosomal DNA (rDNA) in *Tetrahymena thermophelia* terminated in 20-70 repeats of hexameric 5'-CCCCAA-3' (Blackburn 1978). It took another ten years, but in 1988 Robert Moyazis and his colleagues identified the sequence of human telomeres as 5'-$(TTAGGG)_n$-3' (Moyzis 1988). Shortly thereafter, *in vitro* hybridization studies revealed this to be the telomeric sequence for all vertebrates, indicating a high degree of evolutionary conservation (Meyne 1989). Another major advancement in the field came in 1985 when Blackburn and a graduate student, Carol Greider, identified telomere terminal transferase activity in extracts from *Tetrahymena* (Greider 1985). They identified a ribonucleoprotein, thereafter known as telomerase, and cloned the RNA component that specifies the telomere

sequence in *Tetrahymena* (Greider 1987; Greider 1989). These studies provided the first evidence for a mechanism by which cells could bypass the end-replication problem.

Following on this groundbreaking work, a study published in 1995 identified hTR as the template component of human telomerase (Feng 1995). This gene provides the 5'-CUAACCCUAAC-3' template sequence required for telomere synthesis. Expression studies revealed similar levels of hTR in mortal and immortal cells (Feng 1995). Site-directed mutagenesis of the template in Hela cells resulted in mutant telomere sequences, telomere shortening, and cell death (Feng 1995). Two years later, Tomas Cech and scientists at the Geron Corporation identified the catalytic component of human telomerase, hTERT (Nakamura 1997). In that same year, a collaborative study between scientists at Geron and the Shay/Wright lab demonstrated that *in vitro* synthesized hTR and hTERT could be combined to reconstitute telomerase activity, implicating them as the minimal components required for functional telomerase (Weinrich 1997). The existence of a polymerase that could specifically maintain telomeric DNA explains how germ cells maintain telomere lengths for offspring, as well as the limitless replicative potential characteristic of all cancers.

In 1989, Greg Morin showed that extracts from an immortal human cancer cell line, Hela, possessed telomere terminal transferase activity (Morin 1989). This led to the hypothesis that immortal human cells bypassed the end-replication

problem by maintaining telomere lengths using telomerase and, conversely, telomeres in mortal cells would shorten progressively. Carol Greider and Calvin Harley provided experimental evidence for this idea by showing that cultured fibroblasts did in fact show decreasing telomere lengths over time (Harley 1990). This study, although only correlative, provided the first demonstration that telomeres could be the internal counting mechanism theorized by Olovnikov. In 1998, after the discovery of hTR and hTERT, Jerry Shay and Woodring Wright conclusively demonstrated this to be the case. They introduced hTERT into two telomerase-negative, mortal cell types (retinal pigment epithelial cells and foreskin fibroblasts). As expected, non-transfected cells underwent a finite number of doublings, showed telomere shortening and became senescent. By contrast, clones expressing telomerase were able to elongate telomeres and exceeded their expected life span by many population doublings (Bodnar 1998). This study established a causal relationship between telomere shortening and cellular senescence, vaulting telomere biology to the forefront of research in cancer and aging.

Telomere Structure and the Shelterin Complex

As previously mentioned, mammalian telomeres consist of long stretches of 5'-$(TTAGGG)_n$-3'. While this particular sequence has been conserved evolutionarily, telomere length varies considerably among different mammals. Studies examining the length of fetal human telomeres have shown the average to be 10-15 kilobases while mouse telomeres are more than double that at 20-50 kilobases (de Lange 1990; Kipling 1990). Eukaryotic telomeres terminate in a single stranded, G-rich 3'-overhang. Early studies suggested that this well conserved feature averaged around 200 nucleotides in length (Makarov 1997). Electron microscopy studies revealed that telomeres could be packaged in a lariat structure called a T-loop, presumably formed by the 3'-overhang invading the double-stranded region of the telomere (Griffith 1999). The invading strand binds with C-rich strand forming double stranded telomeric DNA, while the displaced G-rich strand forms a small single stranded loop, known as the D-loop (displacement loop) (Griffith 1999). For any organism with linear chromosomes, distinguishing chromosome ends from random double strand breaks poses a critical concern. The evolutionarily conserved T-loop structure contributes to solving this problem by helping hide the chromosome end from DNA damage repair machinery, a process that requires specific interactions with telomere binding proteins

Mammalian telomeres interact with a six-protein complex known as shelterin. This interaction contributes to the secondary structure of telomeres, protecting them from DNA repair processes, and regulates telomere length. The first telomeric protein identified, TRF1 (telomeric repeat binding factor 1), was identified based on its ability to specifically bind duplex telomeric repeats (Zhong 1992). TRF1 binds with high specificity to TTAGGG repeats and does so as a homodimer. Furthermore, TRF1 bends telomeric DNA, facilitating formation of the T-loop structure (Bianchi 1997). In the context of telomerase expression, TRF1 negatively affects telomere length. Overexpression of TRF1 in the human cancer cell line HT1080 led to progressive telomere shortening. Conversely, introduction of a dominant negative version of the protein caused telomere elongation (van Steensel 1997). This negative regulation of telomere length likely results from TRF1 (alone or as part of the shelterin complex) affecting the recruitment of telomerase to the ends or affecting the so-called "open configuration" during which physical access to the end is possible.

TRF2, the other direct telomere ds binding protein, was identified based on sequence similarity to TRF1. Like TRF1, TRF2 forms a homodimer and binds telomere DNA through a Myb domain (Broccoli 1997). However, TRF1 and TRF2 seem to be functionally distinct. Whereas TRF1 plays a critical role in establishing higher order structure of telomeres, TRF2's main function is shielding the telomere from being recognized as DNA damage. Introduction of a

dominant negative TRF2 led to p53/ATM dependent apoptosis, suggesting activation of a DNA damage response (Karlseder 1999). Furthermore, the DNA damage signal originated at telomeres as shown by the formation of telomere dysfunction-induced foci (TIFs), which are defined by the colocalization of canonical DNA damage foci with telomeric proteins (Takai 2003). TIFs have become a hallmark of dysfunctional telomeres and appear at all telomeres in cells depleted of TRF2 (Konishi 2008). The ability of TRF2 to suppress DNA damage signaling is related to both its function in forming T-loops and its ability to suppress ATM signaling (Karlseder 2004). In addition to their contributions as homodimers influencing telomere structure and maintenance, TRF1 and TRF2 also serve as scaffolds by which other shelterin proteins are assembled at telomeres.

Rap1 does not directly interact with telomeres. Rap1 binds directly to TRF2, an interaction required for the telomeric localization and stability of Rap1 (Celli 2005). This binding occurs between the Rap1 C-terminal domain and the TRF2 hinge domain (Reviewed in Palm 2008). Interestingly, Rap1 also contains a Myb domain, which may be involved in recruiting some as of yet identified binding partner to telomeres (Hanaoka 2001). Recent studies have also implicated Rap1 in suppressing inappropriate recombination between telomere sequences, a phenomenon with implications for a telomerase-independent method of telomere maintenance (Sfeir 2010).

Two proteins in the shelterin complex, TIN2 and TPP1, do not directly interact with telomeres. These proteins bind other shelterin proteins and seem to play important roles in stabilizing their telomeric localization. TIN2 binds TRF1 and TRF2 directly and simultaneously (Ye, Donigian et al 2004). Furthermore, inhibition of TIN2 leads to a depletion of telomeric TRF1 and subsequent telomere elongation (Ye and de Lange 2004). In addition to its role in telomere length regulation, TIN2 recruits TPP1 (and by extension POT1) to telomeres (O'Connor 2006; Liu D 2004). Indeed, deletion of TPP1 or expression of mutants lacking POT1-binding capabilities leads to diminished telomeric POT1 as well as telomere phenotypes consistent with POT1 deletion (Hockemeyer 2007).

The shelterin complex contains a third protein that directly binds telomeric DNA, Pot1. Unlike TRF1 and TRF2, however, Pot1 binds G-rich, single-stranded telomeric DNA (Baumann 2001). Pot1 was first identified by sequence similarity to the telomere-capping complex in *Oxytricha nova*, TEBPα/β (Baumann 2001). Similar to TRF1 and TRF2, Pot1 plays a role in masking telomeres from being recognized as damaged DNA. However, whereas TRF1 and TRF2 prevent ATM-dependent DNA damage signals, Pot1 blocks signaling through ATR (Lazzerini 2007). Pot1-dependent suppression of DNA damage signaling requires its interaction with TPP1 and likely functions by blocking RPA (a protein that leaves single-stranded DNA in an open configuration) from binding to telomeres (Hockemeyer 2007; Lazzerini 2007).

Telomeres, Telomerase and Cancer

The discovery of telomere terminal transferase activity in Hela extracts provided the first link between telomere maintenance and the unlimited proliferative capacity (immortality) characteristic of cancer. Limited by the technology of the day, no strong correlation between telomerase activity and cancer could be established. This changed however with the advent of the telomere repeat amplification protocol (TRAP). In 1994, the Shay/Wright laboratory developed a PCR-based strategy for detecting telomerase activity and screened a panel of cultured cells and human biopsies. Their results showed robust telomerase activity in approximately 85% of cancer cell lines and tumor biopsies, whereas mortal cell lines or normal tissue showed no such activity (Kim 1994). This link between human cancers and a single enzyme made telomere biology and anti-telomerase therapy an extraordinarily appealing target for novel ways to treat cancers.

Programmed telomere attrition provides a tumor suppressive role, ensuring that cells that have undergone more rounds of cell divisions accumulating premalignant mutations cease to divide. Once a few telomeres become sufficiently short a cell enters a state of senescence (M1:Mortality Stage 1) In the absence of specific mutations cells can remain in this quiescent state for long periods of time (reviewed in Shay 2011). Some cells acquire mutations in

cell cycle checkpoint genes, such as p53, $p16^{INK4a}$ and pRb, allowing them to escape M1 and continue dividing (Wright 1989). Following escape from M1, cells will continue to divide until many telomeres become critically short. At this point most cells again enter a state of minimal division known as crisis, or M2 (Mortality Stage 2). By the time a cell reaches crisis, critically short telomeres have led to chromosome fusions, breakage events, and rearrangements. This produces genomic instability and further mutation. In the context of this highly mutagenic state, a rare cell may escape crisis by activating a telomere maintenance program (Shay 1991). This ability for a cell to escape crisis by maintaining telomeres has become recognized as one of the hallmarks of human cancer (Hanahan 2000).

As previously discussed, the vast majority of human cancers maintain telomeres by activating telomerase. Thus telomerase inhibition offers an exciting avenue for treating a wide variety of cancers without damaging surrounding healthy (telomerase-negative) tissues. Currently, clinical trials are investigating the efficacy of telomerase inhibition in a variety of tissues via gene therapy, immunotherapy, or small molecule inhibition (Reviewed in Shay 2011). However, this type of therapy does leave the possibility for some unintended consequences. Because of the dynamic and adaptable nature of a cancer cell, prolonged telomerase inhibition may eventually give rise to drug resistant, telomerase-expressing revertants. Additionally, a recombination-based mechanism for

telomere maintenance known as alternative lengthening of telomeres (ALT) offers another means of resistance to telomerase inhibition therapy. Indeed, one study identified a mismatch repair deficient colon cancer cell line capable of engaging the ALT pathway after the introduction of a dominant negative hTERT. Furthermore, after extended culture over many population doublings, the dominant negative became suppressed and the cells were again able to express functional telomerase (Bechter 2004).

Alternative Lengthening of Telomeres

In 1995, Roger Reddel first reported the existence of telomerase-negative immortalized cell lines (Bryan 1995). It has since been shown that roughly 10% of human tumor samples or tumor derived cell lines maintain their telomeres independently of telomerase expression (Shay 1997). ALT cancers occur in a variety of tissue types, primarily bone and soft tissue sarcomas, glioblastomas, and less frequently in carcinomas of the lung, kidney, breast and ovary. While ALT tumors occur less often they tend to have a particularly poor prognosis (Reviewed in Reddel 2003). Furthermore, some hypothesize that tumors could contain a mixture of ALT and telomerase positive cells, reinforcing the notion that ALT provides a viable escape from telomerase inhibition therapy. This possibility greatly highlights the need for a therapeutic strategy that can

specifically target ALT cells. However, no such therapies exist due to our lack of understanding of the molecular mechanism ALT cells use to maintain telomeres.

While the precise ALT mechanism remains elusive, studies have revealed some distinct molecular characteristics of ALT cells. The earliest report of telomerase-negative, immortalized cells observed very heterogeneous telomere lengths, ranging from undetectable to as large as 50 kilobases (Bryan 1995). Furthermore, the length of any single ALT telomere can change much more rapidly than in normal cells (Murnane 1994). ALT cells also contain a specific class of promyelocytic leukemia (PML) nuclear bodies. Known as ALT-associated PML bodies (APBs), these complexes contain telomeric DNA, canonical PML body components, telomere binding proteins, as well as proteins typically involved in DNA recombination (Yeager 1999). Their precise role in the ALT mechanism remains unclear, but some speculate that APBs might provide the molecular platform for ALT activity.

In addition to canonical telomeres, ALT cells contain an abundance of extrachromasomal telomeric repeat DNA. These molecules appear in a variety of structures including double-stranded telomeric circles (t-circles), partially single stranded telomereic circles (C-circles or G-circles, depending on whether the C-rich or G-rich strand forms the complete circle), as well as double-stranded, linear repeats (Nabetani 2009). While t-circles are thought to be involved in normal biology, C-circles appear much more specific to ALT cells. Reddel et al showed

that virtually all ALT lines tested contained C-circles, whereas in telomerase positive or non-immortalized cells C-circles were undetectable above background. Additionally, C-circles appear in ALT cell lines that do not exhibit most of the traditional hallmarks of ALT and are dramatically reduced after ALT inhibition (Henson 2009). These findings suggest that C-circles may somehow be fundamental to the ALT mechanism. Furthermore one study detected C-circles in blood samples of patients with ALT-positive osteosarcomas, implicating that C-circles might become a valuable tool for diagnosing ALT cancers and measuring the effectiveness of treatment (Henson 2009).

While the exact mechanism by which ALT cells maintain telomeres remains unclear, most evidence suggests ALT involves homologous recombination (HR). Telomerase independent telomere maintenance was first demonstrated in yeast and was shown to require the HR protein, RAD52 (Lundblad 1993). The initial observation that human immortalized, telomeras-negative cells underwent rapid telomere elongation and deletion also suggested the involvement of HR in this form of telomere maintenance (Murnane 1994). Another study further implicated HR in ALT by demonstrating that a DNA tag on a single human telomere could be copied to other chromosomes in ALT cells, but not in cells expressing telomerase (Dunham 2000). Furthermore, ALT cells undergo telomere sister chromatid exchange (T-SCE) at a much higher rate than telomerase positive cells, while maintaining comparable rates of SCE at other

genomic loci (Londono-Vallejo 2004). Taken together these data strongly implicate HR as integral to the ALT mechanism but raise the question as to whether ALT cells possess some general defect in HR, allowing for higher rates of recombination across the genome, or whether the phenomenon is telomere specific. Oliver Bechter in the Shay/Wright lab sought to answer this question directly.

In order to investigate the rates of HR in ALT cells vs. non-ALT cells, Bechter el al utilized the pDR-GFP recombination reporter, first developed by Maria Jasin (Pierce 1999). This plasmid contains two inactive GFP genes, and can only express functional protein after digestion with Isce-I in the 5'-GFP and subsequent homologous recombination with the 3'-GFP (Figure 1.1). Using this construct, Bechter showed that the rates of HR were essentially the same in telomerase-positive SW39 cells as in ALT-positive SW26 cells, regardless of whether the integration site was internal or immediately adjacent to the telomere (Table 1.1; Bechter 2003). This study provided the first evidence against the idea that ALT cells possess some defect in the general HR machinery, and in favor of the idea that elevated rates of recombination might be specific to telomeric DNA.

While Bechter's work provided important insights into ALT biology it also highlighted the need for a reporter that could directly measure HR at telomeric sequences. To address this need, my dissertation studies utilized the Tel-Tel HR reporter. The Tel-Tel vector functions similarly to DR-GFP – it

contains two inactive GFP genes and may undergo recombination following induction by I-SceI. The two reporters differ, however, in that the Tel-Tel vector requires that telomeric repeats are used as a recombination substrate and GFP expression requires that the host cell be capable of this type of HR (Figure 1.3). Using this system, I sought to test the hypothesis that telomerase positive cells possessed some specific block to telomeric HR, whereas in ALT cells this suppression has been lost. Furthermore, my work has sought to directly measure the rates of telomeric homologous recombination in a variety of ALT and telomerase positive cell lines. I have validated the Tel-Tel assay as a robust and effective means for measuring ALT activity and screening for proteins that might be involved in the ALT mechanism.

Figure 1.1 DR-GFP Recombination Reporter

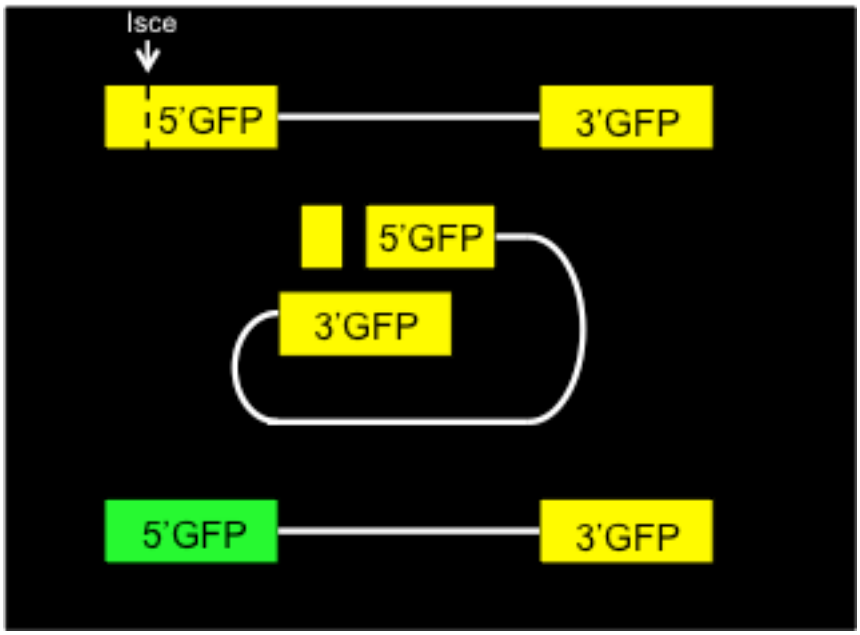
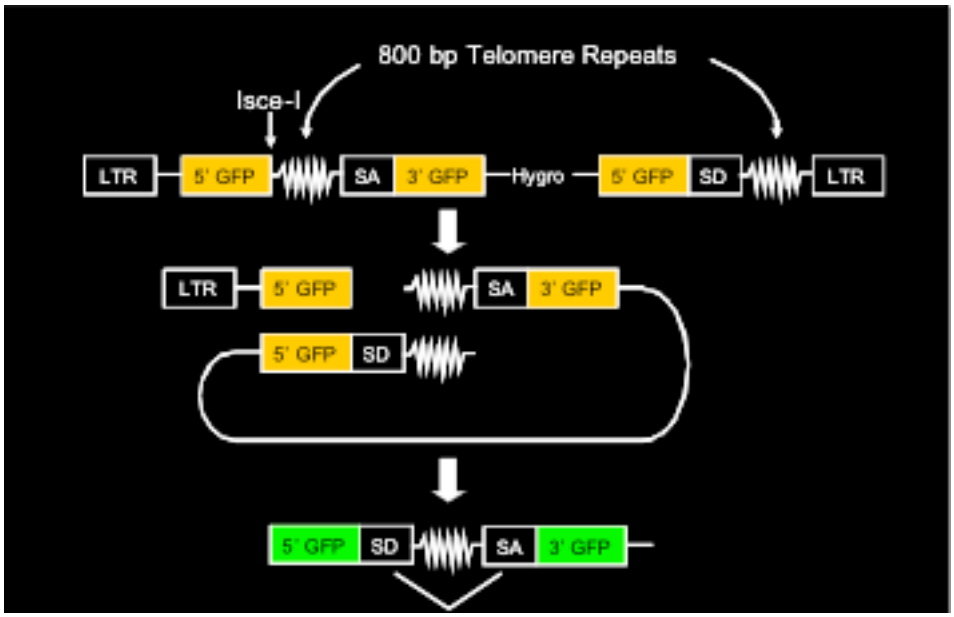


Table 1.1 Average rates of homologous recombination at internal and telomere-adjacent integration sites in ALT and telomerase positive cells.
Average recombination measured as percent of GFP positive cells as measure by FACS after induction of DR-GFP reporter by ISce-I.

Cell Type	Telomere Maintenance	Integration Site	Average Recombination
SW39	Telomerase	Internal	3.5%
SW26	ALT	Internal	3.4%
SW39	Telomerase	Telomeric	2%
SW26	ALT	Telomeric	3.1%

Figure 1.3 Tel-Tel Recombination Reporter measures telomere specific homologous recombination.



Chapter Two

Development of Tel-Tel Reporter Assay in Immortalized Human Cells

Abstract

Over twenty years have passed since the discovery of telomerase-independent telomere maintenance, yet the precise details of the ALT mechanism remain a mystery. A growing body of evidence suggests that ALT cells maintain telomeres by homologous recombination (Reddel 2003 for Review). Groundbreaking work by Oliver Bechter demonstrated that ALT cells and telomerase-positive cells show no difference in the rate of general HR. This study fundamentally shaped our current concept of the ALT mechanism, implying that it involves preferential recombination of telomeric repeats. Since ALT seems to require proteins involved in normal HR, it follows that this telomeric recombination must be suppressed in telomerase positive or normal cells. However, to date there has been no direct evidence to support this hypothesis.

Seeking to investigate the rates of telomere specific recombination, previous work in the Shay and Wright lab utilized the Tel-Tel vector. However, due to the method of integration only a limited number of clones could be

analyzed and no statistically significant conclusions could be made. My work has focused on remedying this limitation. I have developed a strategy for integrating the Tel-Tel vector into a variety of host cell lines and generating a large number of distinct clones for each line. Using this strategy I was able to measure the average rates of telomeric HR for each cell line and provide the first direct evidence that ALT cells show distinctly elevated levels of increased telomere-specific HR. Additionally, I have constructed a control vector which functions in the same manner as Tel-Tel, differing only in that the telomeric repeats are replaced by non-telomeric repeat sequence. Using this vector (referred to as Mut-Mut) and the same incorporation method, I have demonstrated that there is no significant difference in the rates of general HR in ALT and telomerase positive cells. Finally, I used this novel ALT reporter as well as previously established methods to investigate some proteins that may play a central role in the ALT mechanism.

Introduction

The first attempt to specifically measure homologous recombination at telomeric repeats employed the Tel-Tel recombination reporter. In these experiments, two telomerase positive cell lines (Hela and SW39) and one ALT line (SW26) were stably transfected with the Tel-Tel vector and subsequently analyzed by Southern blot to verify copy number and absence of vector rearrangement. After analysis, 9 telomerase-positive (4 Hela and 5 SW39) and 5 ALT (SW26) clones were chosen for the recombination assay. On average, the ALT clones tested were able perform Tel-Tel recombination at least four times higher than the telomerase-positive cell lines. These data, in conjunction with the equivalent rates measured for general recombination, support the hypothesis that ALT cells have lost some suppression of telomere-specific HR. However, clonal variability in Tel-Tel recombination for all three cell types requires cautious interpretation of these data. As shown in Table 2.1, SW39 clones showed differences in recombination rates spanning a full order of magnitude. Additionally, individual telomerase-positive clones gave recombination rates comparable to those measured for some individual ALT clones. These inconsistencies emphasized the need for a method of generating large numbers of clones for each cell type to be tested. Ordinarily, viral packaging would address this concern. However, for reasons that remain unclear, but perhaps due to some

secondary structure, vectors containing telomeric repeats cannot successfully be packaged in a virus. Therefore, to generate large numbers of Tel-Tel clones I employed a Cre/lox exchange strategy.

In the early 1980's, Ronald Hoess and his colleagues reported the discovery of Cre recombinase in bacteriophage P1. This enzyme recognizes specific sites in the P1 genome called loxP sites (locus of X-over of P1) and efficiently catalyzes DNA recombination between pairs of loxP sites (Hoess 1984). Depending on their specific orientation, Cre-mediated recombination between two loxP sites will have a different effect on the intervening DNA. Recombination between loxP sites with the same orientation will result in complete excision of the DNA between them while recombination between loxP sites with opposite orientation causes inversion of the flanked DNA (Sauer 1987). Researchers have long utilized this Cre/loxP system to manipulate mammalian genomes. It has proven particularly useful in the generation of transgenic mice. I have utilized this strategy in a variety of human cell lines to generate large numbers of distinct clones containing single integrations of the Tel-Tel vector and measured the average rates of telomere specific HR for each line.

Results

The precise nature of Cre-mediated recombination at loxP sites depends on the orientation of the loxP sites. Additionally, mutated loxP sites may be employed so as to allow one sequence to be completely replaced by another. For my work, I used this strategy for genomic Tel-Tel integration (Illustrated in Figure 2.1). Each cell line was first infected by lentivirus with a construct referred to as the insertion cassette, which contains a positive and negative selectable marker (blasticidin and thymidine kinase respectively) flanked by two mutated loxP sites. These two loxP sites contain different mutations, making them unable to recombine with each other in the presence of Cre. Blasticidin selection for the insertion cassette produced tens of thousands of colonies for any of the cell lines examined. By controlling viral titer at this step, I could reasonably assume that each clone represented a distinct insertion site. This provided me with a large and diverse population into which I could exchange the Tel-Tel vector. Following selection for the insertion cassette, each cell line was infected with a retroviral Cre-recombinase and subsequently put under puromycin selection. Since the retroviral Cre was used at a high titer, this step did not significantly diminish the total number of clones, thus preserving the diversity of inserting sites. After puromycin selection, cell lines were transfected with a version of the Tel-Tel vector flanked by the same two mutant loxP sites in the original insertion cassette.

In the presence of Cre-recombinase, the mutant loxP sites flanking Tel-Tel will recombine with the mutant loxP sites in the insertion cassette and the Blast-TK construct will be replaced by Tel-Tel. This exchange reaction was performed in eight cell lines and a minimum of 500 Tel-Tel clones was generated for each. Following Tel-Tel exchange, the clones were pooled and grown as a population. The recombination assay is then performed by transfecting this population of cells with an Isce-I expression vector and GFP expression is subsequently measured by FACS. Using this method I measured the average rate of telomere-specific HR for each of the different cell types.

Tel-Tel recombination was measured in four telomerase positive cell lines (DLD1, A549, MDA231 and SW39) and four ALT lines (U2-OS, Saos-2, GM847 and SW13). For each cell type, I performed the recombination assay twice. As illustrated in Figure 2.2, all three ALT lines displayed recombination rates above 1%. This contrasts starkly with the telomerase positive cell lines, for which the highest rate of recombination measured was 0.05%. Using the Tel-Tel assay, I have shown that ALT cells can recombine telomeric repeats at least 20 times higher than telomerase positive cells. In order to further validate these data, I repeated the Tel-Tel exchange reaction and recombination in two ALT cell lines (U2-OS and Saos-2) and one telomerase positive line (A549). Again, the rates of telomeric HR were significantly higher in ALT cells than in telomerase positive cells (Figure 2.3). These data provide direct evidence for ALT cells' ability to

recombine telomeric repeats in a way in which telomerase positive cells are not capable.

Previous studies have shown that telomerase expression in ALT cells can inhibit recombination based telomere maintenance. A study by Lance Ford and colleagues found that expression of hTR and hTERT in an ALT positive, human lung fibroblast cell line led to a significant decrease in the formation of APBs and extrachromasomal telomere repeats characteristic of ALT (Ford 2001). Since the cells in which I measured Tel-Tel recombination fundamentally contrast by the presence or absence of telomerase, I wanted to examine whether telomerase expression itself was sufficient to suppress telomeric HR. To address this question, I introduced hTERT into the hTR-expressing ALT cell line Saos-2 and verified telomerase activity by TRAP (Figure 2.4a). I then performed the Tel-Tel assay side-by-side in parental and telomerase positive Saos-2 cells and found that both showed comparable rates of recombination (Figure 2.4b). Therefore, while telomerase expression may inhibit some ALT phenotypes, it does not appear to block an ALT cell's fundamental ability to use telomeric repeats as an HR substrate.

Materials and Methods

Cell Culture

All cell lines used were cultured in anti-biotic free media containing 10% serum at 37°C in 5% CO_2.

Lentivirus Production and Infection with Insertion Cassette

The insertion cassette vector, containing blasticidin resistance and thymidine kinase genes flanked by mutated loxP sites (loxP5171: 5'-ATAACTTCGTATAATGTGTACTATACGAAGTTAT-3' and loxP2272: 5'-ATAACTTCGTATAAAGTATCCTATACGAAGTTAT-3'), was introduced into cells via lentivral infection. 293FT cells were plated at three million cells per 10 cm^2 culture dish 24 hrs prior to cotransfection with insertion cassette vector and lenitviral packaging vectors PAX and PMD. Transfections were performed by diluting 5 μg insertion cassette vector, 4 μg PAX and 2 μg PMD with water to a final volume of 439 μl. Next, 500 μl of 2X HBS (50 mM Hepes, 10 mM KCl, 12 mM Dextrose, 280 mM NaCl, 1.5 mM $Na_2HPO_4 \bullet 7H_2O$) was added to diluted DNA solution. To form transfection precipitate, 61 μl of 2M $CaCl_2$ was added dropwise to DNA/HBS while gently vortexing. This final 1 ml solution was added to 293FT cells in 9 ml of media. Viral supernatant was collected and 24, 36 and 48 hours post transfection and used to infect individual cell lines. Following

infection, cells were placed under blasticidin selection for 7 days and the resulting clones were trypsinized and pooled (See Table 2.2 for blasticidin selection doses).

Retroviral Production and Infection with Cre Recombinase

The retroviral packaging cell line ΦE was transfected with pBABE-Cre-puromycin vector using Transit-LT1 reagent (Mirus). One million cells were seeded in a 10 cm^2 culture dish 24 hours prior to transfection. For the formation of transfection complex, 45 µl of Transit-LT1 reagent were diluted in 1.5ml of serum free media and this mixture was incubated at room temperature for 20 minutes. Next, 15 µg of pBABE-Cre-puromycin was added and this mixture was incubated for an additional 20 minutes at room temperature before being added dropwise to ΦE cells. At 24, 48, and 72 hours the media was collected from ΦE and transferred to PA317 cells, previously plated at 1 X 10^6 cells per 10 cm^2 dish. PA317 cells were then put under puromycin selection at 4 µg/ml. After successful puromycin selection, PA317 cells constitutively produce Cre-puro viral supernatant, which was used to infect previously generated insertion-cassette cell lines. Refer to Table 2.3 for puromycin selection doses.

Tel-Tel Exchange and Recombination Assay

Cells containing the insertion cassette vector and Cre recombinase were transfected with the Tel-Tel vector. For each reaction, 5 X 10^6 cells were

resuspended in 0.5 ml of complete media and combined with 30 μg of plasmid DNA in a volume of 100 μl in a 0.4 cm electroporation cuvette. Cells were electroporated at 250 V/960 μF and immediately transferred to 10 ml of fresh media in a 10 cm^2 dish. After 48 hrs of recovery, cells were put under hygromycin selection – positive selection for Tel-Tel. Hygromycin selection doses are listed in Table 2.4. After 10 days of hygromycin selection, resistant clones were treated with 1 mM ganciclovir to eliminate any cells retaining the original insertion cassette. Following successful ganciclovir treatment, remaining clones are pooled and grown as a population. Recombination assays were performed by electroporating 5 X 10^6 cells with 30 μg of an Isce-I expression vector. 72 hours post Isce-I transfection, cells are analyzed for GFP expression by FACS.

Discussion

The mechanism underlying the alternative lengthening telomeres has long been believed to involve homologous recombination between telomere repeats. So far the best experimental evidence for this hypothesis has been merely suggestive. My work sought to establish a system for directly measuring the frequency of telomere-specific HR in ALT and telomerase positive cells. The Tel-Tel vector contains two tracts of telomere repeats and GFP expression requires that the host cell be capable of recombining these repeats. Using a Cre/loxP exchange strategy I was able to generate cell lines composed of a large number clones with single integrations of the Tel-Tel vector in distinct insertion sites. Previous methods of Tel-Tel incorporation relied on stable transfection, and verifying single insertion and vector integrity required huge investments of time and labor. Furthermore, this method produced only a small number of clones for each cell line tested and clonal variation within each cell line made interpretation of the data difficult. By measuring Tel-Tel recombination in a pool of hundreds of distinct clones I was able to make a more reliable measurement of the average rates of recombination for each cell line tested.

Using these methods, I have shown that Tel-Tel recombination occurs at a frequency at least 20 times higher in ALT as compared to telomerase positive cells. These data provide the first direct evidence that telomere specific HR

occurs at elevated levels in ALT cells. Furthermore, whereas Tel-Tel recombination occurs at background levels in telomerase positive cells, the frequency of Tel-Tel recombination measured in ALT cells is comparable to those measured for general recombination in both types of cells (Bechter 2003). Comparing these data supports the model that under normal circumstances human cells specifically suppress HR at telomeres. This implies that telomere specific HR as part of the ALT mechanism must involve a loss of this type of suppression. This work provides a major step forward in our understanding of the ALT pathway and solidifies the hypothesis that the underlying mechanism requires homologous recombination of telomeres.

Prior work examined the effect of telomerase expression in ALT cells and found that it could inhibit some canonical ALT phenotypes. The presence of critically short but heterogeneous telomeres is a well-characterized hallmark of ALT. In the presence of telomerase, these chromosome ends will be maintained above threshold levels, eliminating the damage signals that likely trigger elongation by recombination. Therefore, the diminished ALT phenotypes previously observed likely result from a decreased number of critically short telomeres, however, the dysregulation that makes telomere HR possible remains. In the hTR-expressing ALT cell line Saos-2, introduction of hTERT alone is sufficient for telomerase activity and has no effect on Tel-Tel recombination. These data show that ALT cells retain their fundamental ability to recombine

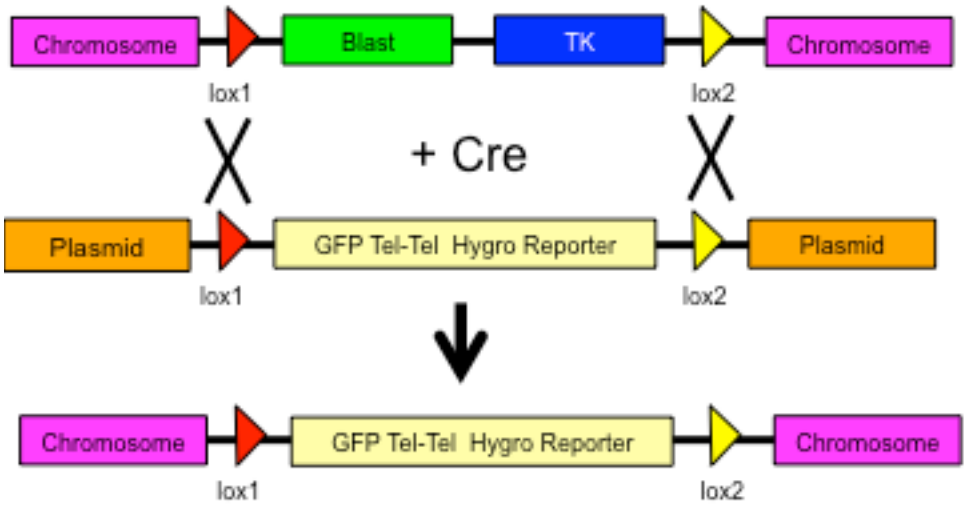
telomeric DNA regardless of whether they are actively doing so to maintain telomeres. In addition to providing information about the basic biology of the ALT pathway, this work makes a strong case for the Tel-Tel reporter as a valuable tool for further experimental investigations. The earlier work examining the effect of telomerase expression in ALT cells employed traditional assays for ALT: heterogeneity of telomere length, APB formation, and presence or absence of extrachromasomal telomere repeats. These assays should be thought of as measures of active telomere maintenance by ALT. By contrast, the Tel-Tel assay directly examines a cell's ability to recombine telomere repeats, the fundamental biology central to the ALT mechanism.

In addition to its utility as an experimental tool, the Tel-Tel assay may also have some clinical relevance. Some researchers have hypothesized the possibility of human tumors that contain a mixture of ALT and telomerase positive cells. This also raises the possibility that some telomerase positive tumor cells could lack the normal suppression of telomeric HR, but for reasons mentioned earlier, traditional assays for ALT would fail to detect these cells. The Tel-Tel assay identifies a cell's ALT competence, which may provide a useful means for detecting cells more likely to develop resistance to telomerase inhibition therapy.

Table 2.1 Tel-Tel recombination the telomerase-positive cell lines, Hela and SW39, and the ALT cell line, SW26. Cell lines were generated by stable transfection with the Tel-Tel vector. Recombination rates given as percentage of GFP positive cells as measured by FACS after induction by ISce-I.

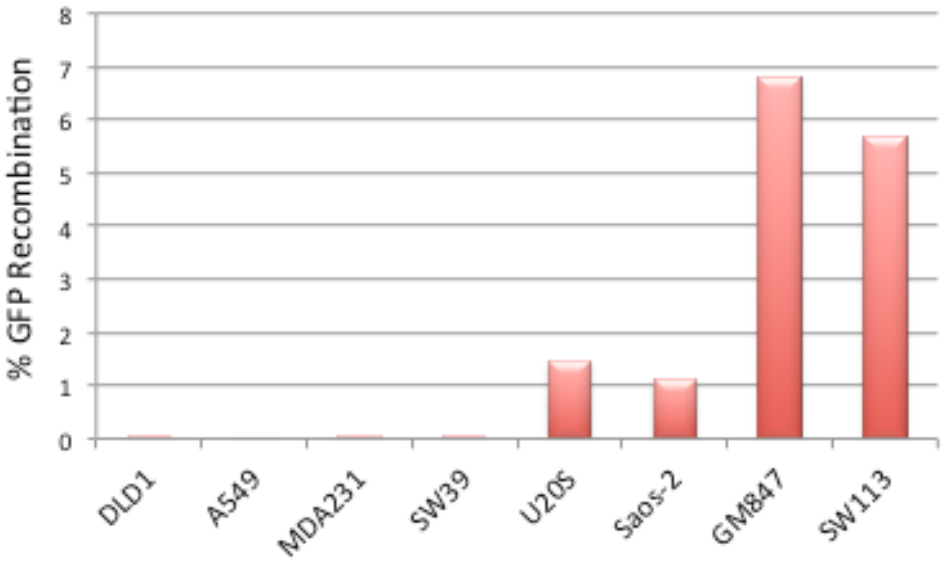
Hela clones (telomerase+)	SW39 clones (telomerase +)	SW26 clones (ALT)
0.04	0.1	1
0.9	0.3	1.3
0	0.4	2
0	1	2.4
	1.4	5.4
Average = 0.2%	Average = 0.6%	Average = 2.4%

igure 2.1 Cre/loxP exchange strategy for generating large number of Tel-el clones with distinct integration sites.



i-SCE1 transfection and FACS analyze for GFP positive

Figure 2.2 Tel-Tel recombination in a panel of telomerase-positive and ALT cell lines. Recombination rates for each cell line were measured in a pool of thousands of Tel-Tel clones with unique insertion sites. Recombination measured as percentage of GFP positive cells as assessed by FACS after induction by ISce-I.



**igure 2.3 Increased Tel-Tel recombination in ALT cells demonstrated
a second experiment.**

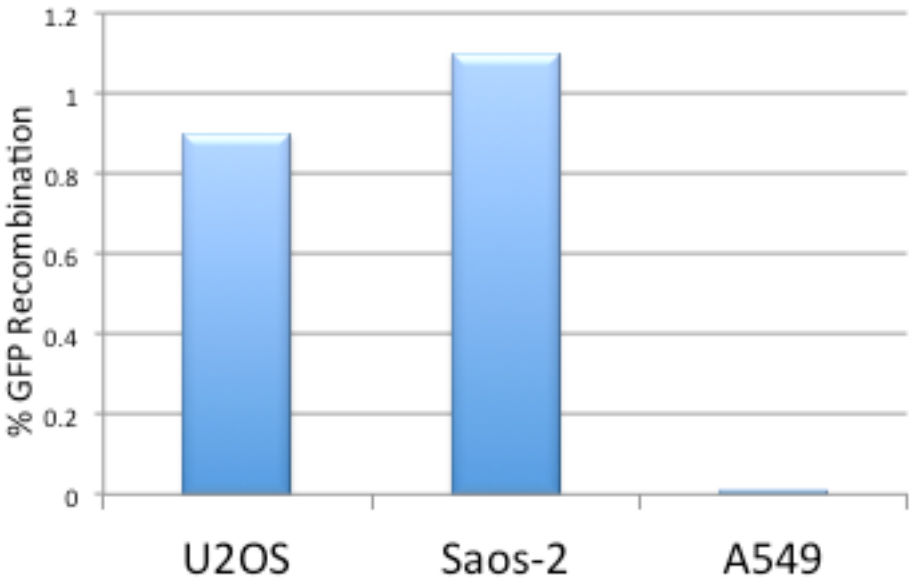


Figure 2.4 Telomerase expression does not affect Tel-Tel recombination. hTERT was expressed in the hTR-expressing ALT cell line, Saos-2. Telomerase activity was verified by the TRAP assay (a). Tel-Tel recombination was the same for normal or telomerase positive Saos-2 cells (b).

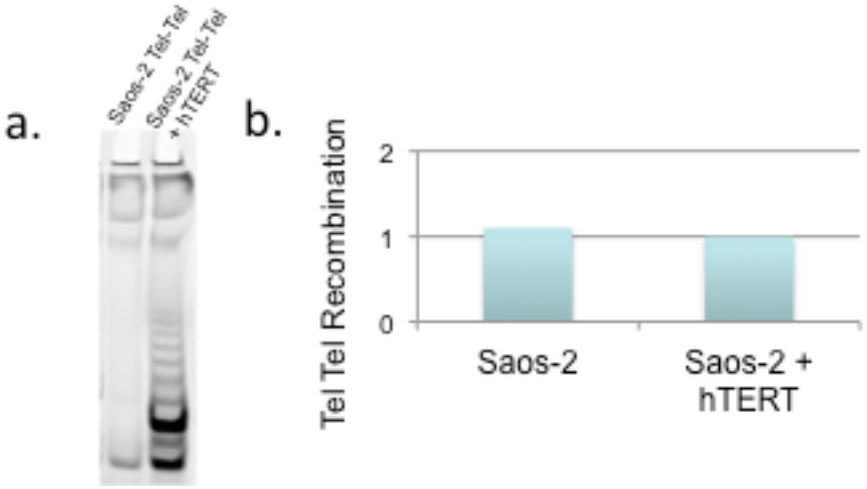


Table 2.2 Blasticidin doses used for selection of insertion cassette.

Cell Type	Selective Dose (µg/ml)
DLD1	10
A549	10
MDA231	10
SW39	5
U2-OS	6.5
Saos-2	7.5
GM847	7.5
SW13	5

Table 2.3 Puromycin doses used for selection of Cre recombinase.

Cell Type	Selective Dose (µg/ml)
DLD1	2.5
A549	0.75
MDA231	0.5
SW39	0.5
U2-OS	0.5
Saos-2	0.5
GM847	0.5
SW13	0.5

Table 2.4 Hygromycin doses used for selection of the Tel-Tel vector.

Cell Type	Selective Dose (µg/ml)
DLD1	584
A549	1945
MDA231	389
SW39	292
U2-OS	292
Saos-2	389
GM847	292
SW13	292

Chapter Three

Construction of Non-Telomeric Recombination Reporter

Introduction

The data generated using the Tel-Tel reporter showed significantly increased levels of telomeric HR in ALT cells as compared to telomerase positive cells. These data, in conjunction with previous experiments showing comparable rates of general HR for ALT and telomerase positive cells, suggests a natural suppression of HR at telomeric repeats. The ALT mechanism must therefore involve a loss of this suppression. While the data certainly do not refute this model, the general recombination studies by Bechter et al do not serve as the most appropriate control for the Tel-Tel experiments. The rates of general recombination measured in this study also used a GFP-based reporter that requires Isce-I-induced breakage, however, this system used the GFP-sequence itself as a recombination substrate (Bechter 2004). The Tel-Tel reporter differs in that it requires recombination of telomeric DNA, which can then be spliced out to generate a functional GFP (See Figure 1.3). In order to more rigorously demonstrate that ALT involves a loss of suppression of telomere-specific HR, I sought to construct a recombination reporter that functions in the same manner as

Tel-Tel but uses non-telomeric DNA as a recombination substrate. Since the recombination block is likely specific to telomeric DNA, I hypothesized that replacing the telomere repeats in the Tel-Tel vector with any sequence should result in the same rates of recombination in both ALT and telomerase positive cells.

In order to construct this type of control vector, I developed a multi-step cloning protocol for replacing the telomere repeats in Tel-Tel with any sequence of interest (Figure 3.1). Because both tracts of telomere repeats have a SacI site immediately adjacent to their 5' end, they must be separated via subcloning before excision and replacement. The entire functional region of the Tel-Tel vector lies between a HindIII site and a BamHI site (Figure 3.1a). Digestion with either HindIII and PstI or PstI and BamHI allows separation and subcloning fragments of the Tel-Tel containing the upstream or downstream telomere repeats respectively (Figure 3.1b). The intermediate vectors generated at this step now contain the telomere repeats flanked by unique restriction sites. Enzyme digestion and gel purification produces upstream and downstream intermediates without the telomere repeats, into which any DNA of interest can be inserted via standard cloning methods (Figure 3.1c). Following this step, the PstI/BamHI fragment containing the downstream half of the reporter can be excised and cloned into the PstI/BamHI sites in the upstream intermediate vector. This cloning step aligns the

two pieces of the original reporter immediately adjacent to one and other, a configuration that reconstitutes the reporter's functionality (Figure 3.2).

Using this protocol I constructed several vectors containing non-telomeric recombination substrates. In this chapter I will outline the rationale for each of the sequences selected and the method used to generate inserts of the appropriate size. I will also discuss the results of the different sequences as well as some of the biological implications.

Results

The final step in the construction of the non-telomeric recombination reporters involves reassembly of the upstream (HindIII/PstI) and downstream (PstI/BamHI) fragments in the pUC-19 backbone. In order to verify that I could reconstitute original vector function in this context the upstream and downstream fragments of the original Tel-Tel vector were cloned into the multiple cloning site of pUC19. The resulting vector was examined by restriction digest and sequenced and found to contain all functional elements of the original Tel-Tel reporter. In transient co-transfections with an Isce-I expression vector, the pUC19 Tel-Tel showed no significant difference in recombination rate, as compared to the original Tel-Tel vector (pUC-19 Tel-Tel 8%; original Tel-Tel 10%). This experiment demonstrated that the cloning procedure itself would not interfere

with the reporter function and I was able to proceed with replacing the telomere repeats.

Because ALT and telomerase positive cells are predicted to recombine non-telomeric DNA with the same frequency a non-telomeric recombination reporter should function similarly in either cell type. The first sequence examined in this context was taken from firefly luciferase. This sequence was essentially chosen arbitrarily, but more specifically chosen due to its non-human origin. Since this reporter must be integrated into human cancer cells, homology to other parts of the genome might interfere with specific recombination within the reporter. In order to generate fragments of luciferase appropriate for cloning, I designed PCR primers to amplify a 750 bp fragment of luciferase and introduce a 5' SacI site and 3' Bsu36I/NotI sites. PCR products were amplified, purified and digested with the appropriate enzymes for cloning into the upstream or downstream intermediate vectors. The resulting plasmids, referred to as pUC-19 Luc-Upstream or pUC-19 Luc-Downstream, were sequenced and shown to contain all functional elements and the predicted luciferase sequence in place of the telomere repeats. Furthermore, side-by side restriction digest of the telomeric intermediates and the luciferase intermediates showed the predicted pattern (Figure 3.3). Lastly, the downstream luciferase-containing fragment was cloned into pUC-19 Luc-Upstream, completing construction of the Luc-Luc plasmid. Restriction digest analysis of this construct and pUC-19 Tel-Tel showed the same

results, confirming that Luc-Luc had indeed been assembled as predicted (Figure 3.4).

Once the Luc-Luc reporter was successfully assembled, I sought to test its functionality in a transient assay. I performed two co-transfections with an ISce-I expression vector in U2OS cells: the Luc-Luc reporter as well as pUC-19 Tel-Tel as a positive control. While the Tel-Tel vector gave the predicted ~8% recombination, the Luc-Luc reporter failed to show any recombination above background. This transient assay was repeated and again the Luc-Luc reporter showed no recombination. Following this very surprising result, another non-human sequence was selected for cloning. Using the same PCR strategy employed to generate luciferase fragments, I was able to generate 750 bp cloning substrates from the kanamycin resistance gene. The same cloning procedure was followed and again all plasmids were verified by sequencing and restriction digest. Following completion of the Kan-Kan vector, transient co-transfection with ISce-I again failed to produce any detectable recombination.

After discovering that luciferase and kanamycin sequences were not functional in the context of my reporter, I sought to examine whether I could construct a functional reporter containing non-telomeric repetitive sequence. The sequence chosen first was similar to human telomeres, but contained two point mutations (T**G**AG**T**G instead of TTAGGG). Because no template existed to amplify 750 bases of this repetitive sequence, an alternative strategy was

developed. First, a commercially synthesized $(TGAGTG)_{13}$ oligo was enzymatically circularized and annealed to a short, linear complimentary oligo (5'– CACTCA –3'). This complex was then incubated with Φ29, which extended the linear oligo and created a very long CACTCA single strand via rolling circle amplification (RCA) (Figure 3.5). Annealing short TGAGTG oligos to the RCA product and elongating with a non-displacing polymerase generated the complementary strand, producing a fully double stranded version of the double mutant. Next, sonication generated smaller fragments, which were then blunted before the addition of a single deoxyadenine to each 3' end (Figure 3.5). At this point the repeat DNA fragments were suitable for Topo-TA cloning.

Topo TA cloning makes use of a linearized plasmid with two 5' deoxythymidine overhangs conjugated to topoisomerase I. In the presence of linear DNA with 3' deoxyadenine overhangs, topoisomerase will efficiently catalyze ligation of the two DNA substrates (Figure 3.6a). Using this method, I was able to generate a plasmid (henceforth referred to as pCR-II Mut2) containing approximately 600 bp of the double mutant sequence (Figure 3.6b). Before isolating inserts to be cloned into the upstream and downstream intermediate vectors, I had to make a several modifications to the pCR-II Mut2 plasmid. The pCR-II backbone contains a SacI site located on the 5' side of the insert and a 3' NotI site but lacks the 3' Bsu36I site required for cloning of the upstream intermediate (Figure 3.7). A small oligo containing a Bsu36I flanked by NotI and

XhoI sites site was ligated into NotI and XhoI sites in pCR-II Mut2 and the resulting plasmid was confirmed by sequencing and restriction digest. Additionally, the pCR-II Mut2 plasmid contained a BamHI site and a PstI site, the presence of which would interfere with subsequent cloning steps. These restriction sites were individually eliminated by digestion with the appropriate enzyme followed by enzymatic fill in or resection of the resulting overhang and re-ligation of the blunted ends. After introduction/elimination of the necessary restriction sites, the double mutant insert was cloned into the upstream and downstream intermediate vectors, followed by assembly of the full Mut2-Mut2 reporter. This final construct was verified by sequencing and restriction digest (Figure 3.8).

After successfully assembling the Mut2-Mut2 vector, I sought to verify its functionality in a transient assay. I again performed two co-tansfections with in U2-OS: Tel-Tel/Isce-I and Mut2-Mut2/Isce-I. After 48 hrs the cells were collected and analyzed by FACS. The Tel-Tel control gave the expected ~8% and the Mut2-Mut2 showed ~4.5% recombination (Figure 3.9). This experiment verifies that the Mut2-Mut2 recombination reporter is indeed functional, and experiments to examine its recombination rate after stable integration are ongoing.

Materials and Methods

Preparation of Intermediate Cloning Vectors

The Tel-Tel vector was digested with the appropriate enzymes for either upstream or downstream cloning. In each case, 2 μg of the Tel-Tel plasmid was digested with 10 U of each restriction enzyme. Double digests were performed in the buffer recommended by New England Biolabs. Following digestion, the fragments were run on a 1%/1X TAE gel and purified using QIAGEN's gel extraction kit according to manufacturer protocol. Purified inserts were then ligated into the MCS of pUC-19 (Invitrogen) at a 3:1 molar ration of insert to backbone. Ligations were performed using the FastlLink DNA ligation kit for 1hr at room temperature (Epicentre). Following ligation, a commercially available strain of competent *E. coli* cells, OneShot Stbl3 (Invitrogen), were transformed with 5 μl of the ligation reaction. Resulting clones were grown in ampicillin containing media and plasmids were extracted using QIAGEN miniprep kit. Sequencing primers used for verification are listed in Table 3.1.

PCR Generation of Luciferase and Kanamycin Inserts for Cloning

Plasmids containing either luciferase or kanamycin were used as PCR templates. For either sequence, 30 ng of plasmid was used as input for the PCR reaction. Primers were used at a final concentration of 0.2 μM and the sequences are listed

in Table 3.1. For luciferase, the following cycling conditions were used: *95℃/10 min → [95℃/30s → 50℃/30s → 72℃/30s] X 15 cycles → 72℃/10 min*. The cycling conditions for kanamycin were the same with the exception of the annealing temperature, which was 62°C. PCR products were run on a 1% agarose/1X TAE gel and purified using QIAGEN's gel extraction kit according to manufacturer's protocol.

Generation of Non-Telomeric Repeat Cloning Substrates

A custom 5'-PO_4-(T**G**AG**T**G)$_{12}$ (double-mutant) was generated by Sigma's oligo synthesis service. This oligo was then circularized with T4 ligase in manufacture provided buffer for 1 hr at 37°C (Fermentas). Following circularization, the circular template was incubated with the linear, complimentary 5'- CACTCA -3' oligo, ϕ29 polymerase, and dNTP's for RCA. The single stranded RCA product was the annealed with shorter 5'- TGAGTG -3' oligo by heating a mixture of the two DNA species to 95°C and allowing to gradually cool to room temperature (~1 hr). This partially double-stranded DNA was incubated with T4 DNA polymerase and dNTPs to fill in any single stranded gaps. The purified DNA was then sheared by sonication (horn-cup style sonicator, Misonix 3000; p2, 30w, 90seconds sonication followed by 90seconds on ice, twice) to a smear of 200-1000bp according to agarose gel analysis. Following sonication DNA was precipitated

with sodium-acetate and ethanol, hydrated ($T_{10}E_1$ buffer) and the concentration determined by spectrophotometry. To prepare the fragmented DNA for TOPO TA cloning (Invitrogen, TOPO-TA pCRII) the DNA ends were polished (T4 DNA polymerase, 12ºC for 15min) and then a single dATP was added to the 3' ends of the fragments (Klenow (3'-5' exo – only in the presence of dATP). The cloning ready DNA was then column purified (Qiagen, min-elute kit).

Discussion

The elevated rates of telomere-specific homologous recombination observed in ALT cells likely results from a loss of suppression that remains intact in telomerase positive and normal cells. This suppression, however, does not affect general HR and telomerase positive and ALT cells have been shown to exhibit comparable rates of general recombination. I sought to construct a control vector that functioned in the same manner as the Tel-Tel reporter but containing non-telomeric DNA as a recombination substrate. Since no block to general recombination exists in either cell type, I predicted that the telomere repeats in Tel-Tel could be replaced with any arbitrary sequence and the resulting vector would function equally in either cell type. I successfully constructed two recombination reporters, one that used luciferase sequence as a recombination substrate and a second that used kanamycin sequence. Unexpectedly, neither of

these vectors produced functional GFP expression after transient co-transfection with an ISce-I expression vector. The exact reasons for the lack of functionality in these two vectors remains unclear but is unlikely due to a fundamental inability to recombine these two particular substrates. The nature of this type of reporter requires recombination followed by splicing of the sequence of interest before the synthesis of functional GFP. It could be that the regions of luciferase or kanamycin selected for cloning introduced some unknown splicing factors, which may in turn have blocked one of the steps required for the reporter to function.

Following the failure of the Luc-Luc and Kan-Kan vectors, I wanted to specifically examine repetitive, non-telomeric sequence in the context of this reporter system. With the help of a fellow lab member, Andrew Ludlow, an RCA strategy was employed for generating fragments of mutant telomere sequence of the appropriate size for constructing a recombination reporter analogous to Tel-Tel. The Mut2-Mut2 vector was successfully constructed and shown to be functional in a transient assay. This experiment demonstrated that a sequence other than telomere repeats could function in a Tel-Tel-style recombination reporter. The most immediate experiment needed to follow this work is stable incorporation of the functional Mut2-Mut2 in an ALT and a telomerase positive cell line using the Cre/loxP exchange strategy outlined in Chapter 2. The prediction is that it should recombine at similar rates in either cell type. These data, in conjunction with my Tel-Tel data, will provide the first conclusive

evidence that telomere-specific HR is suppressed in telomerase-positive cells and that suppression is absent in ALT cells.

In addition to examining recombination of the "double mutant" TGAGTG repeats, work has begun on generating a so called "single mutant" version of the reporter which uses TGAGGG repeats as the recombination substrate. The triplet G's common to mammalian telomeres can form G-quartets. This unique secondary chromatin structure may play an important role in the recruitment of factors important in the suppression of recombination at telomeres. The single mutant would preserve the triplet G's and address some interesting questions about their role in telomere biology.

igure 3.1 Cloning strategy for isolating and excising telomer repeats om Tel-Tel vector.

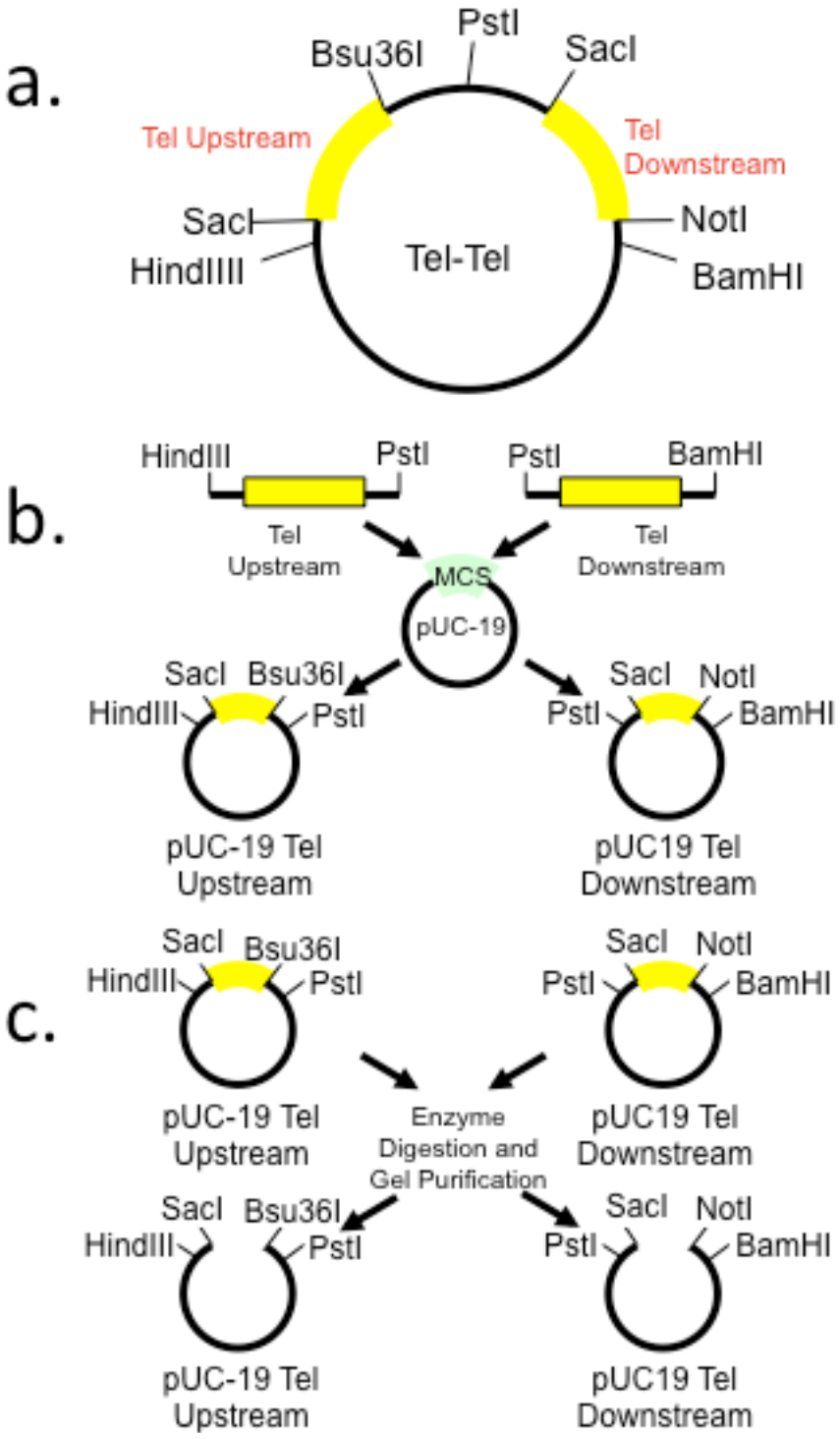
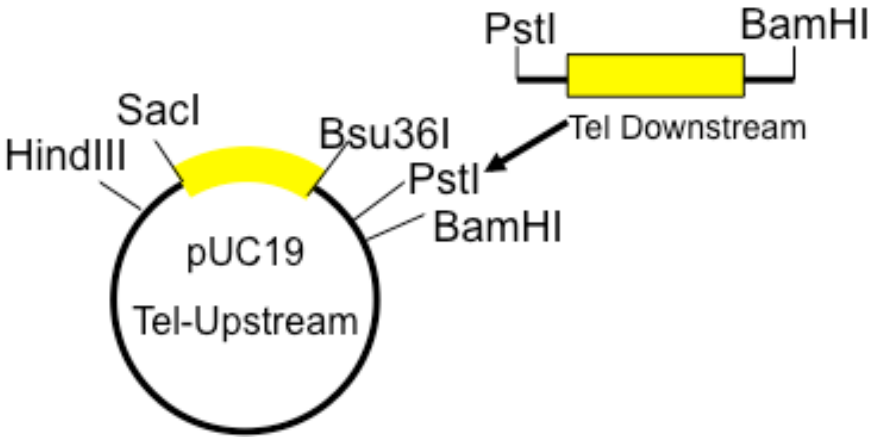
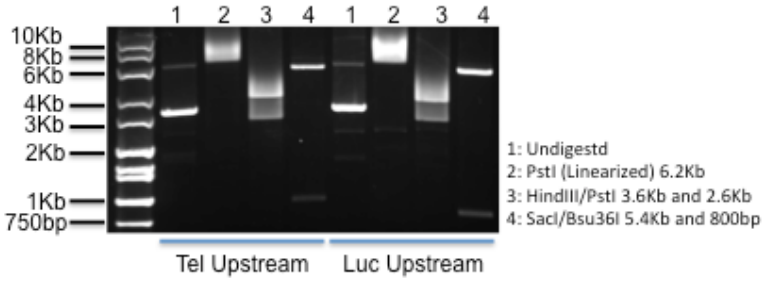
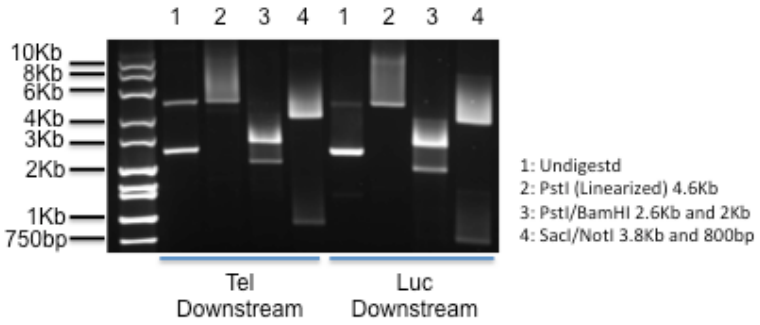


Figure 3.2 Ligation of the downstream fragment into the upstream intermediate plasmid reconstitutes the functional reporter. This reaction serves as the final cloning step in the generation of all non-telomeric recombination reporters generated.

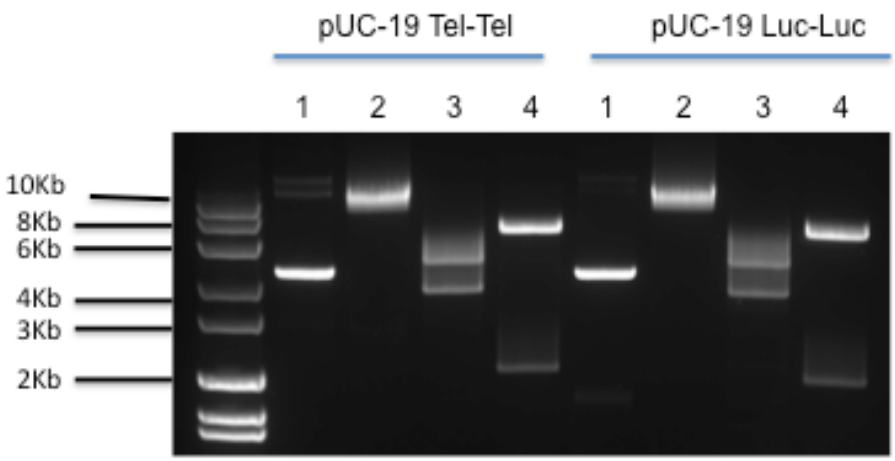


termediates. Restriction digest shows successful cloning of Luc termediates.





enzymes used in cloning shows identical pattern.



1: Undigested
2: PstI (Linearize) 8Kb
3: HindIII/PstI 4.5Kb and 3.5Kb
4: PstI/BamHI 6Kb and 2Kb

repeats in the Tel-Tel vector and generating the Mut-Mut vector.

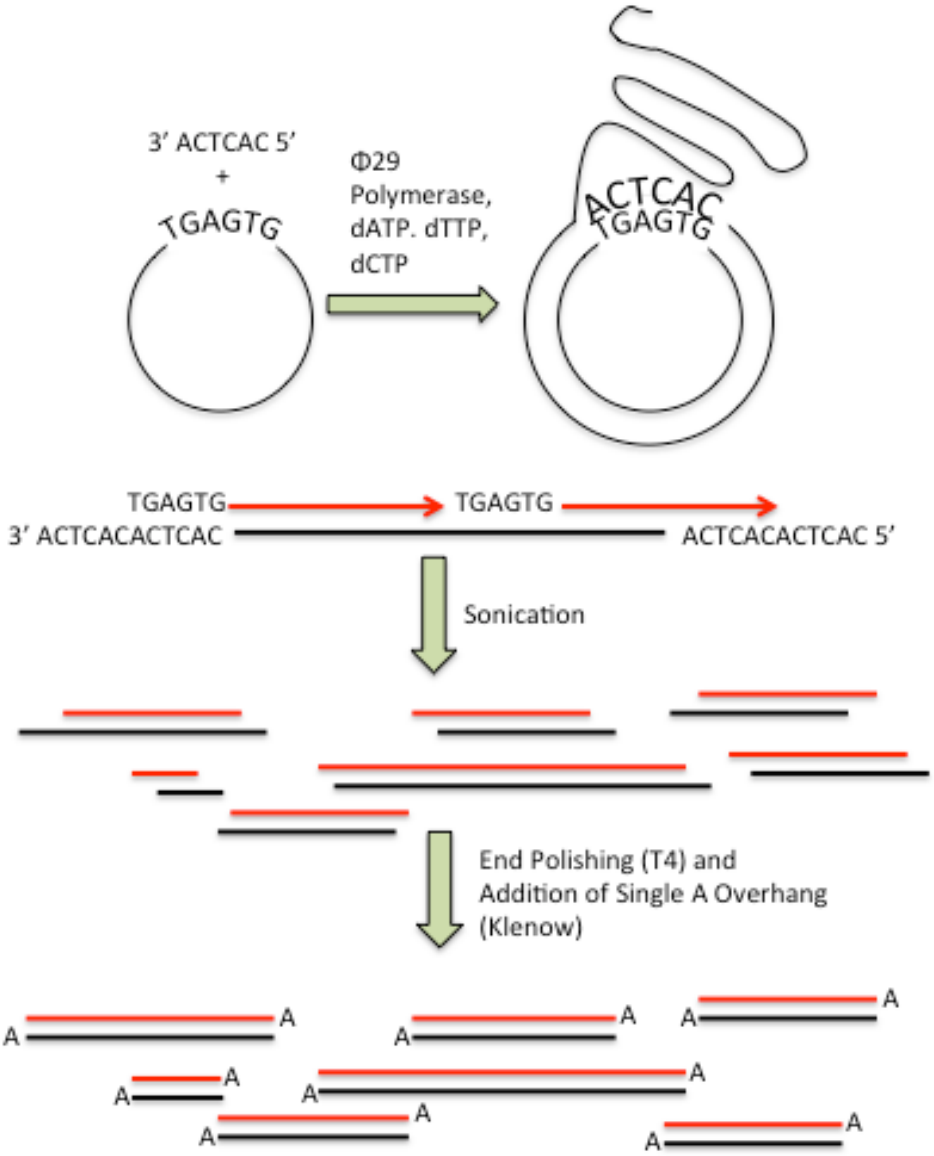


Figure 3.6 Topo-TA cloning succesfully generated a plasmid containing ~600 bp of TGAGTG repeats. (a) DNA of interest can be incorporated into the pCRII vector in a reaction catalyzed by Topoisomerase. (b) A single clone was identified as containing an insert of an appropriate size (red arrow) for use in further cloning.

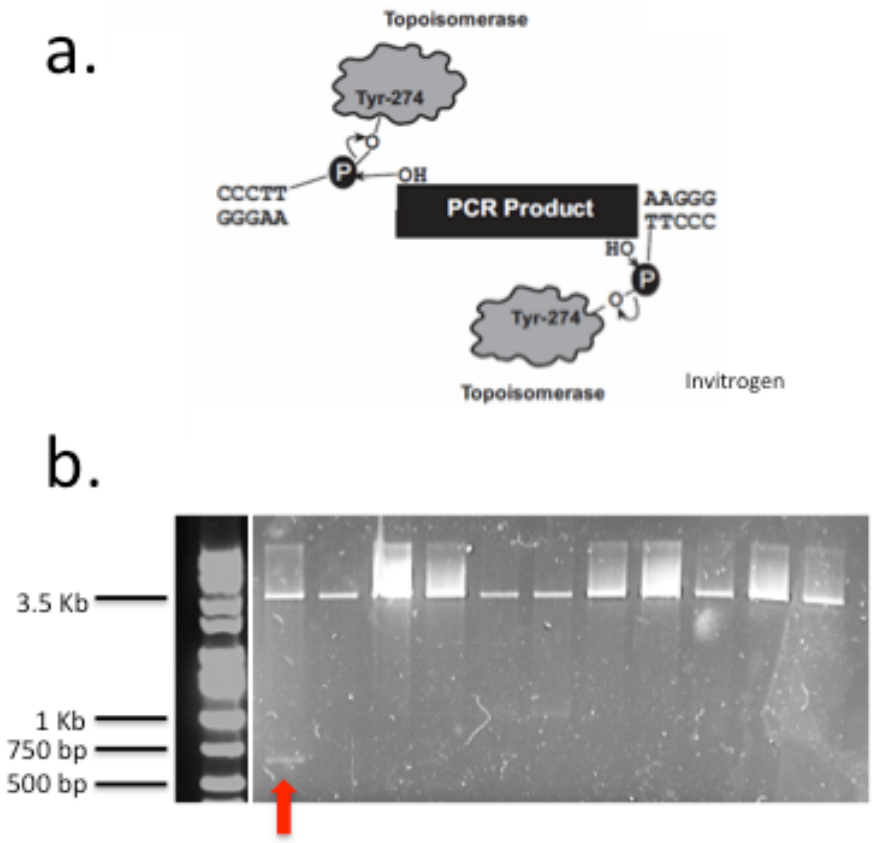


Figure 3.7 pCRII-Topo vector requires modification before use in subsequent cloning steps. Restriction sites indicated in blue are required for cloning with intermediate vectors and present in unmodified pCRII-Topo. Restriction sites outlined in red were eliminated to avoid interference with subsequent cloning steps. Green arrow indicates location of Bsu36I site inserted by ligation with a short oligo.

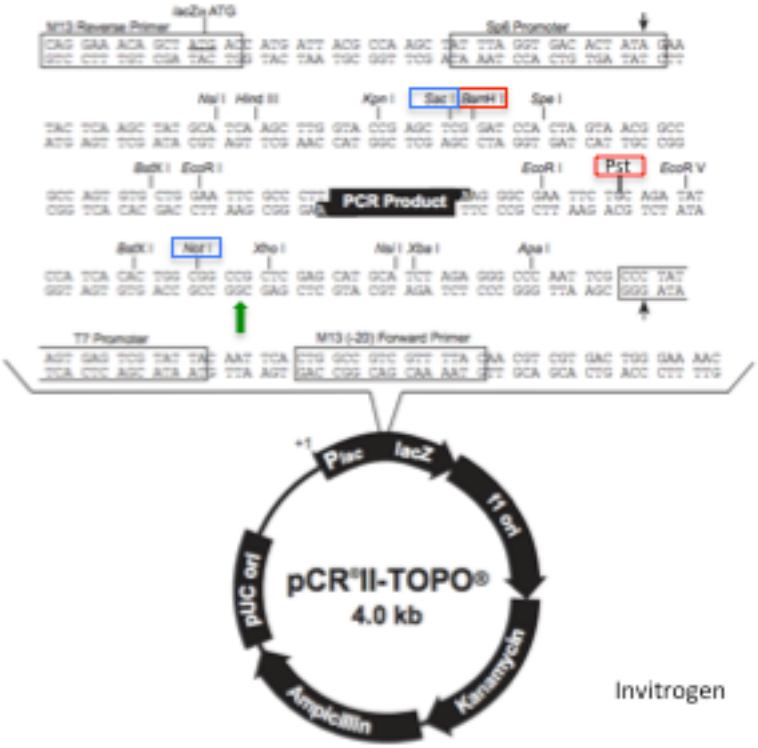
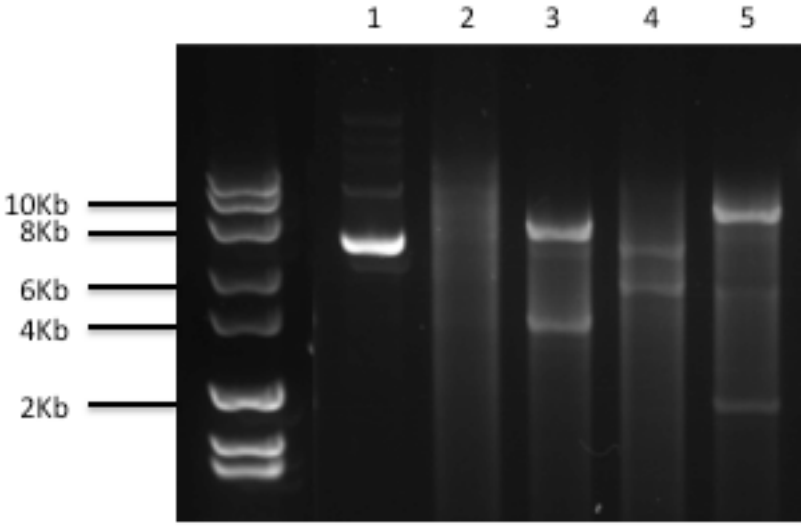
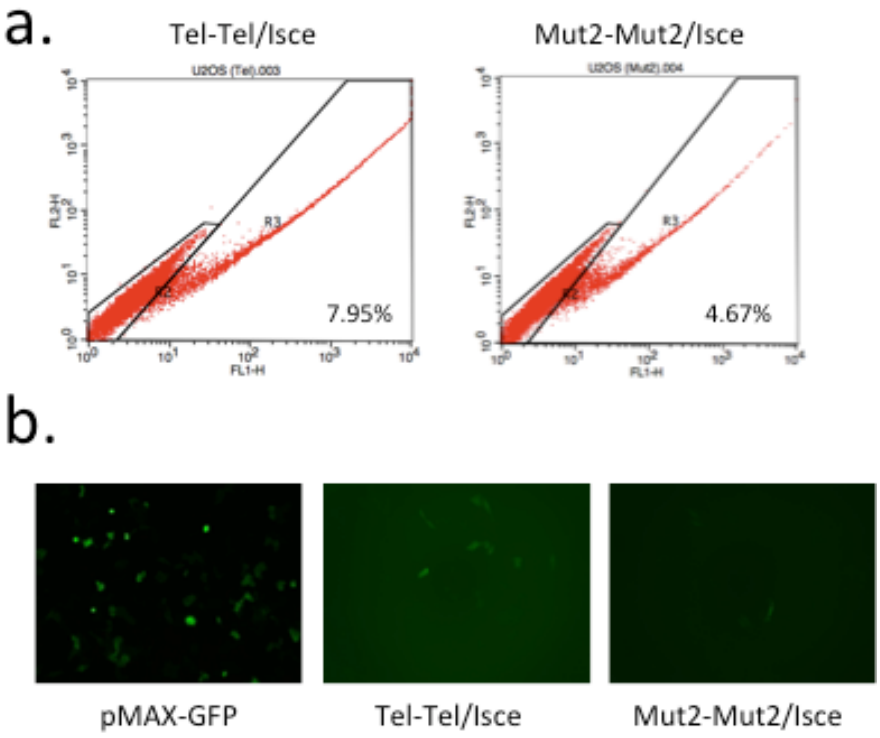


Figure 3.8 Restriction digest analysis of final Mut2-Mut2 vector shows predicted pattern.



1: Undigested
2: PstI (Linearize) 8kb
3: HindIII/BamHI 5.5kb and 2.7Kb
4: HindIII/PstI 4.4Kb and 3.7Kb
5: PstI/BamHI 6Kb and 2Kb

Figure 3.9 The Mut2-Mut2 recombination reporter is functional in a transient assay. (a.) FACS analysis shows the Mut2-Mut2 reporter to recombine at 4.67% in a transient assay. The Tel-Tel vector recombines at nearly 8% in a transient assay performed side-by-side. (b.) Fluorescence microscopy also shows functional recombination of the Mut2-Mut2 reporter, as indicated by the presence of green cells.



Chapter Four

A Candidate Approach to Validating the Tel-Tel Reporter

Many years of research have led to the working hypothesis that the ALT pathway involves homologous recombination of telomere repeats. However, the data have been insufficient to develop a comprehensive model for the molecular details that permit this type of recombination. A number of studies have examined the roles that various proteins play in the regulation of ALT-related phenotypes, such as PML body formation, T-SCE, and telomere length. Unsurprisingly, a number of proteins that interact with telomeres directly or indirectly via interactions with shelterin components affect these commonly accepted ALT assays. While these types of analyses provide good suggestive evidence regarding the molecular details of ALT, they suffer from the major limitation that they measure characteristics related to the ALT mechanism, rather than the biology intrinsic to the mechanism itself. The Tel-Tel assay directly measures a cell's ability to recombine telomeric DNA, the phenomenon most fundamentally at the heart of the ALT pathway. Therefore, the Tel-Tel reporter serves as a more informative and useful tool for examining a protein's involvement in the ALT mechanism. Drawing from previous reports implicating specific proteins in the

regulation of recombination phenotypes, I sought use the Tel-Tel recombination assay to examine the effect of mutant versions of the shelterin component Rap1.

Results

As mentioned in Chapter 1, recent work has implicated Rap1 in suppressing telomeric recombination (Sfeir 2010). It has previously been shown that in several species of yeast Rap1 orthologs suppress non-homologous end-joining (NHEJ) or homology directed repair (HDR) (Cesare 2008; Marcand 2008). Sfeir et al demonstrated that conditional deletions or shRNA knockdowns that removed Rap1 from mouse telomeres resulted in a significant increase in T-SCEs. Their data led them to postulate a model wherein Rap1 binds to the shelterin complex and suppresses HDR at telomeres. They further suggested that Rap1 might recruit other unknown proteins to telomeres via interaction with its BRCT domains. In order to investigate the role Rap1 plays in permitting Tel-Tel recombination, I examined the effect of two mutant forms of Rap1.

A549 Tel-Tel and U2-OS Tel-Tel cells were infected with a version of Rap1 deleted either for its Myb (Rap1 ΔMyb) domain or its BRCT domain (Rap1 ΔBRCT). Following retro-viral infection, western blot was employed to verify the presence of the mutant Rap1 construct. As shown in Figure 4.1, the Rap1 ΔBRCT was successfully introduced in both cell types. However, for reasons that remain

unclear, repeated attempts to express the Rap1 ΔMyb construct were unsuccessful. It should be noted that the Rap1 antibody used for western blot is not specific to the Rap1 myb domain. Next I examined the effect of the Rap1 mutant construct on the Tel-Tel assay. As shown in Figure 4.2, U2-OS cells expressing only wild type Rap1 gave the predicted ~1% Tel-Tel recombination, whereas expression of the Rap1 ΔBRCT produced an increase in Tel-Tel recombination of approximately 50%. The lack of any detectable change in recombination for the Rap1 ΔMyb mutant comes as no surprise considering it could not be detected by western blot.

Following the observation of increased Tel-Tel recombination, I wanted to examine whether the Rap1 mutant had any effect on C-cirles. Introduction of the Rap1 ΔBRCT led to a doubling of C-circles as compared to uninfected controls (Figure 4.3). Despite the variable nature of the C-circle assay, statistical analysis revealed this difference to be significant ($p = 0.02$). Again, the Rap1 ΔMyb cells showed no difference from controls due most likely to an absence of the mutant construct. In the telomerase positive cell line, A549, no change in Tel-Tel recombination was observed after introduction of Rap1 mutants. Interestingly, A549 cells expressing Rap1 ΔBRCT showed a statistically significant increase in C-circles (Figure 4.4; $p = 0.02$). It should be noted, however, that this increased level of C-circles is <1% of the level observed in wild type ALT cells.

Materials and Methods

C-Circle Assay

Genomic DNA was isolated from 5 X 10^6 cells using commercially available kit from Roche and digested for 8 hrs with the restriction enzymes EcoRI and BamHI. 32 ng of digested genomic DNA was used as input for C-circle assay. DNA was incubated at 30°C overnight in 40 ng/ul BSA, 1 mM dATP, 1 mM dTTP, 1 mM dGTP, .1% TWEEN-20, 1X ϕ29 Buffer (New England Biolabs) and 1U ϕ29 polymerase (New England Biolabs). Following incubation, samples were hybridized to Zeta probe blotting membrane (Biorad) by slot blot. The blot was then probed with radiolabeld CCCTAA probe overnight at 42°C. Following incubation the blow was washed in 2X SSC for 15 min, two ten minute washes in 0.1X SSC/0.1% SDS for 10 minutes, and a final wash in 2X SSC.

Western Blot for hRap1

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% TritonX-100, 1% Sodium deoxycholic acid, 0.1% SDS and 1X Roche Protease inhibitor). 30 μg of lysate was run on a 15% SDS-PAGE gel. Western blot was performed with a mouse monoclonal anti-hRap1 antibody (abcam ab14404) at a dilution of 1:1000.

Tissue culture and retroviral infection followed protocol described in chapter 2.

Discussion

Despite a lack of comprehensive model for the mechanism underlying ALT, a growing collection of proteins have been implicated in regulating recombination phenotypes related to ALT. Among them, the shelterin component Rap1 has been suggested to specifically suppress telomere-specific homologous recombination. Since the Tel-Tel assay directly measures a cell's ability to recombine telomeric DNA, I examined the effect of overexpression of mutant versions of the Rap1 protein. I chose two versions of Rap1, each deleted for one of its protein-protein binding domains. The Myb domain allows Rap1 to locate to telomeres through interaction with the Myb domain of TRF2. The BRCT domain of Rap1 theoretically facilitates additional protein-protein interactions, although no binding partner for this domain has been identified. Unfortunately, for reasons that cannot be explained, I was unable to successfully express the ΔMyb mutant in either cell line tested. Expression of the ΔBRCT mutant, however, resulted in increased Tel-Tel recombination as well as an increase in the common ALT marker, C-circles.

The working hypothesis for how ALT cells maintain their telomeres by homologous recombination involves a loss of some suppressive element that is maintained in normal and telomerase positive cells. One can easily imagine that this suppressive element must be recruited to and maintained at telomeres. The observation that a loss of Rap1 leads to an increase in ALT-phenotypes, suggests that it may serve as a scaffold for recruiting an unidentified protein to telomeres, which in turn suppresses homologous recombination. In this scenario, a mutant version of Rap1 lacking the putative protein binding BRCT domain would displace a fraction of wild type Rap1 from proteins. As a result, a lesser fraction any protein requiring interaction with the Rap1 BRCT domain would localize to telomeres. The increased Tel-Tel recombination and C-circle formation observed in the presence of Rap1 ΔBRCT may be due to an unknown protein responsible for suppressing recombination experiencing diminished telomeric localization. Future experiments should examine the effect of full Rap1 knockdown on Tel-Tel recombination. One would expect this to result in recombination rates even higher than those observed in the presence of mutant Rap1. Furthermore, if the BRCT domain is responsible for recruiting the suppressive factor, I would expect the expression of a Rap1 ΔBRCT mutant after complete knockdown would fail to return recombination rates to their normal level.

While the data collected from the Tel-Tel assay, further implicates Rap1 as important to the ALT mechanism, it offers little in the way of precise

mechanistic insight. The most important conclusions to draw from these experiments are that they validate the Tel-Tel reporter as a biologically relevant ALT reporter. Demonstrating that the Tel-Tel system can be experimentally manipulated to recapitulate the previously known ALT biology, strengthens the case for its use as a new measure of ALT. Since the Tel-Tel reporter provides a measure of the most basic biology involved in ALT, telomere-specific homologous recombination, future studies utilizing knockdowns, chemical inhibitors, over-expressions or other perturbations, will provide valuable insight into the precise mechanism of this telomere maintenance mechanism.

Figure 4.1 Western blot for hRap1. 30 μg of lysate was run on a 15% SDS-PAGE gel. Western blot was performed with a mouse monoclonal anti-hRap1 antibody (abcam ab14404) at a dilution of 1:1000.

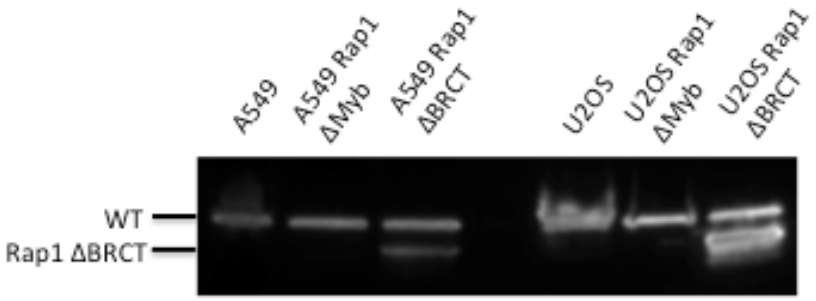


Figure 4.2 Tel-Tel recombination in the presence of a mutant Rap1.

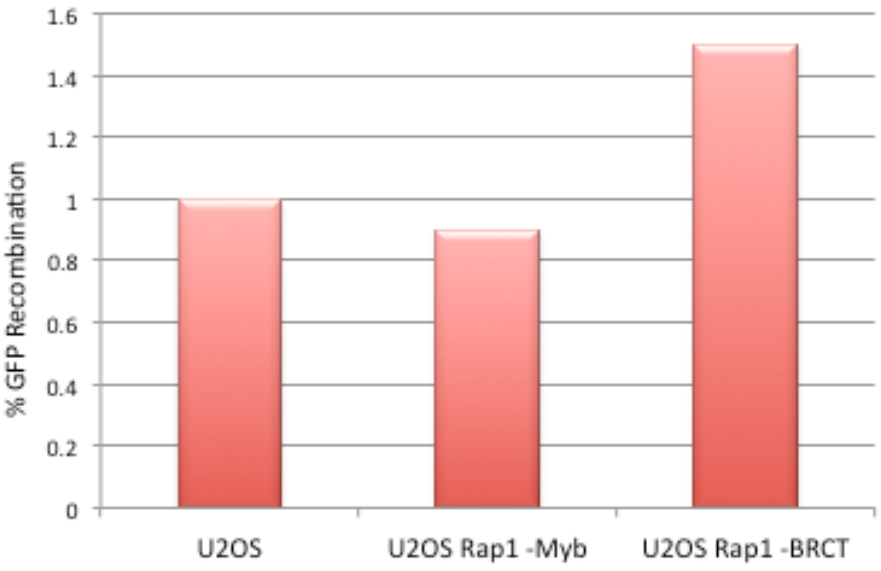


Figure 4.3 Expression of ΔBRCT mutant Rap1 leads to an increase in C-circle formation in U2-OS. n = 6, p = 0.02

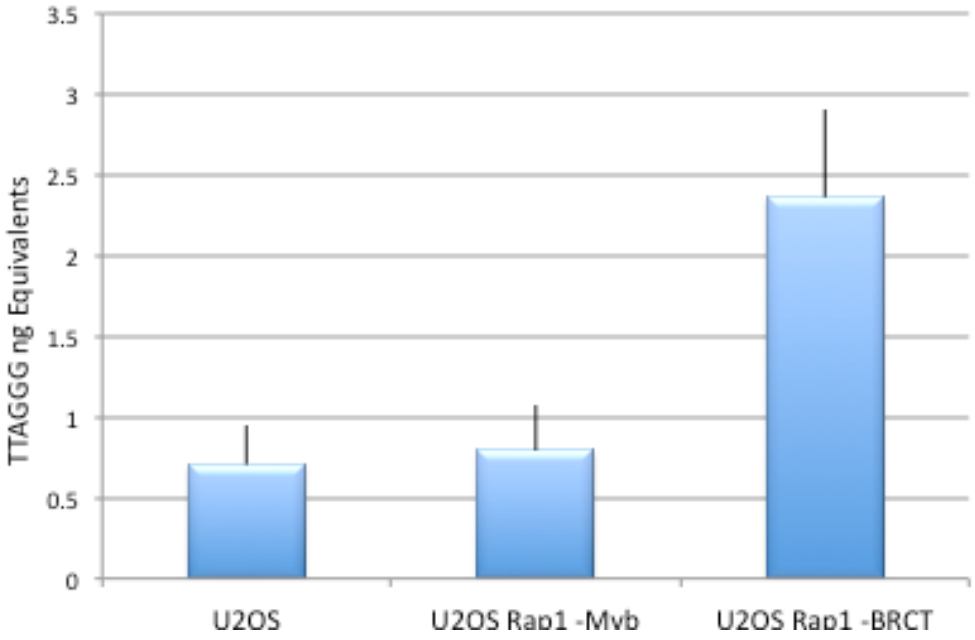
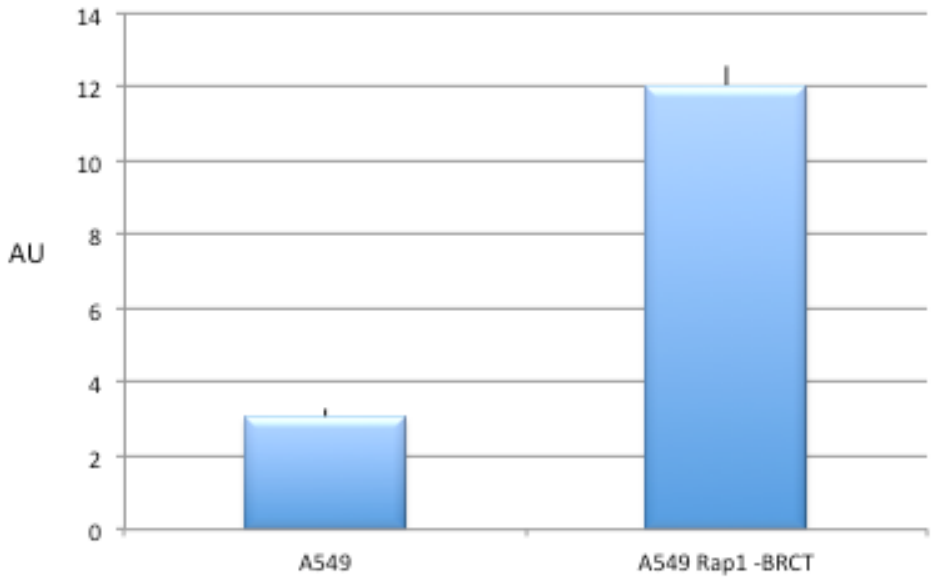


Figure 4.4 Expression of ΔBRCT mutant Rap1 leads to an increase in C-circle formation in A549. n = 6, p = 0.02



Chapter 5

Discussion and Future Directions

For my dissertation research, I sought to investigate the molecular mechanism of the ALT pathway. This telomere maintenance program has long been believed to require homolous recombination of telomere repeats but the precise molecular details have remained a mystery. My work made use of a reporter that specifically measures a cell's ability to recombine telomeric DNA. Using this Tel-Tel reporter I have provided the first concrete evidence that ALT cells are capable of specifically recombining telomere repeats, whereas this type of recombination is blocked in telomerase positive cells. I addition to providing valuable information about the basic biology of ALT, this work provides a valuable new tool for further investigations of the ALT mechanism. Previously established experimental tools measure some of the established phenotypes associated with ALT but do not actually measure the telomere specific homologous recombination fundamental to the ALT pathway. Future work using the Tel-Tel system will allow investigators to examine factors that specifically contribute to or prevent homologous recombination at telomeres. Two proteins of particular interest are the chromatin remodeling proteins ATRX and DAXX.

ATRX and DAXX have previously been shown to localize to telomeres where they are required for the formation of the histone variant H3.3 (Lewis 2010). This led a group of researchers to examine the telomere status of a panel of pancreatic neuroendocrine tumors (PanNETs). Their results found that all samples mutated for either ATRX or DAXX showed the heterogeneous telomere length characteristic of ALT cells (Heaphy 2011). Furthermore, they found that the prototypical ALT cell line U2-OS carries a homozygous deletion of exons 2 through 19 in ATRX (Heaphy 2011). The strong correlation between ALT phenotypes and mutations or deletions for ATRX and/or DAXX makes them very intriguing candidates for further study. Future studies with the Tel-Tel system should investigate the effect of ATRX/DAXX knockdown in telomerase positive cells. This type of experiment would address whether ATRX and DAXX are necessary for the suppression of telomeric HR. Additionally, overexpression studies in ALT cell lines would provide information regarding whether or not ATRX and DAXX are sufficient to suppress telomere HR. The Tel-Tel reporter is ideal for these studies, as it would be directly influenced by the chromatin modifying properties of ATRX and DAX.

In addition to developing the Tel-Tel reporter system, I was also able to construct a non-telomeric repeat control reporter that functions in a transient assay. The most obvious and immediate follow up to this work will be to stably integrate this vector using the Cre/loxP exchange strategy employed for the Tel-

Tel experiments. Since general recombination should function similarly in both cell types, I fully expect that this reporter will give comparable rates of recombination in telomerase positive or ALT cells. In addition to the "double mutant" sequence, current work to construct a so-called "single mutant" is ongoing. Whereas the double mutant contains two variations to canonical telomeric sequence (TGAGTG) the single mutant will maintain the characteristic triplet G's found in telomeres (T**G**AGGG). It has been speculated that the secondary structure formed by this type of G-rich sequence may have important biological consequences. This reporter will again be integrated into telomerase positive and ALT cells although the results are a bit more difficult to predict. It may be that the single mutation will be sufficient to disrupt the suppression of telomeric HR, and therefore it would recombine equally in either cell type. Alternatively, if the triplet G's are involved in the suppression, it would behave similarly to the Tel-Tel reporter and fail to recombine in telomerase positive cells. Interestingly, literature regarding chromatin localization of ATRX and DAXX suggest that these proteins preferentially bind to G-rich repetitive regions such as telomeres (Law 2010).

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