Functional Studies of Human Telomerase Holoenzyme

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ABSTRACT

Telomeres are the DNA structures that cap the ends of linear chromosomes. In humans, telomeres are composed of the hexameric repeats TTAGGG. The reverse transcriptase, telomerase maintains the telomeres by adding the TTAGGG repeats to the chromosomes during cell division. In the absence of telomerase, telomere length limits the self-renewal capabilities of cells. Telomerase is expressed in approximately 90% of all human cancers, making it an almost universal therapeutic target as well as an important player in the progression from healthy to cancerous cell.

The biophysical studies discussed in this thesis focus on expanding the understanding of the function and enzymology of human telomerase. We have developed a working hypothesis that the dimeric telomerase behaves as a single-pass enzyme, which needs to be reactivated after its processive extension reaction on a substrate. We used different biochemical approaches in conjunction with a highly quantitative activity assay to test this hypothesis. We first observed that in both gel based TRAP and the digital droplet TRAP (ddTRAP) assays, telomerase exhibited catalysis-dependent inactivation. The qPCR and ddPCR analysis found that the enzyme was stable without catalysis. In sequential extension

experiments, telomerase showed both fast-acting and slow-acting sites and these two types of active sites had different substrate affinities and acted in tandem. The sequential action of these two active sites required that both must be harbored by individual dimeric telomerase complexes. This suggests that the two active sites in each enzyme are asymmetrical, one fast and one slow, and after two passes of reaction, a dimeric enzyme becomes inactive. Furthermore, we discovered the potential of many cancerous and non-cancerous cell lysates with and without telomerase activity to reactivate the catalytically exhausted telomerase complexes. Taken together, the data support the single-pass hypothesis, provide a new catalytic mechanism for telomerase holoenzyme, and suggest a novel regulatory mechanism of its activity in a catalysis-dependent manner.

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PRIOR PUBLICATIONS

Ludlow, A.T., Robin, J.D., **Sayed, M.,** Litterst, C.M., Shelton, D.N., Shay, J.W. and Wright, W.E. Quantitative telomerase enzyme activity determination using droplet digital PCR with single cell resolution. *Nucleic Acids Res*, 42(13), 2014. e104doi: 10.1093/nar/gku439. Epub 2014 May 26. PMID: 24861623

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Ao Cheng, **Mohammed Sayed**, Woodring E. Wright and Qiu-Xing Jiang* (2016). Novel 3-Step Purification of Active Human Telomerase Complex
(In preparation)

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LIST OF ABBREVIATIONS

ALT- Alternative Lengthening of Telomeres

AVG- Average

BP- Basepair

DNA- Deoxyribonucleic Acid

ddPCR- Droplet Digital Polymerase Chain Reaction

ddTRAP- Droplet Digital Telomeric Repeat Amplification Protocol

hTERT- Human Telomerase Reverse Transcriptase

hTR- Human Telomerase RNA

mRNA- Messenger RNA

M & M- Michaelis-Menten

nt- nucleotides

PAGE- Polyacrylamide Gel Electrophoresis

PCR- Polymerase Chain Reaction

qPCR- Quantitative Polymerase Chain Reaction

RNA- Ribonucleic Acid

RNP- Ribonucleoprotein

RRL- Rabbit Reticulocyte Lysate

SDS- Sodium Dodecyl Sulfate

siRNA- Short interfering RNA

TRAP- Telomeric Repeat Amplification Protocol

UPL- Universal Probe Library

I. Introduction into Telomere Biology

History of Telomere Biology and the End Replication Problem

Telomeres were predicted to exist as early as 1938 by Herman Müller, who along with Barbara McClintock (1941) discovered that these capping structures were able to protect the linear ends of chromosomes from fusing. This early observation demonstrated just one of many important roles played by the telomeres in cells. In 1961, Leonard Hayflick and Paul Moorhead were able to show that cells in culture grew for a finite amount of time. The time limit on the cells ability to proliferate in culture became quite predictable to them and was later called the "Hayflick limit". This introduction of a molecular clock that pre-determined the cells ability to proliferate in culture went against the widely held view at the time that cells should be immortal in culture, as long as the optimized culture conditions were met. It was almost two decades later that the fundamental role of the telomere was realized.

The discovery of the structure of DNA as well as how DNA was replicated and the passing of genetic material from a single parental cell into daughter cells forever changed biology (Watson and Crick 1953). This seminal discovery paved the way for modern molecular biology we all know today. It was quickly realized however, that the linear nature of DNA along with the ability of its replication machinery to move only unidirectionally, 5' to 3', would lead to a loss in genetic material at the lagging strand of DNA synthesis. This phenomenon was dubbed "the end replication problem" (Watson 1972). It was further characterized by Olovnikov in 1971 and 1973 that a loss in DNA would occur after each round of cell division and that loss would be at least equal to the length of the last Okazaki fragment used in that particular replication event on the 3' end of the telomeric lagging strand. The concept of the end replication problem along with the previous observations by Hayflick and Moorhead formed the beginning of a molecular basis for our understanding of replicative senescence. The loss of DNA in the telomere region of the chromosome after each round of cellular replication/division was the molecular clock (or replicometer) that governed the lifespan of the cells in culture. It has been realized that the

telomeres do not just play a protective role in the cell, but a fundamental role in cellular replication/ reproduction and cellular aging/death.

In 1978 Elizabeth Blackburn and Joseph Gall discovered the sequence of the telomeres in *Tetrahymena thermoplhilia*. It was a hexameric array of TTGGGG repeats on both strands of the each chromosome. Blackburn predicted that telomeres were the solution to the end replication problem because although the telomeres would shorten after every cell division, the loss of this TTGGGG repeat sequence would not be detrimental to the cells ability to pass on vital genetic information to its offspring's if there was a mechanism protecting the germline from losing telomeric repeats. The telomeres were then shown to protect the integrity of the DNA in a fashion very different from the original observation by Möeller and McClintock decades earlier. Because *Tetrahymena* could grow in culture indefinitely, Blackburn predicted that there must be a mechanism by which the telomeres were maintained. This hypothesis turned out to be true and almost a decade later Blackburn and Greider discovered the enzyme that maintained the telomeres, called terminal telomere transferase, now known as telomerase. Telomerase was found to be a ribonucleoprotein complex which contained its own template RNA (TR). The template RNA allowed telomerase to avoid the need for an RNA primer to extend the DNA strand like most polymerases. This *de-novo* synthesis of hexameric repeats allowed the organism to maintain the ends of the DNA even after the loss occurring from incomplete DNA replication during cell division. Together with Jack Szostak, who found that this mechanism also existed in yeast, Blackburn and Greider would share the Nobel Prize (2009) for discovering the fundamental roles of telomeres and telomerase in cells.

Modern Biology of Telomeres and Telomerase in Humans

Telomeres are dynamic DNA-protein complexes at the end of chromosomes. Human telomeres are composed of an array of TTAGGG hexameric repeats which vary in length between 15-20 kilobases in human at birth. Human telomerase, the RNP enzyme that maintains the telomeres, was first identified in HeLa cell lysates (cancer cell line) (Morin et al. 1989). However, it was shown that telomerase was not

expressed in somatic cells (Harley et al. 1990) and the lack of telomerase rendered these cells unable to maintain their telomeres to prevent the ~ 50-100 base pairs lost after each round of replication (Wu et al. 2012). The lack of telomerase in all human tissues spurred researchers in the 1990s to understand this mysterious enzyme. Human telomerase was found to comprise of two key components, human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR or hTERC) (Nakamura et al. 1997 and Feng et al. 1995). Together, hTERT and hTR were enough to reconstitute telomerase activity in vitro (Weinrich et al. 1997). Re-expressing telomerase in cells that lacked telomerase was shown to prevent the shortening of telomeres and therefore replicative senescence (Kim et al 1994; Shay and Bacchetti et al. 1997). Furthermore, the addition of only hTERT to cells that did not express telomerase was enough for the cells to bypass the "Hayflick limit" and continue to divide in culture (Bodner et al. 1998). These findings cemented the role of telomere length as the limiting factor for cells ability to grow indefinitely.

Most human somatic tissues have no detectable telomerase activity, but it is widely believed that at one point in time these tissues did have some telomerase activity. Stem cells as well as proliferating progenitor cells were shown to maintain telomerase expression and activity (Wright et al. 1996). Telomerase activity has also been detected in highly proliferative somatic cells such as suprabasal skin cells (Taylor et al. 1996) and transient amplifying colorectal stem-like cells (Tahara et al. 1999). Peripheral blood mononuclear cells such as lymphocytes have also been shown to have telomerase activity albeit transiently after stimulation (Chebel at al. 2009). Because telomere shortening plays a major role in the ability of cells to replicate and divide, and telomerase plays a role to combat the replicative senescence, telomerase has evolved as a key player in the field of regenerative medicine and anti-aging therapies.

Telomere and Telomerase-Related Diseases

Telomere length in humans is heritable and only very recently has it been shown to play a critical role in a wide spectrum of diseases known as "telomeropathies" (Holohan et al. 2014). These

telomeropathies range from diseases such as cardiovascular to even degenerative neurological diseases such as Alzheimer's and Parkinson's (Bojessen et al. 2013). Using a candidate gene approach, it was shown that a number of mutations in telomere and telomerase genes were responsible for these disease phenotypes. The first such mutation identified was in the *DKC1* gene, a gene that encodes for Dyskerin (Heiss et al. 1998). Dyskerin is a protein that plays a critical role in the telomerase holoenzyme by binding to and stabilizing hTR, the RNA component. The DKC1 gene mutation led to a rare genetic disorder, 1 in a million, known as dyskeratosis congenita. The symptoms of dyskeratosis congenita include skin hyperpigmentation, oral leukoplakia and nail dystrophy, with the leading cause of mortality being bone marrow failure and pulmonary fibrosis (de la Fuente et al. 2007). Today it has been shown that a variety of mutations in different genes play a clear role in many age-related diseases. The underlying cause is the inability of the cells to maintain their telomere length, leading to progressively shorter telomeres. Examples include mutations in TERT, CTC1 and TINF2, leading to diseases such as familial idiopathic pulmonary fibrosis, Coats plus syndrome and Hoyeraal-Hreiderasson syndrome, respectively (Armanios et al. 2013). Historically, androgens have been used as a treatment for diseases such as bone marrow failure and pulmonary fibrosis. The mechanism for why such a treatment was so effective was discovered recently. Danazol, a synthetic estrogen analog, was found to increase telomere length and telomerase activity in patients with telomere related diseases (Townsley et al. 2016). With the advent of next generation sequencing technologies and the increase of telomere length measurement assays in clinical settings, it is likely that we will see a more translational approach to the field of telomere biology.

Telomerase and Cancer

The vast majority of cells in humans are telomerase negative and this leads to progressive telomere shortening after every round of cell division. The telomeres shorten until they reach a critical length to where the cells will not divide anymore. This stage is known as M1 (Mortality Stage 1) or senescence (Shay & Wright 1989). The few surviving cells continue to divide, incurring a plethora of DNA damage

including but not limited to chromosomal bridge, breakage and fusions events; all of which are passed onto the next generation. Eventually the telomeres get so critically short that the cells enter the crisis stage, also known as M2 (Mortality Stage 2). The damage in the M2 stage results is the vast majority of cells undergoing apoptosis with only a rare chance for cell escaping. It is in this stage that the rare escaped cell (~1 in 10^{-7}) activates a mechanism to escape crisis, which is almost universally accomplished by the upregulation or reactivation of telomerase. Cells escaping crisis could thereafter maintain the telomeres and prevent themselves from dying (Shay & Wright et el. 1991). Telomerase re-activation has essentially immortalized these now pre-cancerous cells and is why telomerase is believed to be one of the hallmarks of cancer (Hanahan and Weingberg et al. 2000).

Advanced malignant cancer cells by definition must be able to divide indefinitely, and in order for cancer cells to achieve such immortality, they upregulate/reactivate telomerase. It has been shown that approximately 90% of all cancers have telomerase activity (Bacchetti and Shay et al. 1997). Through high selective pressures --- pressures that largely remain unknown, cancer cells develop two defining traits, short telomeres and telomerase reactivation. It would seem counterintuitive for a cancer cell to have short telomeres. One would think that since they have managed to hijack the telomerase, their telomeres would be very long. There seems to be a lack of selective pressure for cancer cells to have excess levels of telomerase, which would result in cancer cells having longer telomeres. It has been observed that ~90% cancers display telomere lengths equal to or less than those in cells of adjacent healthy tissues (Hiyama K et al. 2009). Cancer cells have developed this remarkable ability to express just enough telomerase for them to continue to divide indefinitely suggesting an unknown cytosolic mechanism for finely controlled telomerase catalysis. As always, there are exceptions to this. The 10% of telomerase positive cancers that have long telomere lengths, with respect to the adjacent normal tissue, appear no different from those with short telomeres. Since about 90% of all cancer cells are telomerase positive, the remainder that do not have telomerase could be cells that have acquired sufficient alterations to become cancerous, but remain normal (mortal). This notion could explain the small percent of adult cancers that spontaneously undergo

remission. The other mechanism by which cells can continue to divide without telomerase is ALT (alternative lengthening of telomeres) (Bryan et al. 1997).

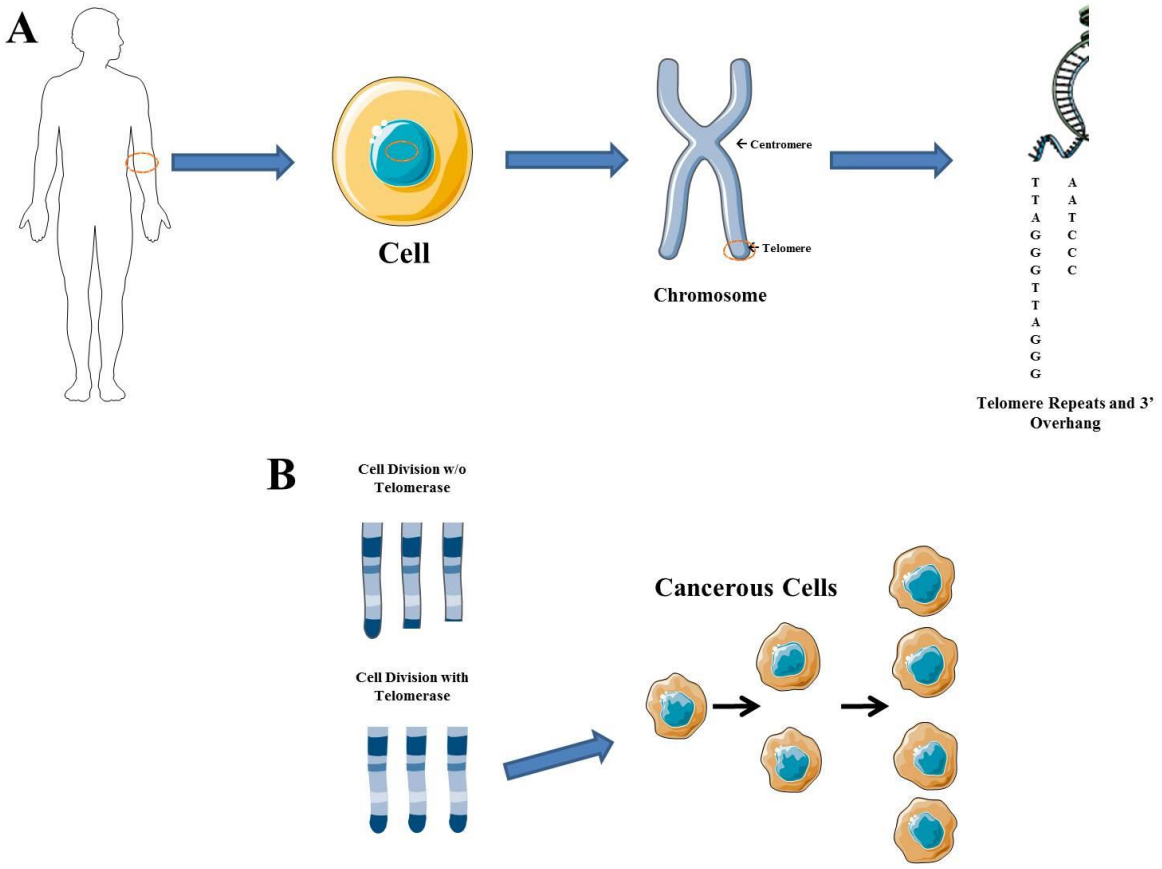

Figure 1-1: ***(A)*** Telomeres are located in the nucleus of the cell. Human telomeres contain TTAGGG repeats. ***(B)*** Telomerase and Cancer. Image depicts cell division with and without the presence of telomerase. Telomeres get shorter after each round of cellular division in normal cells. When telomerase is activated, telomere length is maintained. This allowed cancer cells to continuously divide.

II. Telomerase

Molecular Biology of Telomerase

Telomerase is a ribonucleoprotein which is comprised of two main components, the catalytic protein subunit, TERT, and the template RNA, TR or TERC. In humans the two components are often referred to as hTERT and hTR. Although the holoenzyme telomerase complex *in vivo* contains many more accessory proteins, hTERT and hTR are the minimally required components for telomerase activity in vitro (Beattie et al. 1998) (Weinrich et al. 1997). In vitro reconstitution experiments have shown that p23 and Hsp90, chaperone proteins, are also required for the proper assembly of the active telomerase enzyme, in vitro (Holt et al. 1999). The addition of gledanamycin, an Hsp90 inhibitor, to the IVT mix results in a decrease in telomerase activity. The telomerase complex in vivo is estimated to range anywhere between 500 kDa to 1 MDa, making it one of the larger molecular complexes in the cell. Table (1-1) shows a list of the other well-known accessory components of the telomerase holoenzyme (Egan and Collins et al. 2010). In vivo telomerase is assembled in Cajal bodies, small nuclear organelles that contain proteins and RNAs. It has remained unclear the exact mechanism of its assembly in Cajal bodies, but recently it has been shown that TCAB1, a nuclear transport protein, is involved in recruitment of the unassembled telomerase to Cajal bodies for proper assembly (Stern et al. 2012).This further established the point that telomerase activity in a cell does not equate to telomere lengthening in-vivo (Ouellette et al. 1999).

The telomerase RNA component, TR, is what truly sets telomerase apart from other reverse transcriptase's found in nature. The telomerase RNA component has been identified in a variety of species including 32 vertebrate species (Chen J-L et al. 2000). The size of the RNA ranges from 1544 nts in budding yeast to as small as 146 nts in ciliated protozoans (Tzfati et al. 2000) (Romero et al. 1991). Human telomerase RNA, hTR, is 451 nts long.. Similar to the TRs in other vertebrate species, they share the template region. The template region is what allows for telomerase to recognize the telomere sequence

Protein	Interacting Region	Function
TEP1	hTERT and hTR	Telomerase Processivity
p23	hTERT	Assembly
Hsp90	hTERT	Assembly
Hsp70	hTERT	Assembly
L22	hTR (nt 64-222)	Processing and localization
hGAR1	hTR (H/ACA Domain)	Stability, maturation and localization
Dyskerin	hTR (H/ACA Domain)	Stability, maturation and localization
hNOP10	hTR (H/ACA Domain)	Unknown
hnRNP C1/C2	hTR (nt 33-147)	Stability, maturation and localization
La	hTR (nt 1-205 and 250-451)	Unknown
hNHP2	hTR (H/ACA Domain)	Stability, maturation and localization
hStau	hTR (nt 64-222)	Unknown
L22	hTR (nt 64-222)	Processing and localization
TCAB1	hTERT and hTR	Biogenesis and nuclear localization
PARN	hTR (Poly A tail)	Processing

Table 1-1: Table of known telomerase accessory factors and their functions.

of the chromosomes and de-novo synthesizes the DNA hexameric repeats on the telomeres. The template region of hTR is 5'- CUAACCCUAAC-3'. It is located near the 5' end of hTR (nucleotides 46-53). Along with providing the template for telomerase, hTR is also a key structural element of the telomerase holoenzyme complex (Nakamura et al. 1997). Various conserved structural elements within hTR play a key role in telomerase processivity and telomerase assembly as a whole. There are eight highly conserved regions (CRs), including a pseudoknot domain, the CR4-CR5 domain, a Box H/ACA domain characteristic of small nucleolar RNPs (snoRNPs) and the CR7 domain (Chen J-L et al. 2000). The majority of telomerase accessory proteins in fact bind not to hTERT but to these hTR domains. A prime

example of this is the H/ACA box. The H/ACA box has been shown to play a role in hTR accumulation, hTR 3' end processing and telomerase activity (Mitchell et al. 1999), but it is also recognized and bound by the accessory proteins dyskerin, NOP10 and NHP2. The critical importance of the RNA component to the telomerase enzyme is well understood. However, hTR is not the limiting factor of the telomerase enzyme. Human telomerase RNA is expressed in almost all cells at reasonably high levels. What limits telomerase assembly in the cell is in fact the protein catalytic subunit hTERT.

Structure and Function of Telomerase

Human TERT, hTERT, is composed of three domains: C-terminal domain or CTE, N-Terminal domain and reverse transcriptase domain or RT domain. The RT domain is what provides TERT with its catalytic ability. All reverse transcriptases found in nature (HIV-1 RT, M-MLV RT, AMV RT etc.) share seven universally conserved RT motifs. All seven of these motifs are highly conserved in hTERT (Lingner et al. 1997) and shown in Figure (1-2) as A-E and 1-2. What distinguishes hTERT, as well as all of the other 40+ TERTs, from other RTs are the template RNA and its enzymatic processivity. The first difference is straightforward; while other RTs require a primer to reverse transcribe, telomerase contains its own template provided by hTR. The second difference, enzymatic processivity, can be attributed to the very large N-terminal domain, although lack of structural data of the human telomerase holoenzyme has limited researcher's ability to consolidate this hypothesis. Most RTs will bind their substrates, catalyze the reaction and then dissociate from the newly formed products. However, human telomerase is able to bind the substrate, synthesize the TTAGGG repeats, translocate and then repeat the synthesis for another TTAGGG repeat. This phenomenon is called repeat addition processivity and is the major enzymatic property that distinguishes telomerase from other RTs. It is widely believed that the large N-terminal domain, along with a long and flexible linker region, gives telomerase the flexibility to translocate while attached to the substrate. This remarkable ability of telomerase allows for four simultaneous interactions: RNA-DNA, TERT-DNA, TERT-RNA and TERT-TERT.

Structural studies on the telomerase complex have been complicated by the low abundance of the enzyme in cells. It has been estimated that there are between 50-100 catalytically active molecules per cell (Cohen et al. 2007). This is far from the amount needed for classical structural elucidation methods such as X-ray crystallography and nuclear magnetic resonance (NMR) Researchers have turned to overexpressing and purifying domains of the beetle *Tribolium castaneum* TERT subunit. These studies, using x-ray crystallography, revealed a conserved palm, finger and thumb domain shared by most polymerases that are necessary for nucleic acid recognition and incorporation (Gillis et al. 2008). More recently, electron microscopy (EM) studies have shown the human telomerase structure to a resolution of 21 Å (Sauerwald et al. 2013). The structure revealed a bilobal architecture with a flexible linker connecting the two monomers forming an active dimer. While this is a big step forward, the structure still lacks the resolution to decipher the structural relationship between hTR and hTERT. The recent EM structure supports the hypothesis of telomerase as an active dimer (Cohen et al. 2007) (Wenz et al. 2001) (Beattie et al. 2001) (Gardano et al. 2012) yet there is also evidence to support telomerase being an active monomer (Errington et al. 2008) (Alves et al. 2008). Biochemical studies on telomerase activity have shown two opposing models for its interaction and extension of the substrate. The first, parallel model, shows an enzyme that has both subunits of the dimer interacting with and extending the DNA substrate simultaneously (Prescott and Blackburn et al. 1997a). The second model, sequential model, shows only one subunit of telomerase interacting with the DNA substrate at a time while the other subunit lies in a dormant state (Wenz et al. 2001). Other studies have also shown telomerase dimeric molecules behaving in a dominant negative fashion (Saurwald et al. 2013) (Nguyen et al. 2009). Hopefully with the new "resolution revolution" in the field of cryo-EM, researchers will be able to collect data and obtain a higher resolution structure of the active human telomerase complex including hTR and the telomeric substrate. This structure would not only go a long way in the fields of telomere and telomerase biology, but also aid in the structure-based drug design for telomerase activators and inhibitors.

Therapeutic Targeting of Telomerase

Telomerase's unique property makes it a prime target for cancer therapy. As mentioned earlier, cancer cells almost universally activate telomerase (~90%). More importantly, with the exception of some progenitor cells and stem cells, telomerase is lacking in most normal human cells. This discrimination power makes telomerase an attractive and universal target for cancer therapeutics. To date there have been a variety of therapies targeting telomerase and while initially the results were promising in tissue culture, they did not translate in the clinical trials. These therapies included small molecule inhibitors, natural compounds, antisense oligonucleotides and RNA interference. The targeting approaches shared a common theme, inhibiting telomerase activity inside the cells. Imetelstat (GRN163L), the most promising of the therapies, made it to phase two clinical trials, and only till recently failed in the treatment of solid tumors due to hematological toxicities leading to thrombocytopenia (low platelets counts). When patients have low platelet counts they have to go off therapy and during the time that platelets are returning to normal the telomeres in cancer cells start to regain some length. Imetelstat is currently being refashioned for the treatment of myeloproliferative diseases including patients with a too high platelet count. Imetelstat is a modified oligonucleotide that binds to and inhibits the template region of the hTR component of telomerase, making it an exceptionally potent active site inhibitor (Marian et al. 2010).

The latest avenue for telomerase therapeutics is designed to take advantage of telomerase catalytic abilities. Telomerase is used as a vehicle for the delivery of toxic drugs. Since telomerase is expressed only in the cancerous cells, healthy cells are spared. Although early in its clinical development, the drug 6'-thio dG is a prime example (Mender et al. 2015). 6'-thio dG is a modified nucleoside that is preferentially recognized by telomerase and incorporated into the G rich telomeric regions during cell divisions. Unlike normal nucleosides, 6'-thio dG interferes with the cell's ability to bind shelterin proteins and once incorporated into telomeres they are recognized as DNA damage. This accumulation of DNA damage culminates in cell death. Optimization of 6'-thio dG is still required, but the data showing rapid and highly selective cell death are quite promising. In the end it is understood that telomerase therapeutics

would make a great secondary therapy in combination or following traditional (first-line) cancer treatments. New and more specific regulators for telomerase activity will likely be better target for cancer therapy.

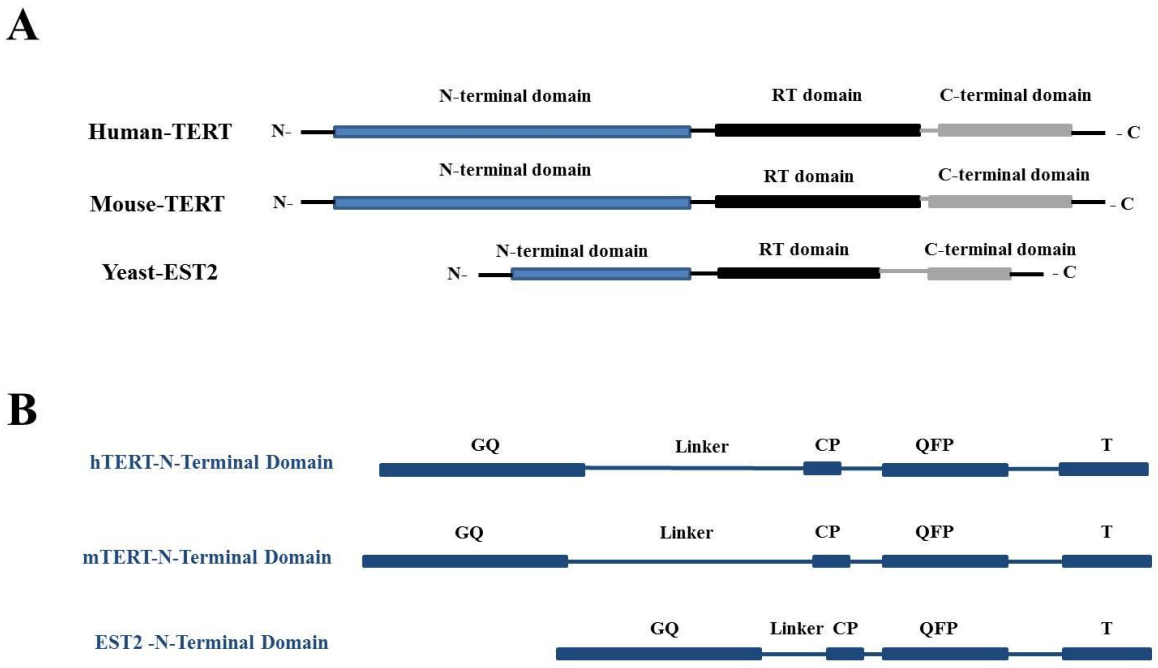

Figure 1-2: ***(A)*** Diagram of human, mouse and yeast TERTs. ***(B)*** N-Terminal domain regions of human mouse and yeast.

III. Regulation of Telomerase

Chromatin Level Regulation: Epigenetic and Telomere Position Effect (TPE)

Telomerase activity needs to be carefully maintained and monitored during human development and then eventually shut down at an appropriate time. When this balance is not achieved, telomerase upregulation and reactivation becomes a primary hallmark in cancers. For this reason, telomerase is one of the most highly regulated proteins in the cell in all levels, with the expression of full length hTERT protein being the limiting factor. The human hTERT gene is approximately 41kb long and contains 16 exons and 15 introns (Cong et al. 1999) (Wick et al. 1999). The hTERT gene is present in most cells on chromosome 5p15.33 as a single copy (Bryce et al. 2000) (Meyerson et al. 1997). Although epigenetic regulation does not represent the major mechanism of telomerase regulation, evidence has shown that it does contribute a small part. For example, treatment with the DNA methylation inhibitor 5-azacytidine produces detectable levels of hTERT transcripts in two telomerase-negative ALT cell lines (GM847 and SUSM-1) (Dessain et al. 2000) (Deveraux et al. 1999). Another example included the histone deacetylase inhibitor, Trichostatin A, which also produces an upregulation of hTERT transcription in telomerase negative cells (Cong et al. 2000) (Xu et al. 2001) (Takakura et al. 2001). Human hTERT epigenetic regulation may also be cell type-dependent. An increase in methylation of the hTERT promotor correlates with a significant decrease in telomerase activity in B-cell lymphocytic leukemia (Bechter et al 2002). CpG islands within the genome are key methylation sites. CpG sites simply consist of regions in the DNA where cytosine is followed by a guanine. It has been estimated that 70-80% of CpG sites are methylated in mammals (Jabbari et al. 2004). A cluster of these CpG sites can be found within the hTERT promotor region and may be able to explain the correlation between levels of methylation and telomerase activity/expression, but no consensus has been established.

The position of the hTERT gene may also play an important role in its regulation. It has been shown that the expression patterns of genes in yeast as well as humans can be altered by the shortening or

lengthening of telomeres (Baur et al. 2001). However, the genes must be located within certain proximity of the telomeres. This phenomenon is called the telomere position effect (TPE). The hTERT gene has been mapped to the sub-telomeric region of chromosome 5 and is approximately 2 MB away from the telomeres (Leem et al. 2002) away from the telomeres. Whether 2MB is sufficient enough for hTERT to be outside of the scope of influence by the telomeres is not known and is currently under research.

Transcriptional Regulation: hTERT Promoter and Alternative Splicing

Transcriptional regulation at the promotor region of a gene is the classic method by which cells can effectively regulate the expression level of a protein in response to a stress or stimulus. Sequence analysis has revealed many potential transcription factor-binding sites within the hTERT promotor (Cong et al. 1999). This wide array of transcription factor binding motifs within the hTERT promotor indicates a complex web of regulation. Several activation factors have been found to be involved in telomerase activation. These include c-Myc, Sp1 and steroid hormones such as estrogen (as reviewed in Cong et al. 2002). Along with activation factors, there are also a number a number of repressors that have been shown to negatively regulate hTERT transcription. These include but are not limited to p53, Mad1, pRB and E2F (as reviewed in Cong et al. 2002). Overall, transcriptional regulation at the hTERT promotor may be one of the major forms of telomerase regulation in human.

Approximately 95% of genes in the human genome are alternatively spliced. Splicing accounts for the high coding capacity of the genome. Interest in alternative splicing has grown as researchers started to implicate this regulatory network in a variety of human diseases such as muscular dystrophies, premature aging disorders and cancer (Bonnal et al. 2012)(Tazi et al. 2009)(Wang et al. 2007). Recently, alternative splicing has shown to be another mechanism of hTERT regulation. The full-length hTERT transcript contains all 16 exons, but this is only one of 22 isoforms that have been found to exist for hTERT (Hrdlickova et al. 2012). Of all the 22 isoforms for hTERT, only the full length containing all 16 exons has been shown to be translated into active telomerase. Telomerase splicing patterns have been

implicated in telomerase regulation. For example, cancer cells have a similar splicing pattern to cells in development as opposed to normal cells (Ulaner et al. 2001)(Wong et al. 2013). The mechanism involved in reverting the splicing patterns of hTERT back to the states of their development remains to be fully elucidated. It is hypothesized that a variety of factors bind along the long pre-mRNA transcript (41kb) and enhance or repress the spliceosome machinery's ability to bind to a particular site. Alternative splicing adds to the already dense and complex regulatory mechanisms of hTERT transcription.

Post-translational Regulation

While transcriptional regulation is the major mechanism for telomerase regulation, post-translational regulation is also an important component in the regulation and recruitment of the telomerase holoenzyme. Telomerase expression levels per se do not necessarily correlate with activity levels of the enzyme (Ulaner et al. 2000). Studies have shown that reversible phosphorylation of the catalytic hTERT subunit at specific tyrosine, serine or threonine residues can alter telomerase activity, structure and localization. For instance, telomerase activity in peripheral blood mononuclear cells can be modulated with the introduction of activators and inhibitors of protein kinase C (Bonder et al. 1996). Protein kinase C has also been shown to modulate telomerase activity in breast cancer PMC42 cells, human nasopharyngeal cancer cells and prostate cancer cells without altering transcriptional levels of telomerase (Li et al. 1997) (Ku et al. 1997) (Jagadeesh et al. 2006). Protein kinase B or Akt has also been implicated in telomerase post-translational regulation. Activated Akt is able to phosphorylate hTERT at two sites and increase activity of telomerase in vitro (Kang et al. 1999) (Breitschopf et al. 2001). Furthermore, telomerase phosphorylation is involved in cellular localization of the holoenzyme to the nucleus. In the case of activated CD4 T-cells, an increase in telomerase transcript levels was not the reason for high telomerase activity. Instead, it was shown that telomerase was translocated from the cytoplasm to the nucleus, whereas previously telomerase activity had only been detected in the cytoplasm (Liu et al. 2001). A more thorough understanding of telomerase regulation would have great impact in the design of novel targeted and comprehensive therapy for a variety of telomere/telomerase related diseases.

Chapter 2: Purification of the Active Human Telomerase Holoenzyme

Introduction

Structural and functional studies of the human telomerase complex are made difficult by the low abundance of endogenous telomerase enzymes in cells. One could argue that for this very reason the telomerase RNP has been shrouded in a cloud of mystery since its discovery 30 years ago. Since the expression of the reverse transcriptase component (hTERT) of the telomerase holoenzyme is rate limiting, we engineered a recombinant hTERT and overexpressed it along with hTR (the functional RNA component of telomerase) in H1299 cells, a human non-small cell lung cancer cell line. Importantly, in collaboration with Dr. Ao Cheng (a former postdoctoral fellow in Jiang Lab), we developed a novel strategy to purify/isolate either recombinant or endogenous enzymes for our functional and structural studies. The multi-step purification procedure, which will be discussed later, allowed us to work with not only a highly enriched sample, but also a catalytically active enzyme. The sensitive telomere repeat amplification protocol, more commonly known as TRAP assay, allowed us to track the enzyme through the multi-step procedure and validate whether our preparation and purification procedures contained an enrichment of telomerase enzymatic activity. The TRAP assay, although highly sensitive, does have some limitations. It was originally designed to test samples from cell lysates, where telomerase levels would be anywhere from 10-100 fold more than the final yields we were able to isolate. The TRAP assay is also gel-based, making quantification and detection of 2-5 fold changes between samples quite difficult. In collaboration with Dr. Andrew Ludlow (Postdoctoral fellow in the Shay/Wright Lab), we set out to convert the TRAP assay into a more quantitative method. We developed a digital, high-throughput and highly sensitive version of the TRAP assay called ddTRAP or droplet digital TRAP (Ludlow et al. 2014). Together with our engineered recombinant enzyme, a novel purification strategy and a highly quantitative and sensitive ddTRAP assay, we were able to proceed with functional and structural studies of the human telomerase complex.

Results

Development and Overexpression of Recombinant Human Telomerase and Generation of the Stable Cell Line Super H1299

The engineered recombinant hTERT contains an in vivo biotinylation sequence, a Tev –protease cutting site, a cMyc tag before the hTERT N-terminus, adding 99 amino acid residues before the hTERT sequence. The added sequence is: M A G K A G E G E I P A P L A G T V S K I L V K E G D T V K A G Q T V L V L E A M K M E T E I N A P T D G K V E K V L V K E R D A V Q G G Q G L I K I G V E N L Y F Q S T M E Q K L I S E E D L E F T. The conserved biotinylated sequence is biotinylated at the conserved MKM site in mammalian cells (Figure 2-1). The modified hTERT plasmid and the exogenous hTR plasmid were packaged in retroviral and lentiviral vectors, respectively and used to transfect and generate a stable cell line, which we called Super H1299. Details of this will be discussed in the methods chapter. After hygromycin selection the cells were grown and harvested on a weekly basis and used for our various experiments.

The validation experiments for the newly formed Super H1299 cells involved the TRAP assay, western blots for hTERT and qPCR/ddPCR for hTR. The gel based TRAP assay revealed a 10-20-fold increase in activity in the Super H1299 cells compared to the parental H1299 cells (Figure 2-2). The activity increase is indicated by the increase in intensity of the TRAP ladder showing telomerase products and the decrease in the internal PCR control (IC), which is a competitive inhibitor in the TRAP assay. Thus, the more telomerase in a sample, the less IC is detected. The 10-20-fold change in activity shows not only a successful transfection, but also proper folding and biogenesis of the active recombinant telomerase complex (hTERT and hTR). Furthermore, we detected another increase in activity when comparing the overexpression of both hTERT and hTR to only that of hTERT (lane 3 vs lane 6 in Fig 2-2). We then confirmed whether or not the recombinant telomerase was completely translated and more importantly if the biotin tag was available for binding to streptavidin. The addition of tags to the hTERT sequence should translate to a recombinant telomerase that is ~ 20 kDa larger than the endogenous

hTERT. As expected, the western blot revealed an hTERT band slightly larger and at a higher level of expression than the expected band for the endogenous 127 kD hTERT (Fig 2-3). Next, we tested the availability of the biotin tag on the surface of the telomerase complex. This test was key towards determining if this engineered protein was suitable for downstream purification and subsequent experiments. The results in Fig 2-4 show a successful pulldown of the recombinant telomerase using streptavidin-coated beads. More importantly, the control sample showed little to no nonspecific pulldown of the endogenous telomerase from the parental H1299 cells.

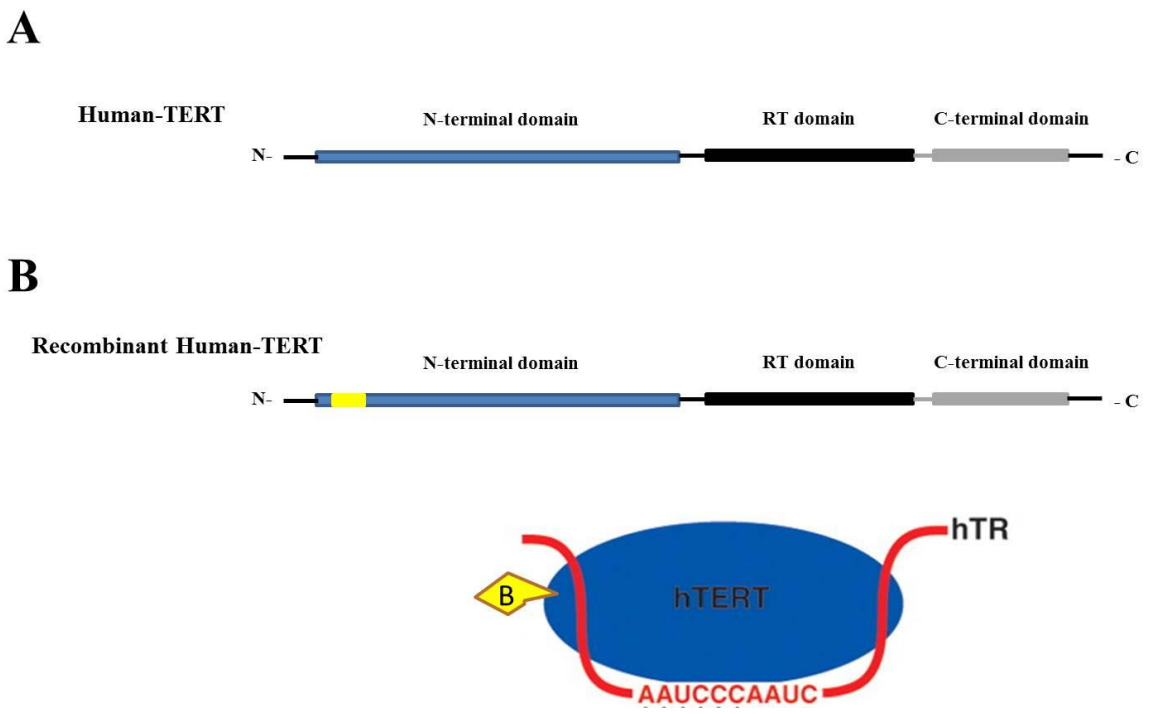

Figure 2-1*(A)* Diagram of Endogenous hTERT gene. ***(B)*** Diagram and cartoon depiction of recombinant hTERT with biotin sequence and tag (yellow).

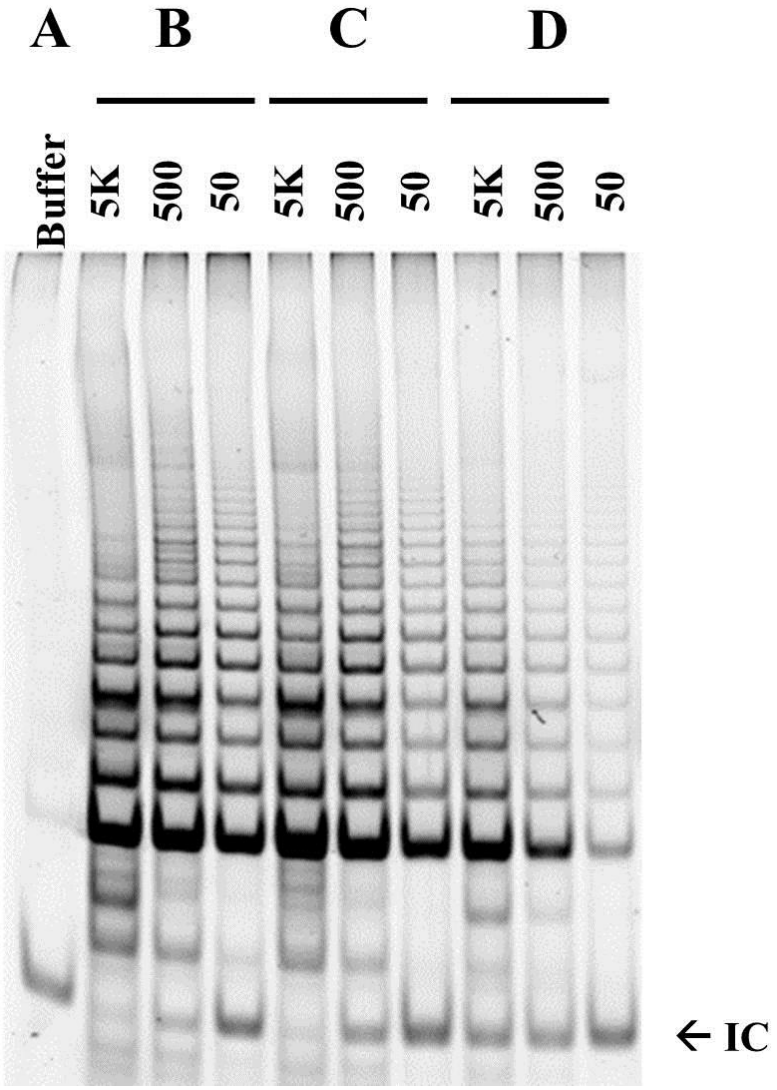

Figure 2-2: TRAP assay to validate activity levels in newly generated stable cell line Super H1299. 5000 cells were lysed and then serially diluted to 500 and 50 cells as indicated above the sample lane. The bottom band on the gel is the internal PCR control (IC). IC determines the relative strength of the TRAP ladder. Since the IC competes with the telomerase extension products in the PCR, it is inversely proportional to activity. If IC band is faint, then telomerase ladder and activity are strong. If IC band is stronger than the telomerase ladder and activity should be weaker. IC is also a control to help prevent false negatives. ***(A)*** Buffer only control. No telomerase ladder and strong IC band show that the PCR worked. ***(B)*** Super H1299 cells with overexpressed recombinant hTERT and hTR at the indicated cell equivalence. ***(C)*** H1299 cells with only overexpressed hTERT and not hTR. ***(D)*** Control sample of H1299 parental cells with no overexpression.

Development of the Droplet Digital TRAP Assay

The work presented in this section has been published in Nucleic Acid Research September 1, 2014; 42(13): e104.

The development of the TRAP assay in the 1990s was instrumental in the identification of the necessary components of the telomerase enzyme as well as for the discovery of telomerase in a wide range of cells and tissues, both normal and cancerous. The original assay used radioactively labeled DNA to detect the telomerase ladder. In 2006, the assay was converted into its modern nonradioactive form (Herbert et al. 2006). By using a fluorescently labeled substrate, users were able to visualize the bands on a gel by simply scanning it and exposing it to the correct excitation wavelength. Keeping up with the trend of developing a new version of the TRAP assay every decade, we set out to move the TRAP from the gel based version, which was limiting in both sample size (20 at a time) and quantification, to a high-throughput version with accurate quantification. The advent and commercialization of digital droplet PCR made this possible.

Although originally conceptualized in the early 1990s (Skyles et al 1992), the digital droplet PCR system began to gain attraction decades later. Today it is hailed as the next step in the evolution of PCR. Gel-based PCR began as a qualitative assay. It then moved to the intermediate form of qPCR, which was able to relatively quantitate DNA and RNA samples. Today ddPCR gives a user absolute quantification of the input material, building upon the sensitivity of qPCR. The key to ddPCR is the sample partitioning. The creation of discrete reactions in the form of droplets before the PCR reaction allows for Poisson statistical data analysis to treat each droplet as an individual PCR reaction. The samples are still detected via fluorescence from the probe, similar to qPCR. However, the droplet fluorescence is now read in a digital fashion, either scored a 0 or 1. This indicates whether the targeted sample was present in the droplet prior to the PCR reaction. After analyzing the 20,000 droplets (typical amount per sample) the reader will give the user an absolute number in the form of molecules/ul. Furthermore, we conducted various control experiments to determine the efficiency of droplet formation and droplet capture of molecules (appendix).

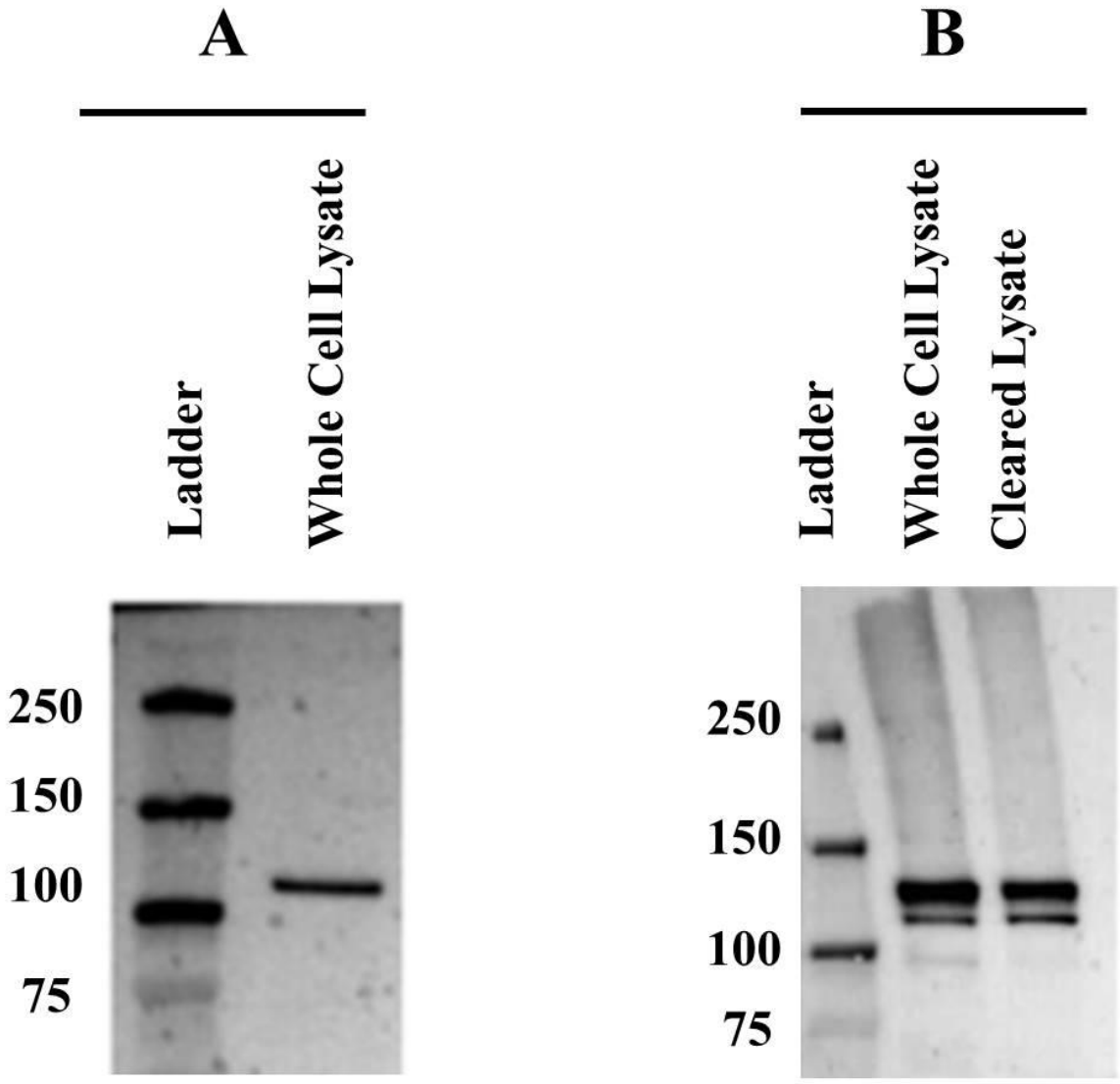

Figure 2-3: Western Blot validation of Super h1299 expression of recombinant hTERT using antibody against hTERT. ***(A)*** Parental H1299 cells showing the control band for endogenous untagged hTERT at ~127 kDa. ***(B)*** Super H1299 cells showing two distinct bands for hTERT. The lower band is the smaller untagged endogenous hTERT, while the upper band shows the overexpressed recombinant tagged hTERT. Tagging makes it larger than the endogenous hTERT and run higher in the gel at about ~130-135 kDa. The recombinant tagged TERT is much higher than the endogenous TERT.

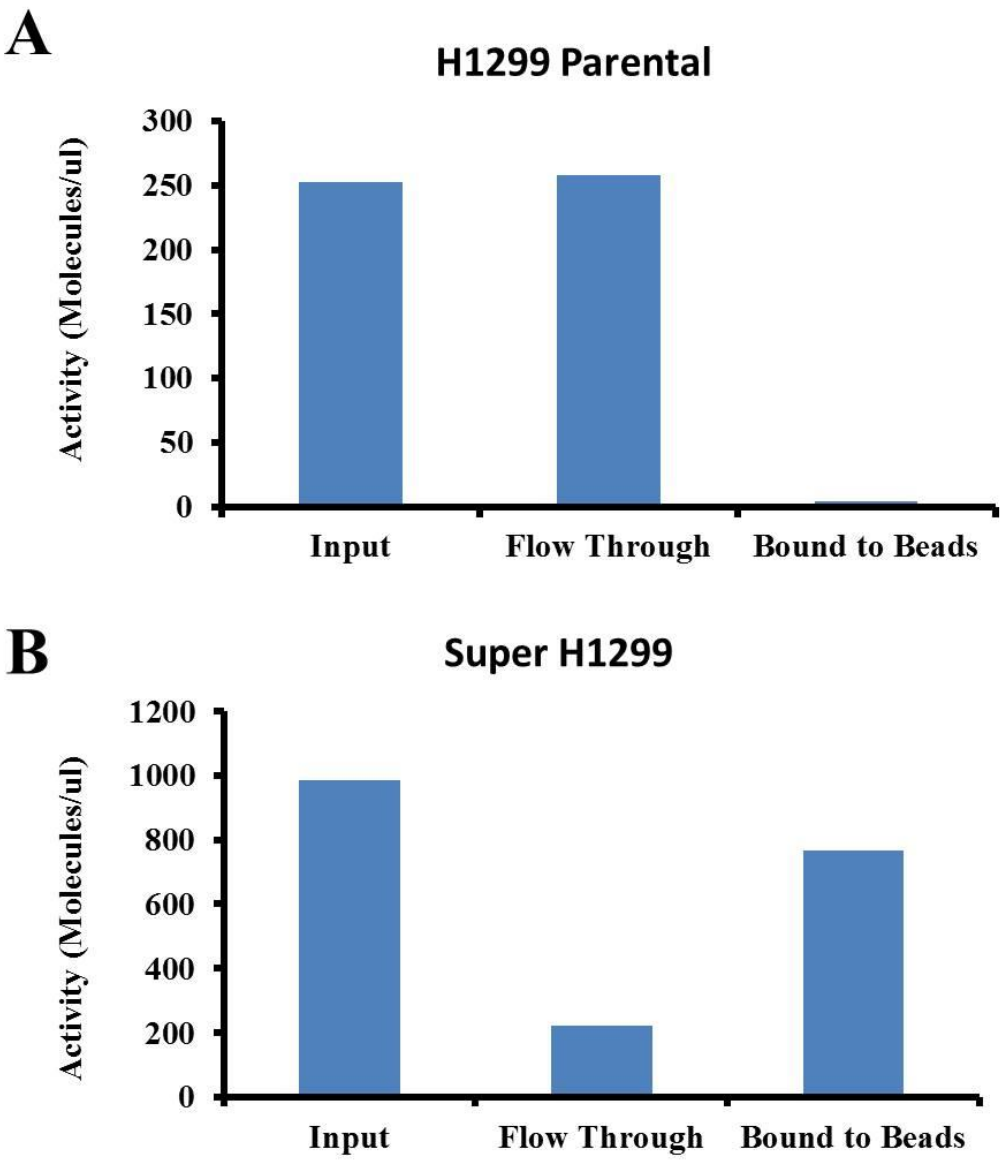

Figure 2-4: (A) H1299 parental cells were lysed and incubated with streptavidin-coated magnetic beads. ddTRAP reveals little to no binding to the beads. This indicated these beads have very low nonspecific binding to endogenous telomerase enzyme. (B) Super H1299 cells were lysed and incubated with streptavidin coated beads. The data shows successful pulldown of recombinant biotinylated telomerase. This validates the availability of the biotin tag on the engineered enzyme.

The workflow for droplet digital TRAP involves an extension reaction prior to the PCR (Fig 2-5). Samples are incubated with the TS substrate oligo and nucleotides. If the sample contains telomerase, telomerase will incorporate the nucleotides and extend the TS substrate. These extended substrates, called telomerase extension products, are then individually partitioned into droplets and allowed to be amplified by PCR. The control sample, BJ cells, contains no telomerase and therefore shows very few "background" positive droplets (Figure 2-5 B). After subtracting the background droplets from the lysis buffer control, the BJ cells show no telomerase activity. The positive control, H1299, in contrast, has high telomerase activity and can be visualized by the separation of the droplet populations in Figure 2-5. More importantly, a robust signal from the H1299 cells was obtained from only 50 cells. This sensitivity and the ability to run up to 96 samples in a 96 well plate are crucial for future experiments involving purification of telomerase.

One-Step Affinity Purification of Endogenous Telomerase from H1299

To study endogenous (untagged) telomerase from the parental H1299 cells we took advantage of telomerase's ability to stably bind and extend the telomeric (TTAGG3) repeats in vitro in order to purify catalytically active complexes (Cohen et al 2007). Our modified procedure did not involve any preselection by using an antibody to pulldown the complexes (Cohen et al 2007). Our one step affinity purification simply involves a telomeric substrate, which we called R3, tethered to beads via a biotin-streptavidin linkage. Telomerase can then stably bind to the triple G at the end of the oligo. This interaction has a half-life of over 10 hours at 4^{o} C (Figure 2-6). The introduction of dATP and dTTP then gives telomerase the nucleotides to catalytically extend the substrate in vitro. By only introducing dTTP and dATP, telomerase is limited to the addition of TTA to the oligo sequence. The DNA-RNA interaction between the TTA sequence and hTR is very weak and has a half-life of about 5 min at room temperature (Figure 2-6). This instability causes the telomerase complex to elute off of the beads by dissociating from the oligo substrate. After analyzing the samples using gel based TRAP, we were able to obtain a 20-25% yield of total active telomerase in the elution when comparing to the whole cell controls (Figure 2-7).

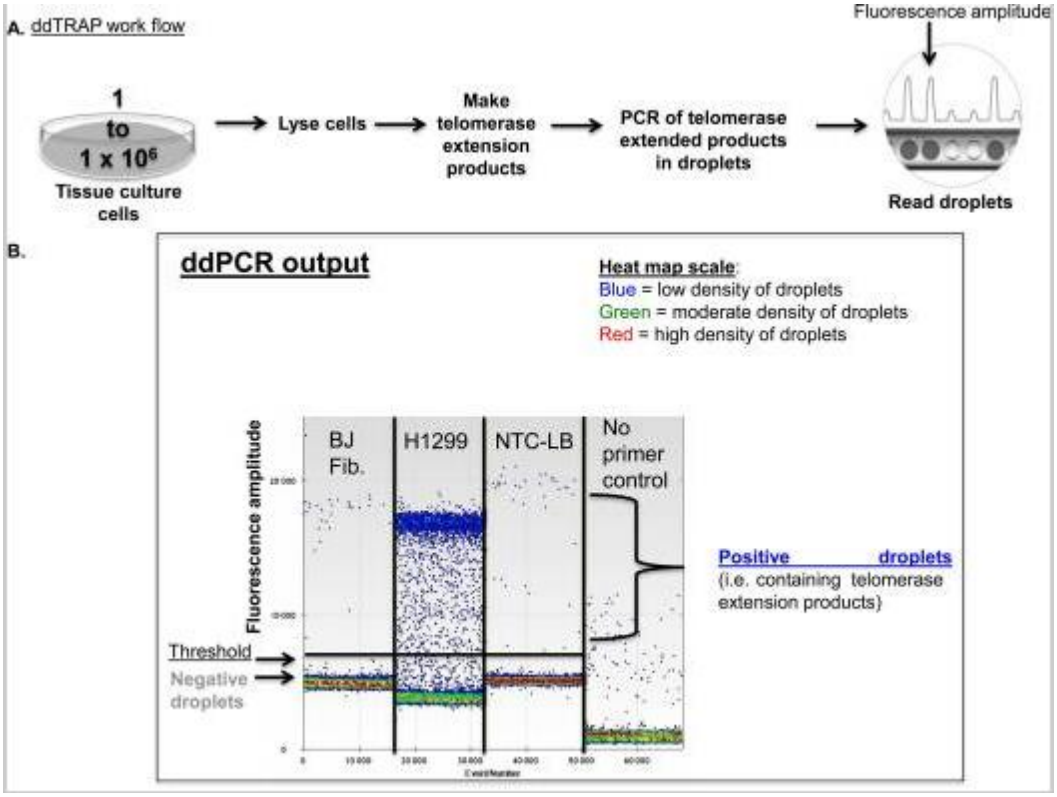

Figure 2-5: ***(A)*** Droplet digital TRAP assay workflow. Cells are harvested and lysed similar to the original TRAP assay. The main difference is the extension reaction is done separately from the PCR and is then partitioned into droplets before proceeding with the PCR. Following the completion of PCR, the samples in each well are read on a flow cytometer. Each droplet is read as a distinct fluorescent event and then plotted by its intensity. ***(B)*** A typical ddTRAP plot showing the droplet fluorescence from each well on the plate. BJ cells show little to no positive droplets after the background is corrected for using sample lysis buffer (NTC-LB). The H1299 cells show two distinct populations and a robust signal for positive droplets from merely 50 cells.

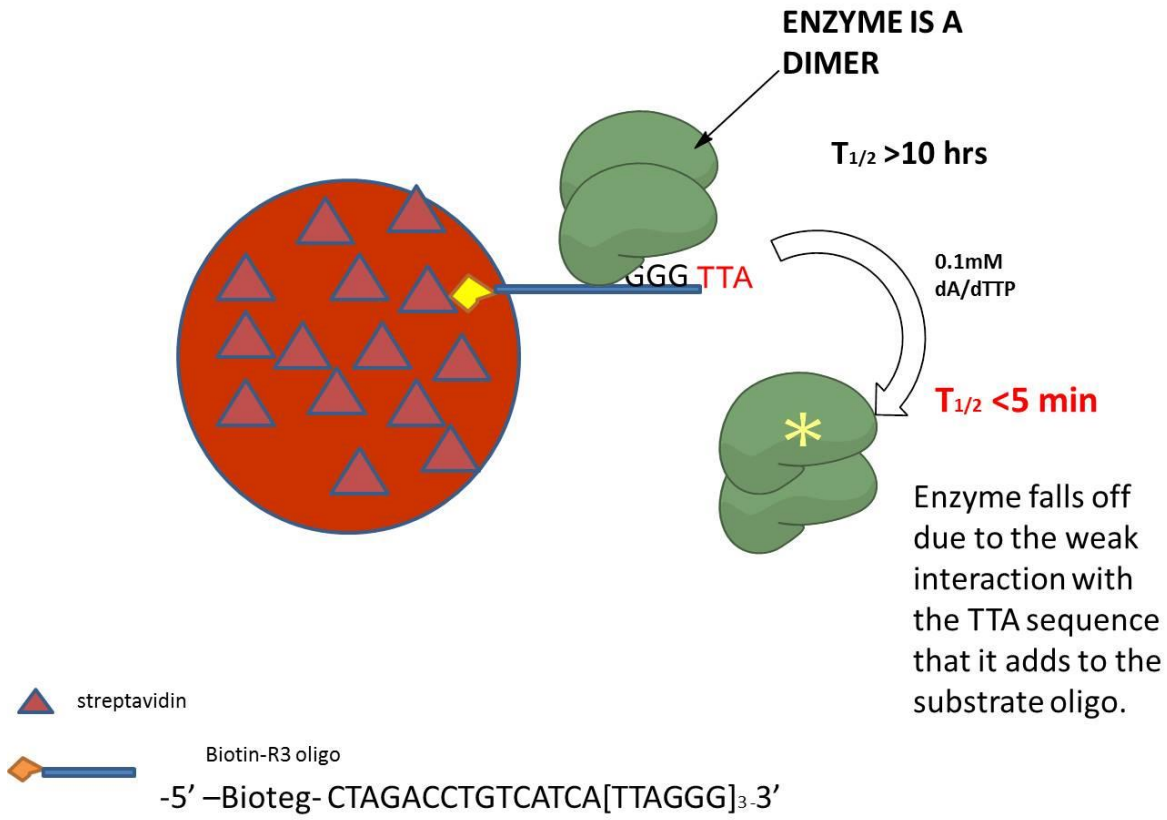

Figure 2-6: Diagram of one-step telomerase affinity purification of endogenous telomerase using R3 oligos on a magnetic bead.

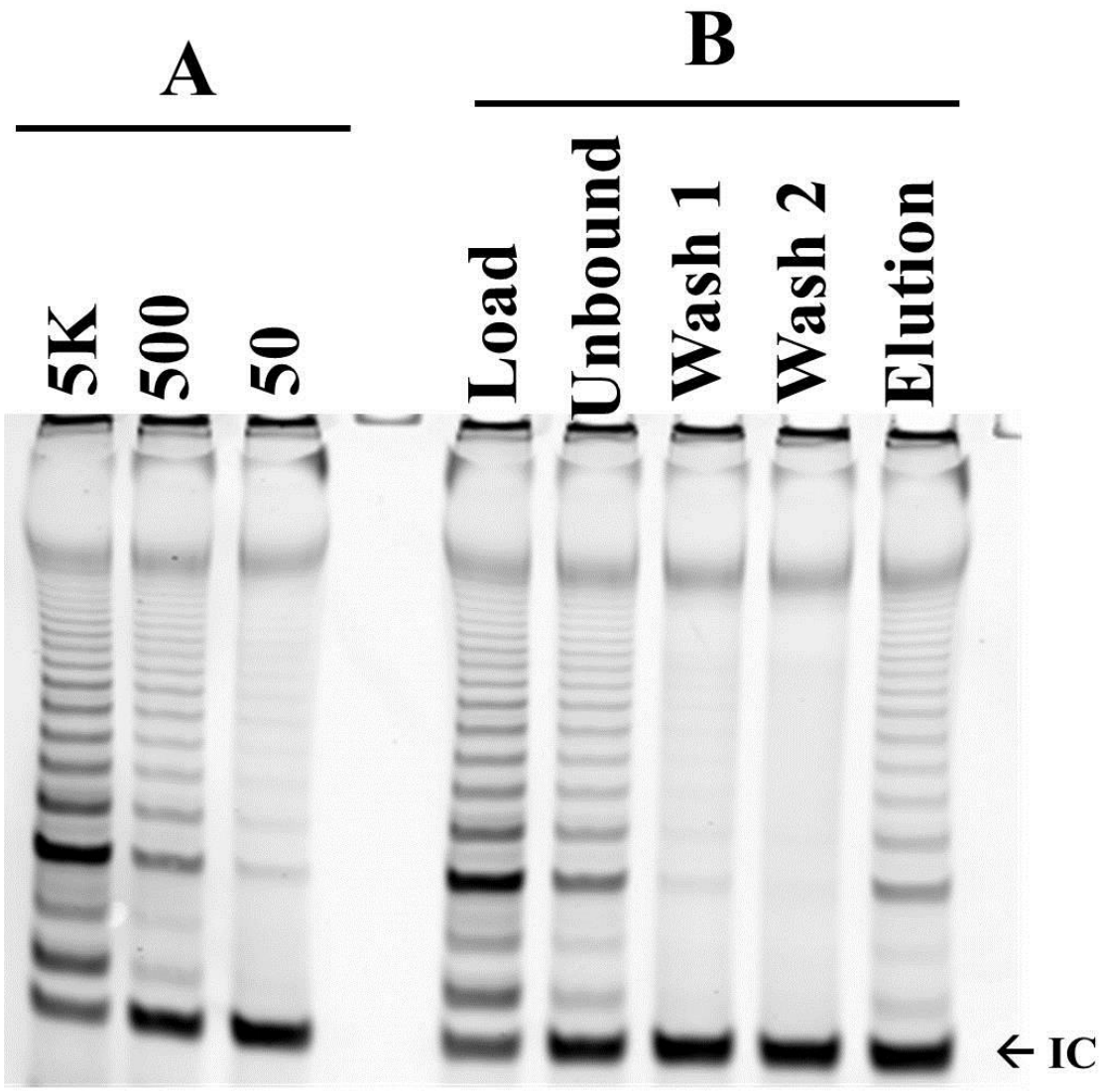

Figure 2-7: TRAP assay on samples from one-step affinity purification of telomerase. ***(A)*** H1299 cell controls at cell equivalence of 5000, 500 and 50. ***(B)*** Samples from purification. All samples are at 5000-cell equivalence. TRAP assay shows ~ 20% of the activity in the elution. The load sample when compared to the 5K-control sample shows similar but slightly less activity. The elution sample on the far right is comparable to activity between 500 and 5000 cells.

Three-Step Purification of Recombinant Telomerase from Super H1299

Affinity purification of our engineered telomerase involves a continuous glycerol gradient, monomeric avidin chromatography, and ion exchange chromatography. In a 10-30% continuous glycerol gradient we took advantage of telomerase's large size and were able to isolate the activity to the bottom 4-5 ml of the gradient (Figure 2-8 and Figure 2-9) from most of the cellular proteins. This allowed us to remove the vast majority of small (less than 200 kDa) protein complexes from the mixture (Figure 2-10). The gradient results indicated to us that the telomerase complex was at least ~600 kDa (Binns et al. 2010). This result correlates well with a dimeric telomerase complex containing at least two hTERT and hTR subunits as well as the other accessory factors. The active fractions of the gradient were then incubated with monomeric avidin beads. In this step we used our biotin tag to pull down telomerase. After binding to the column, the telomerase enzymes were eluted with free biotin (Figure 2-11). In this step we were able to separate recombinant telomerase from the endogenous (untagged) telomerase, as indicated by the western blot in Figure 2-13. Using ddTRAP we calculated the total yield from the avidin column to be ~30% (Figure 2-12). After the avidin column, the three elutions were then pooled together and incubated with an ion exchange column. Again, we took advantage of another property of telomerase. At pH 7.0, the pH of our buffers, telomerase has an overall negative charge. This negative charge on telomerase allowed us to incubate it with a cation exchange column. A salt gradient was used to elute the enzyme. The majority of the telomerase activity was eluted in fractions 2-4, corresponding to a NaCl concentration of 300-500mM (Figure 2-14). Silver stain results on the elutions containing telomerase activity indicated a highly purified sample with about 6-10 distinct protein bands (Fig 2-15). It is reasonable to believe these bands are associated proteins and factors of the telomerase holoenzyme complex.

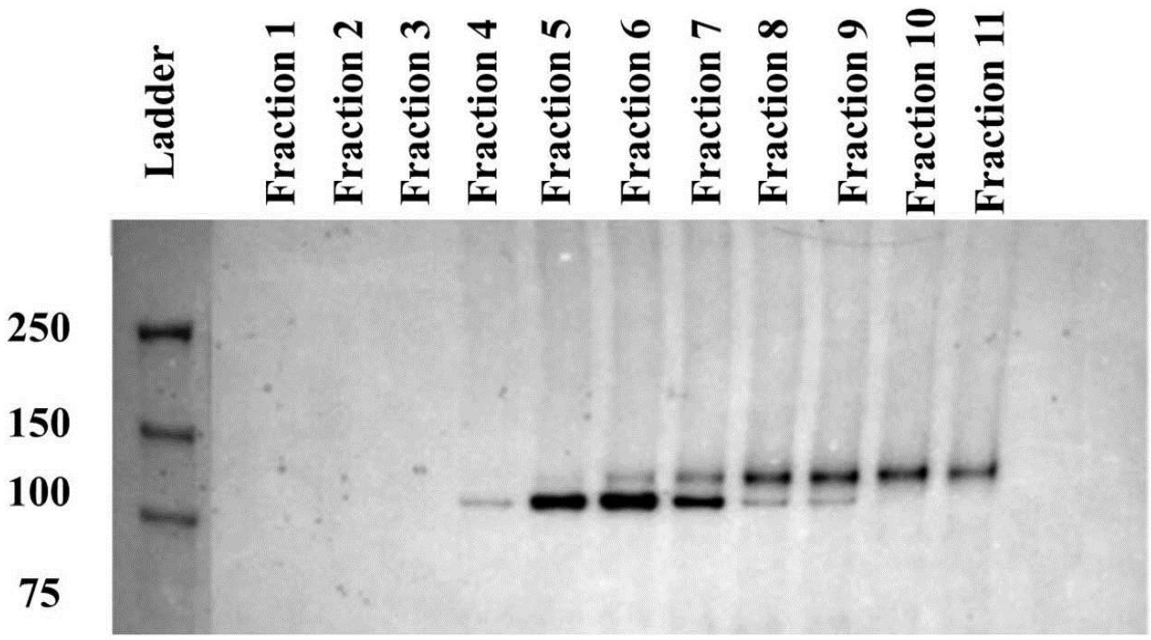

Figure 2-8: Western blot using antibody against hTERT for samples from the glycerol gradient. Further validation that our tagged recombinant telomerase are in the bottom fractions of the gradient (F7-F11).

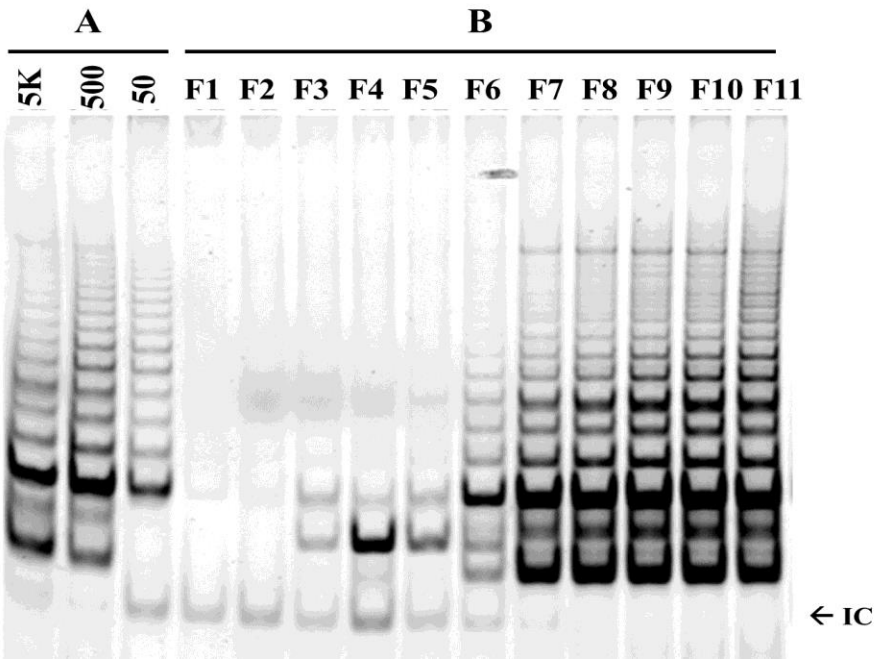

Figure 2-9: TRAP gel for telomerase activity in the10-30% continuous glycerol gradient. ***(A)*** Control 5000, 500 and 50 cells. ***(B)*** Glycerol gradient was divided into 11 fractions of one mL each. Then aliquots equivalent to 5000 cells were analyzed by gel-based TRAP assay. The majority of telomerase activity is in the bottom 5 fractions of the gradient (F7-F11).

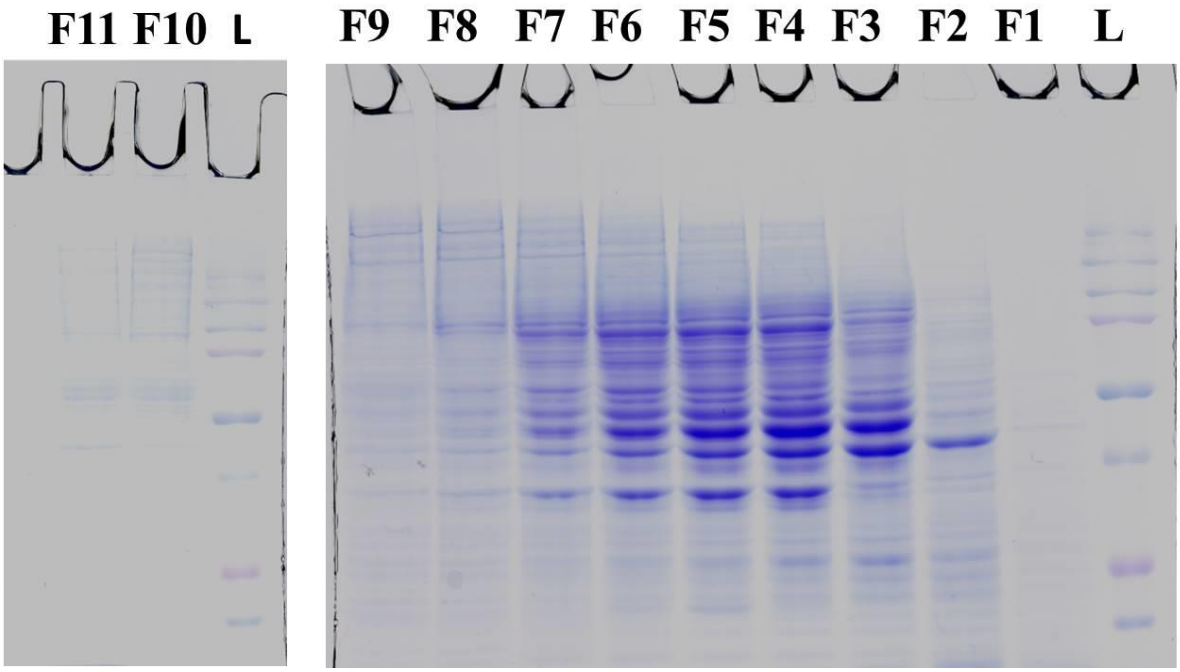

Figure 2-10: SDS-PAGE gel for glycerol gradient fractions (F-F11). Equal volume from each fraction was loaded to SDS-PAGE gel and stained with Coomassie blue dye. F7-F11 are the fractions containing telomerase activity. These fractions were pooled together and used for the next step in the purification.

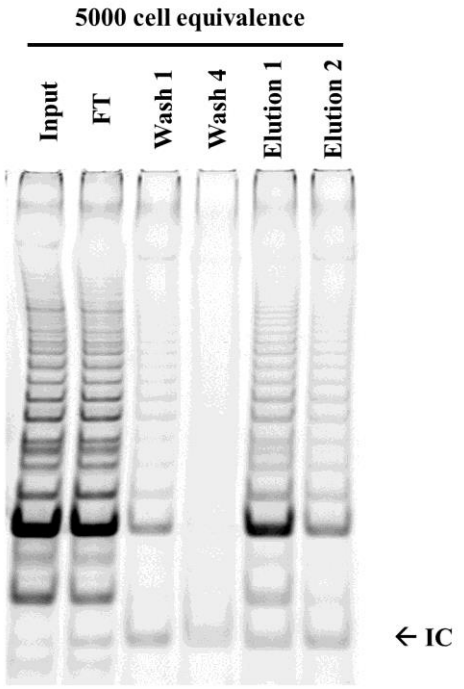

Figure 2-11: TRAP gel for samples from the monomeric avidin pulldown of recombinant telomerase. Data show that biotinylated telomerase can be pulled down and be eluted.

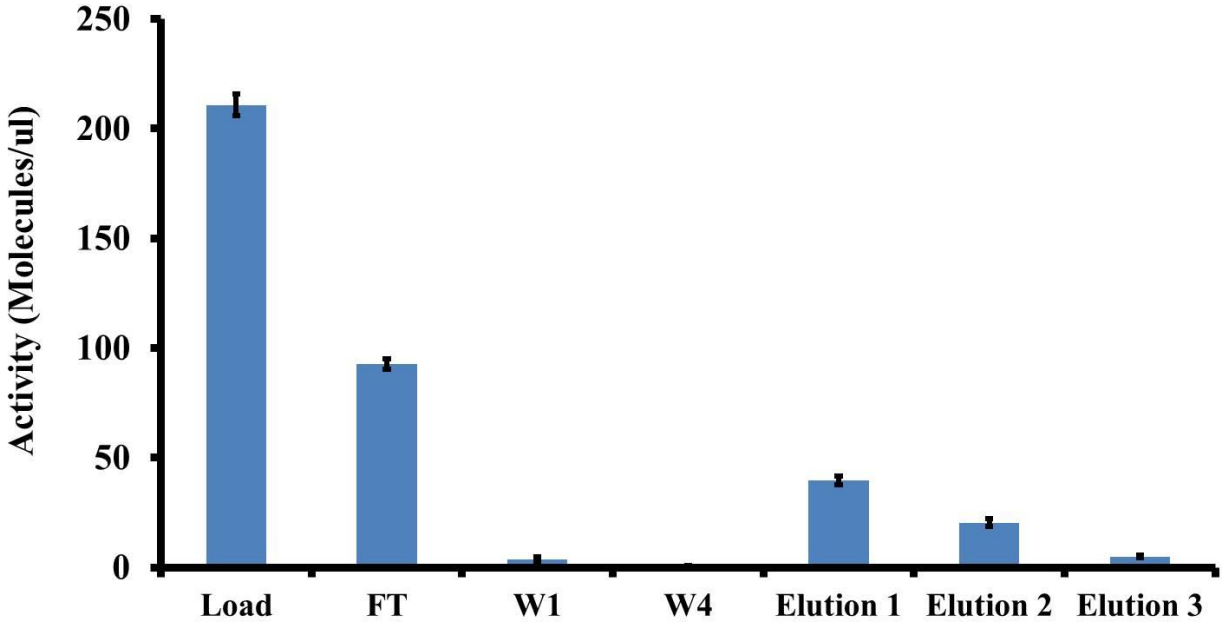

Figure 2-12: ddTRAP assay of samples from the second step of our three-step purification of recombinant telomerase. We ran the samples from the avidin column and used ddTRAP to quantify the activity and calculate the yield. We were able to collect ~30% of the total activity in the three elutions.

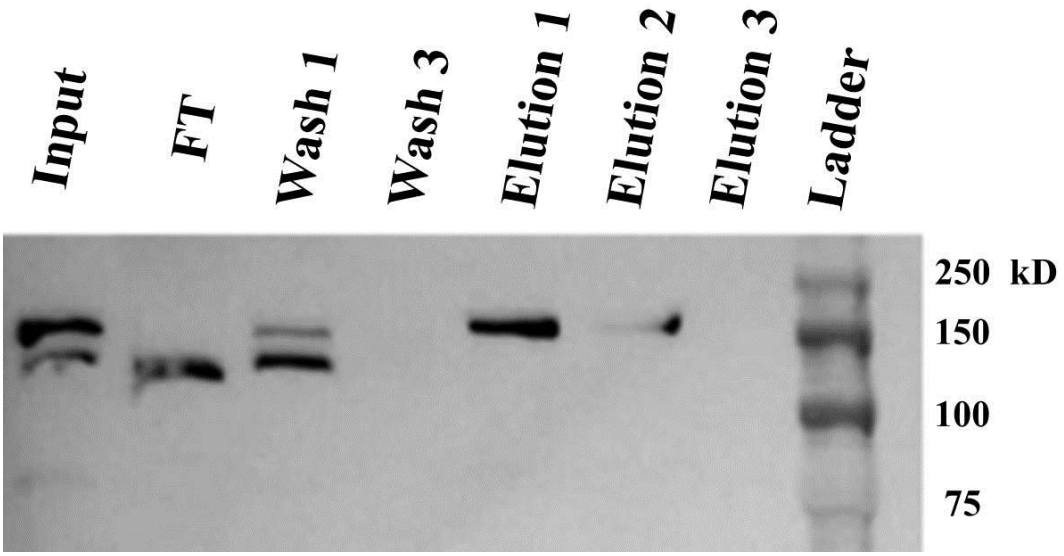

Figure 2-13: Western blot using antibody against telomerase on samples from avidin pulldown. The FT from the column was almost completely composed of untagged endogenous telomerase. The avidin column allowed us to separate the two populations of hTERT and isolate only our recombinant telomerase. The elutions lanes only contain the larger band around 130 kDa. That band corresponds to our recombinant telomerase.

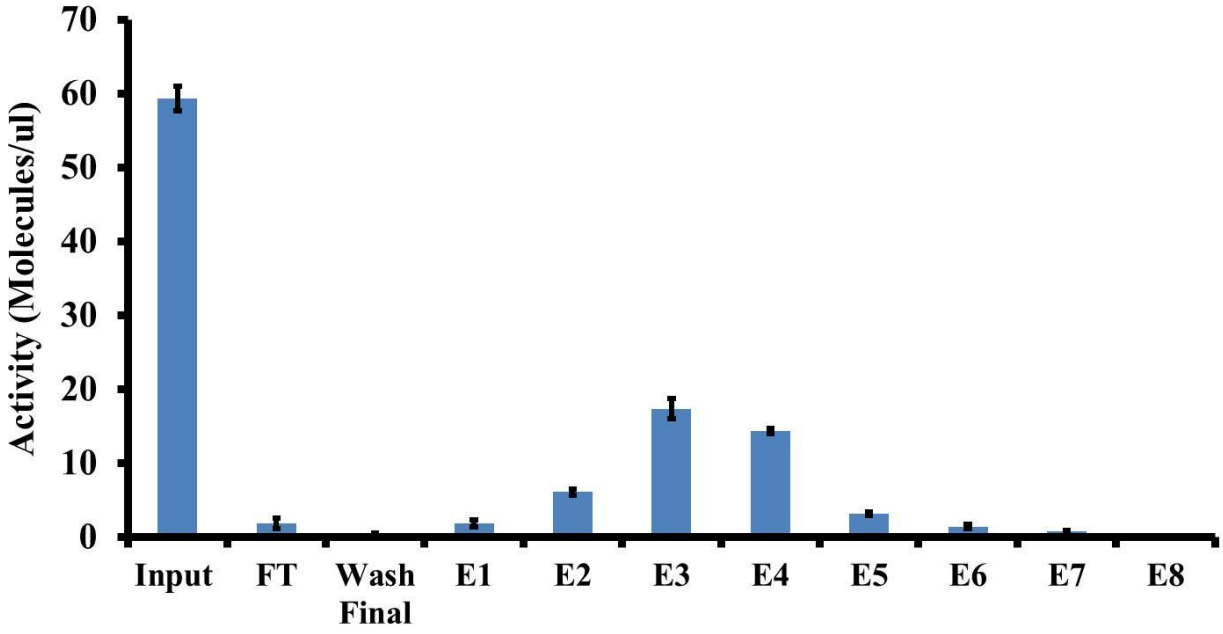

Figure 2-14: DDTRAP on samples from the SPFF column. The elutions from the avidin column were pooled together (input). Recombinant telomerase could bind a cation exchange column and be eluted out with a yield of ~ 76%. The salt concentration in E3 was 400mM NaCl.

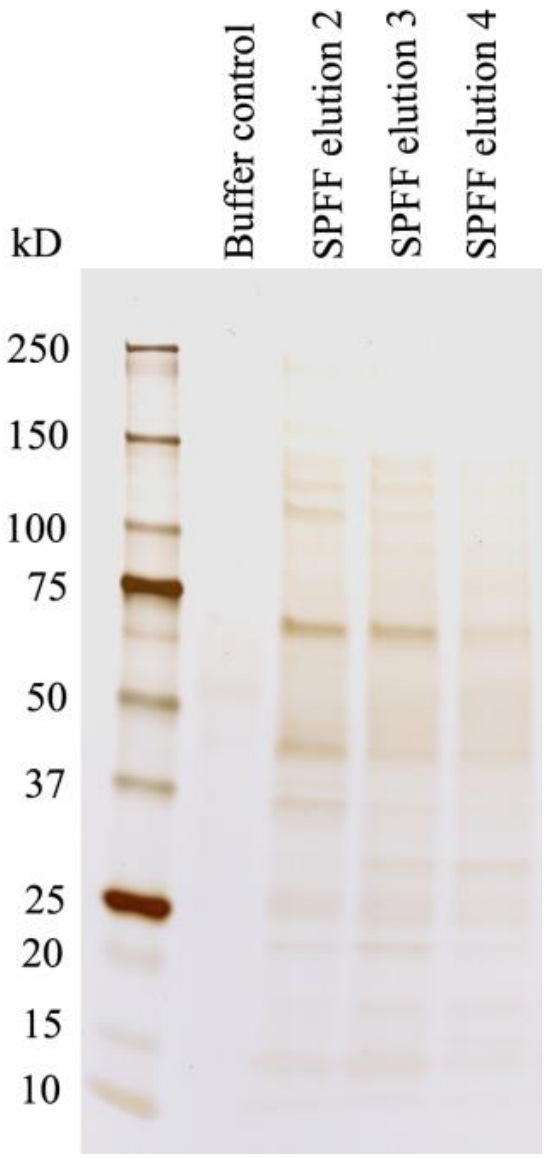

Figure 2-15: Silver stain gel on elution fractions 2, 3 and 4 from SPFF column. These are the final samples from our 3 step purification.

Validation of Active Enzyme on Chemically Modified Carbon Grids

Unlike the one-step affinity purification, the three-step purification of recombinant telomerase complex did not involve an affinity purification step to select for catalytically active telomerase. We merely assayed the samples with TRAP to detect the presence or absence of activity. In order to determine whether the recombinant enzyme could recognize, bind and catalytically extend a telomeric substrate (R3), we chemically modified carbon-coated EM grids (R3-ChemiC grids) to capture telomerase complexes. The purpose was two-fold: Firstly, it was to validate whether our recombinant telomerase was behaving similar to its endogenous counterpart. Secondly, it was a necessary step in capturing telomerase for structural studies via cryo-electron microscopy (not discussed in this thesis). The grids were engineered using carbodiimide chemistry (EDC/Sulfo NHS) to tether our R3 oligo on the surface of carbon-coated grids (Llaguno et al 2014). We tested various conditions for telomerase binding and extension of the R3 oligo on the surface of the R3-ChemiC grids (Figure 2-16). The samples were incubated in the presence of radioactive dGTP [alpa-32P] as well as the other nucleotides to allow telomerase to extend the R3 substrates. After extensive washing, the results revealed a robust signal from incorporation of "hot" dGTP into the R3 oligos on the surface of the ChemiC grids by the highly purified recombinant enzyme (Figure 2-17). The low signal to noise ratio shows in the control groups indicate specific enzymatic recognition and extension of the substrates on the surface of the carbon coated EM grids.

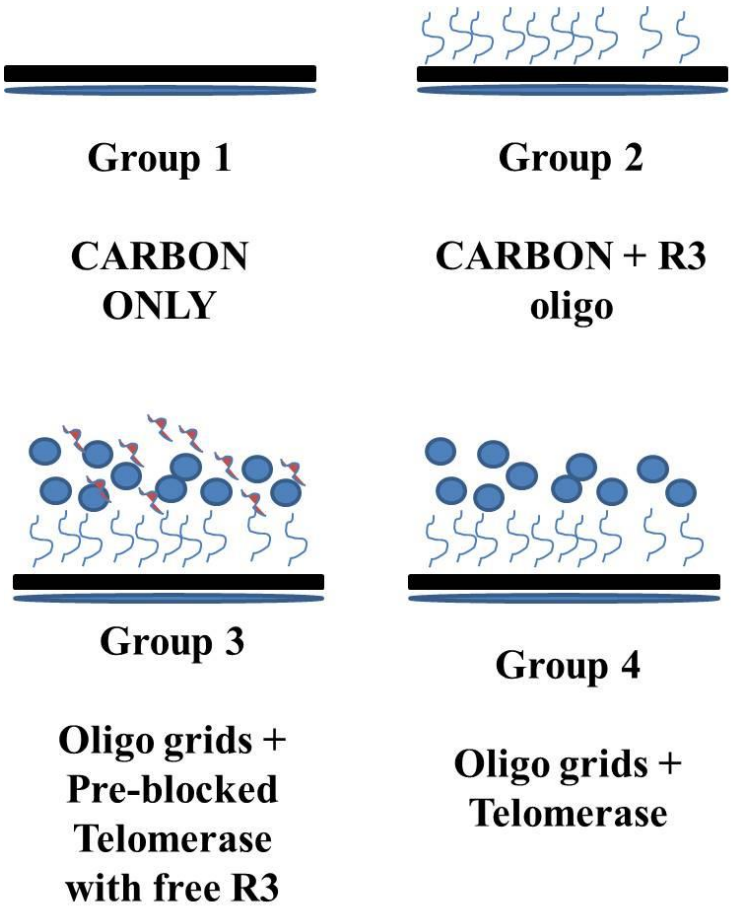

Figure 2-16: Samples and conditions for our extensions on the grid. Group 1 is carbon coated EM grid. Grids from group 2, 3 and 4 have been modified to have R3 oligos attached to their surface to capture our telomerase enzymes. Groups 1 and 2 contain no enzyme in the reaction.

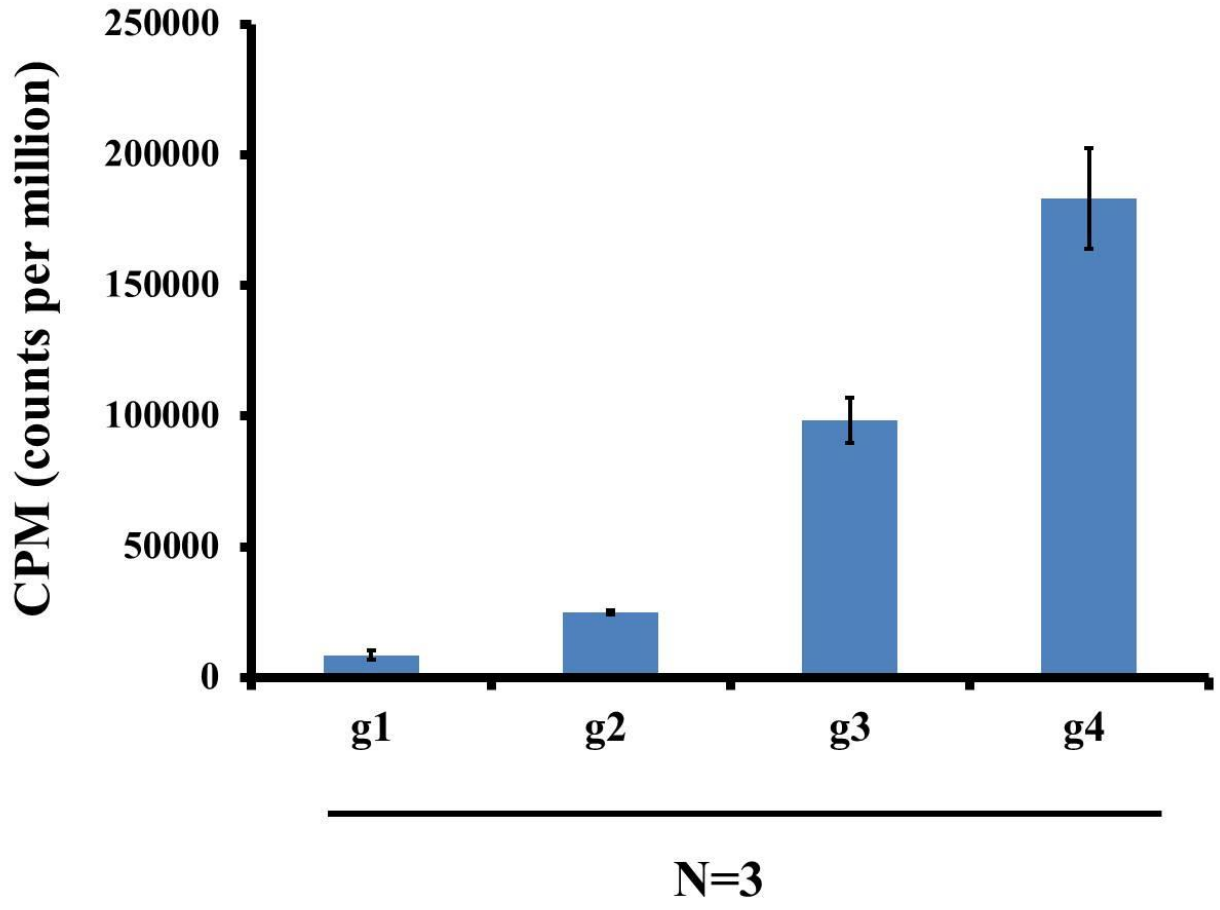

Figure 2-17: Graph of radioactive counts from our telomerase incorporation of alpha ^{32}P-dGTP nucleotides into the R3 substrate oligo. G1 and G2 are negative controls and show us the background radiation. In G4 we can see a robust signal that is 8 fold more than the background levels in G2. In group 3 we were able to show almost 50% reduction in signal by blocking the enzyme with free R3 before incubation with the grids. This is further evidence to the specific binding and extension of our telomerase to the R3 oligos on the grids.

Discussion

The major obstacles to our understanding of the structure and function of human telomerase can be summed up into three parts. The first part is the lack of a good starting material. Even a cancer cell line, such as H1299, a cell line with one of the highest levels of telomerase activity and expression could at most provide ~50-200 fully assembled and active telomerase complexes per cell (Cohen et al 2006 and Xi et al 2014). This amount of starting material would have been magnitudes of order below the micrograms to milligrams range used in conventional studies on proteins. In fact, it is well below even the nanogram range of starting material used for some of the more newly developed techniques such as EM. The second part of the roadblock to telomerase studies was the inability of researchers to purify the complex. Telomerase is a large RNP complex, making it highly unstable and susceptible to RNase degradation. In order to conducting functional studies on an enzyme, one has to be able to purify it to relative purity. Only then can enzymatic properties such as time constants, on- and off-rates and binding constants be obtained by biophysical analysis. The third and final obstacle to telomerase studies was the lack of a robust and quantitative assay to measure its activity. Although the gel based TRAP has come a long way since its initial development, it still lacked the absolute quantification and high-throughput abilities for the studies we intended to carry out. In this thesis research all three obstacles were lifted successfully and summarized below.

We transfected and overexpressed the two main components of telomerase, hTERT and hTR, into H1299 cells. After viral infection and selection we were able to generate a stable cell line, which we called Super H1299. These super H1299 cells displayed 10- to 20-fold increase in activity compared to the parental H1299 cells. The activity increase not only confirmed the successful transfection and expression of our hTERT and hTR vectors, but also the proper assembly of the two components into a functional telomerase enzyme. This allowed us to overcome, to a certain extent, the low abundance of telomerase level in cells. Although the telomere and telomerase field has come a long way, there is still no means of expressing human telomerase in a bacterial system. Therefore it is not possible for us to have

the luxury of collecting micrograms to milligrams of telomerase holoenzymes from bacteria. However, we have made significant progress in generating our model cell line with at least 10 times more activity for us to capture in order to purify telomerase.

Purifying telomerase is not an easy task. In order to purify human telomerase we engineered a novel recombinant version of hTERT, the catalytic protein subunit. Our design allowed for the in-vivo biotinylation of hTERT. We then developed a three-step purification procedure designed to take advantage of three of our engineered telomerase's properties: size, affinity tag and charge. Telomerase's large size allowed isolation after ultracentrifugation in the bottom 4-5 mls of the continuous glycerol gradient. We were able to separate telomerase from the vast majority of contaminating smaller proteins, genomic DNA, membrane proteins and other cellular debris. Furthermore, the location of telomerase activity in the gradient corresponded to a MW of at least ~600 kDa, consistent with at least some of the published literature. This answered a very fundamental and hotly debated question in the telomerase field. As discussed in the chapter 1, the debate still continues on whether telomerase is a functional monomer or higher order oligomer. Our results could be interpreted to contradict the hypothesis that telomerase behaves as a monomer and suggest that it is more likely a dimer or possibly even a higher order oligomer. Next, we used our biotin affinity tag protocol to pulldown telomerase from the pooled gradient fractions. We were successful in our attempts to separate the recombinant tagged enzyme from the untagged endogenous enzyme. From there we were able to bind the purified recombinant telomerase, which is negatively charged, to a cation exchange column and elute from the column with a salt gradient. This final step helped further purify the telomerase enzymes. The novel three-step purification procedure gave a final yield of about 30% of the total activity. Considering the contribution of the endogenous enzymes to the total activity, we could confidently say that our actual yield is well above 30% of the activity.

Finally, the development of the ddTRAP assay allowed us to overcome the final obstacle to the functional telomerase studies. We were able to measure and follow the telomerase activity throughout the purification procedure with ddTRAP. The ddTRAP data confirmed the data from our western blots and

glycerol gradient profiles. We were even able to detect a robust signal from as few as 25 Super H1299 cells. More importantly, we could confidently differentiate samples that had previously looked identical in terms of activity by a gel-based TRAP. The ddTRAP assay would show the 2 -3 fold change in activity, when previously the gel-based TRAP would not because it could only accurately detect 5-10 fold changes. The ddTRAP assay also will liberate us from another key limitation of the gel based TRAP, the limited sample size per assay. We were now able to not only assay every sample in our purification, but also we assayed each sample in triplicates and performed proper statistical analysis of our data. With its absolute quantification, high sensitivity and high-throughput abilities, the ddTRAP assay enables us to tackle the fundamental questions we set to answer in the coming chapters.

Chapter 3: Catalysis Dependent Inactivation of the Human Telomerase Holoenzyme

Introduction

Cellular regulation of gene expression occurs at many levels. Each level of regulation is layered with sophisticated networks of control and response to both internal and external stimuli. To further add to this complexity, virtually every step of the regulatory network can be modulated. The first major level of gene regulation occurs at the DNA. In this level the accessibility of the gene, via structural or chemical modifications, is the main form of regulation. The second major level of gene regulation and possibly the most important is the regulation of transcription. Transcriptional regulation is a vital process in the cell and is governed by access of the gene to RNA polymerase for transcription, the enhancement or repression of the promotor and alternative splicing. The final level of gene regulation is the post-translational modifications of the translated protein. Post-translational modifications can influence the activity, subcellular localization and cellular interactions of the newly translated protein. These finely tuned mechanisms of regulation allow the cell to respond to any stress or stimuli at any given moment within the cell cycle.

Evidence has shown human telomerase to be regulated by all of the above-mentioned levels of regulation (Cong et al 2002), thus making telomerase one of the most highly regulated enzymes in the cell. The initial aims of the study presented in this chapter were more aligned with obtaining and resolving the structure of the human telomerase complex as opposed to addressing its function. While navigating through the data we came across an interesting phenomenon that led to this study embark on a new course towards addressing the functional nature of human telomerase. The key observation was that partially purified telomerase complex was losing activity and becoming inactivated after two rounds of reactions on the R3 substrates. The inactivation however was not due to a lack of stability or sample integrity, but rather due to multiple rounds of catalysis. Telomerase was being catalytically exhausted in

spite of excess levels of substrates. The results in this chapter lead to the proposal that telomerase behave as a single-pass enzyme, meaning that after one-round of catalysis, regardless of the excess of substrate, the particular active sites in telomerase enzymes are rendered inactive.

Results

Double Affinity Pulldown of Human Telomerase Led to the Loss of Activity

Endogenous telomerase was purified from Super H1299 cells with a novel double affinity pulldown. We again took advantage of telomerase's ability to stably bind and extend/elute from the R3 substrate with the addition of dTTP and dATP. In this purification however, the end point was not the first elution, but rather involved a second pulldown and elution from R3 substrate; hence the double affinity pulldown of telomerase (Figure 3-1). In order to deactivate the free nucleotides, dATP and dTTP, from the first elution, we treated the eluate with the diphosphatase apyrase. Apyrase was shown to deactivate the free nucleotides (Figure 3-2). The deactivation of the nucleotides allows telomerase to bind to a new set of R3 substrate oligo. After the second round of binding, telomerase was eluted again with dATP and dTTP. The samples in the purification were then assayed using the gel- based TRAP assay (Figure 3-3). We observed similar binding and elution profiles in the first round of the pulldown; however, the second pulldown profile was not as expected. The second elution had little to no telomerase activity even when the mass of the eluate from 100-fold more cells (cell equivalence) was assayed in the gel-based TRAP assay (Figure 3-3). Following multiple failed attempts to recover active enzymes, we decided to test whether we simply lost the enzyme during the second elution. We quantified the RNA component of telomerase, hTR, a stoichiometric component of the telomerase holoenzyme. The rationale was that by tracking hTR we could determine whether there is telomerase in the samples we employed for the TRAP assay. RT-qPCR on hTR revealed the presence of a significant amount of telomerase in the second eluate (Figure 3-4). qPCR results found that the hTR concentration in the second eluate is equivalent to approximately 8% of the input (Figure 3-4). The enzyme and activity levels indicated by qPCR were well within the detection limits of the gel-based TRAP assay, yet the data showed little to no

activity. The results from qPCR and gel-based TRAP, confirmed that after two rounds of catalysis on the substrates, the telomerase becomes inactive, suggesting the proposal that telomerase was potentially behaving as a single-pass enzyme.

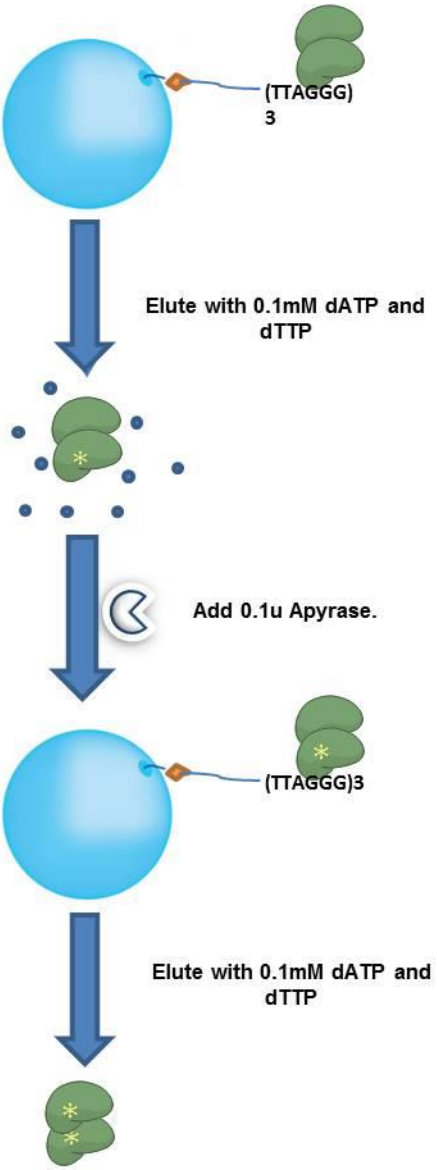

Figure 3-1: Schematic diagram of double affinity pulldown for endogenous telomerase using R3 substrate oligo.

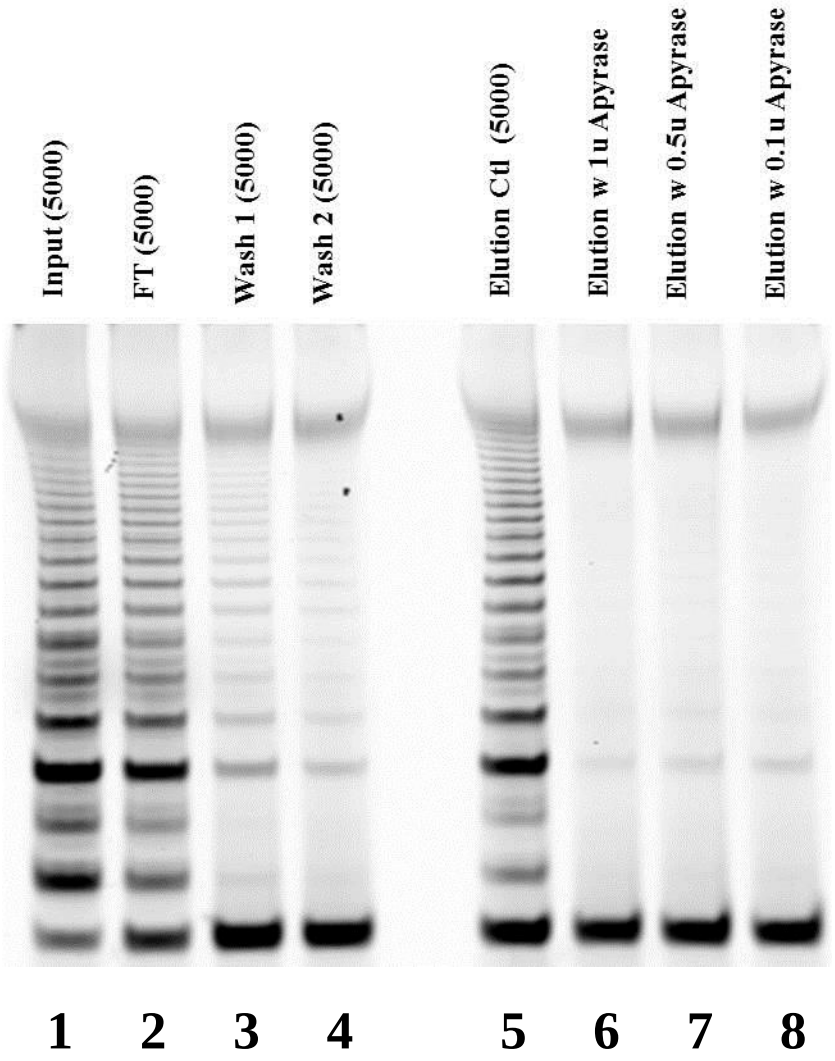

Figure 3-2: Elution buffers containing free dATP and dTTP at 0.1mM were pretreated with 1, 0.5 and 0.1 units of Apyrase. The control lane with no Apyrase shows an elution profile similar to previous results. By deactivating the nucleotides with Apyrase treatment we would expect no telomerase to elute since there are no nucleotides to extend the substrate and generate the TTA sequence. 0.1 u of Apyrase was sufficient to successfully deactivate the nucleotides as indicated by the lack of activity in lane 8.

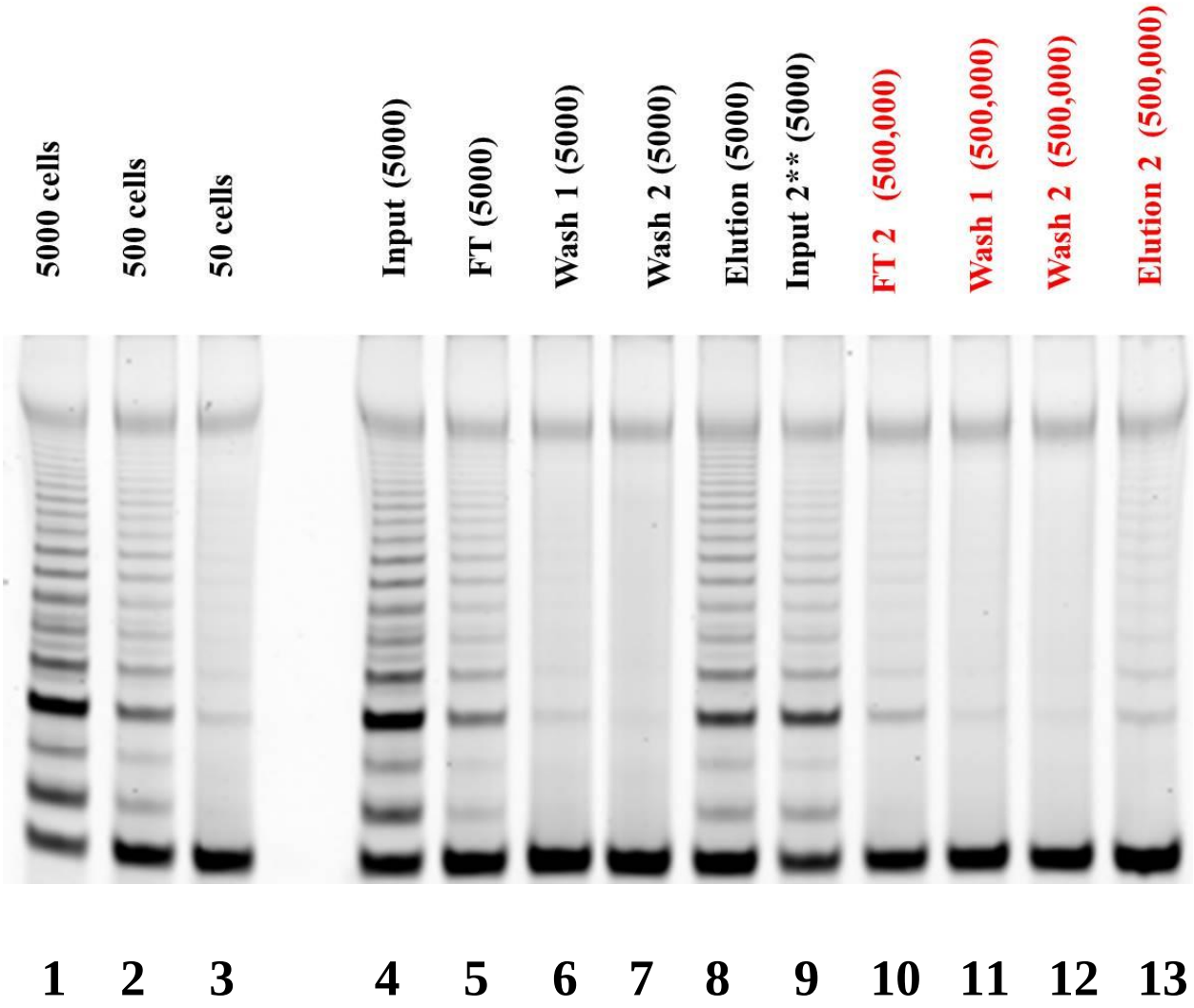

Figure 3-3: Gel-based TRAP activity assay on samples from double affinity pulldown of endogenous human telomerase from H1299 cells. Sample from first elution was treated with 0.1 u of Apyrase for 30 min on ice. Apyrase treatment did not affect the activity as indicated by lanes 8 and 9. The second elution (elution 2) shows little to no activity even though 500K-cell equivalence was used for the TRAP.

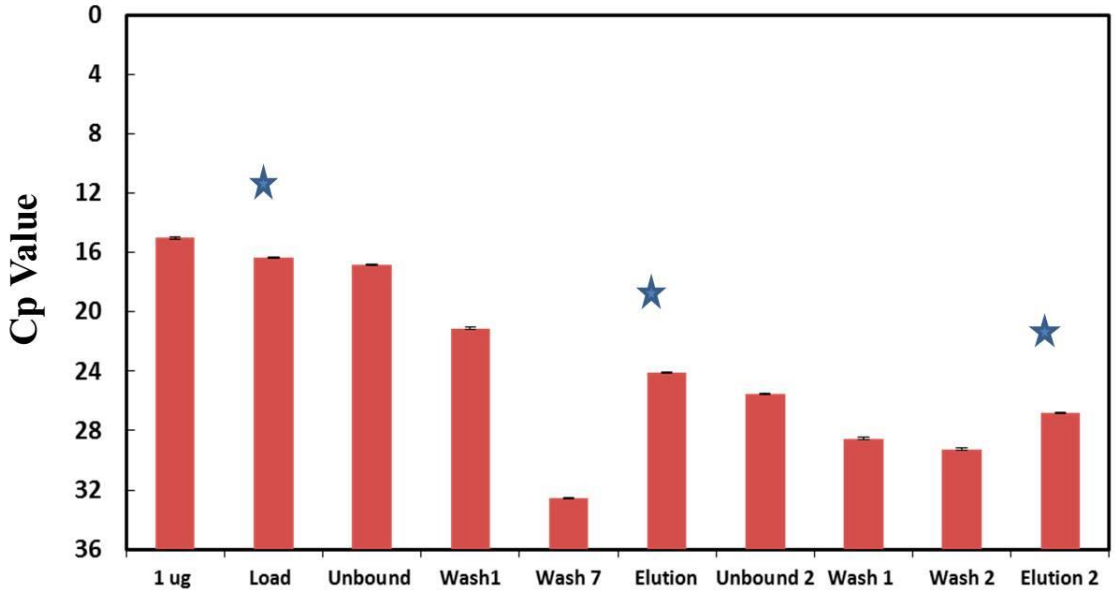

Figure 3-4: RT-qPCR on the RNA component of telomerase, hTR. Samples were collected from the double affinity purification and total RNA was extracted. Seven washes were required to get the hTR levels to baseline. After seven washed the assumption was made that the signal from hTR would correlate to hTR from the telomerase complex as opposed to free hTR. Elution 2 contains approximately 8-10% of the input. Blue stars represent the first load to the column, first elution before apyrase treatment and the second elution from the second set of beads.

Two Rounds of Extension on Tethered Telomerase Complexes Led to its Catalytic Inactivation

To further explore this single-pass hypothesis, we employed the recombinant biotinylated-hTERT. The recombinant hTERT was purified and then tethered to streptavidin-coated beads. Under these conditions, the tethered enzyme could now undergo multiple rounds of extensions as well as encounter fresh substrate and dNTPs all in the same test tube (Fig 3-5). The products from each round of extension were then removed and assayed using the ddTRAP assay. As expected for a single pass enzyme, the relative activity decreases regardless of the excess in substrate (Fig 3-6). In order to test for sample stability, control samples were left at room temperature throughout the course of the experiment. The time delayed control sample was able to account for 90% of the total activity after 270 min at room temperature (Figure 3-6) indicating a stable enzyme. This phenomenon was shown to be the case for the endogenous telomerase as well (Fig 3-7).

The lack of significant activity in extension 3 also helps us exclude the possible product-inhibition of the telomerase because we removed all the products after the second round, and washed thoroughly the enzymes before incubating them with fresh substrates and reaction mixture. The removal of products did not recover any substantial enzyme activity as we expected should the product-inhibition be a sound way to preserve the enzyme active sites.

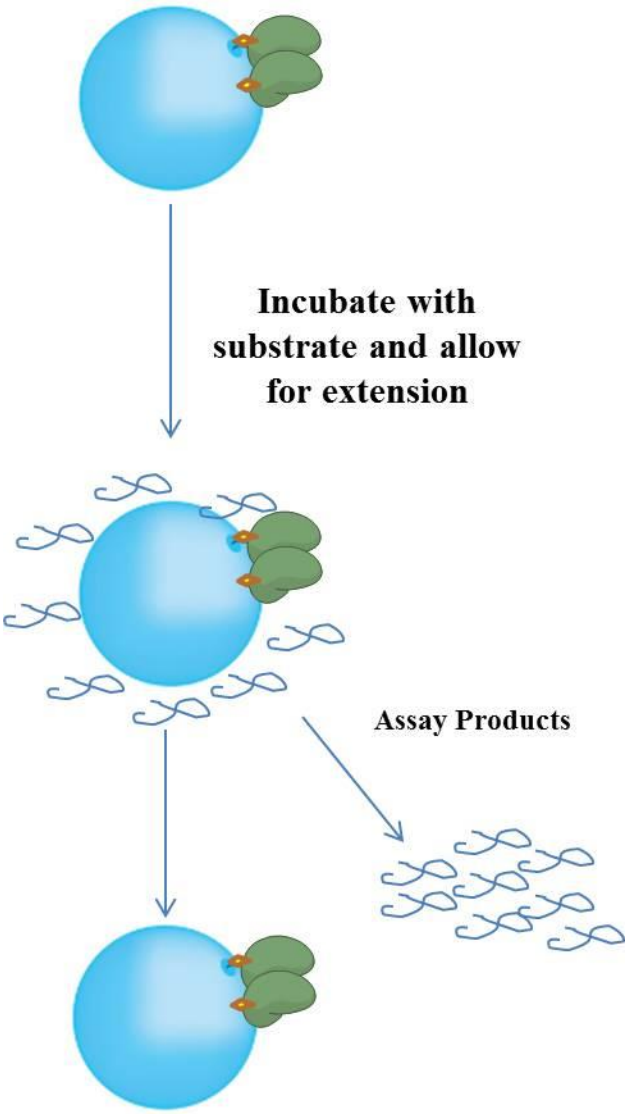

Figure 3-5: Schematic diagram of the tethered telomerase assay. Purified recombinant telomerase was tethered to streptavidin coated magnetic beads. The beads were allowed to incubate with the substrate for 2 hours at room temperature. The reaction was ended by the removal of the solution from the beads. The removed products were then analyzed by ddTRAP. Following two washes, telomerase was then introduced to fresh substrate and the extension cycle was repeated.

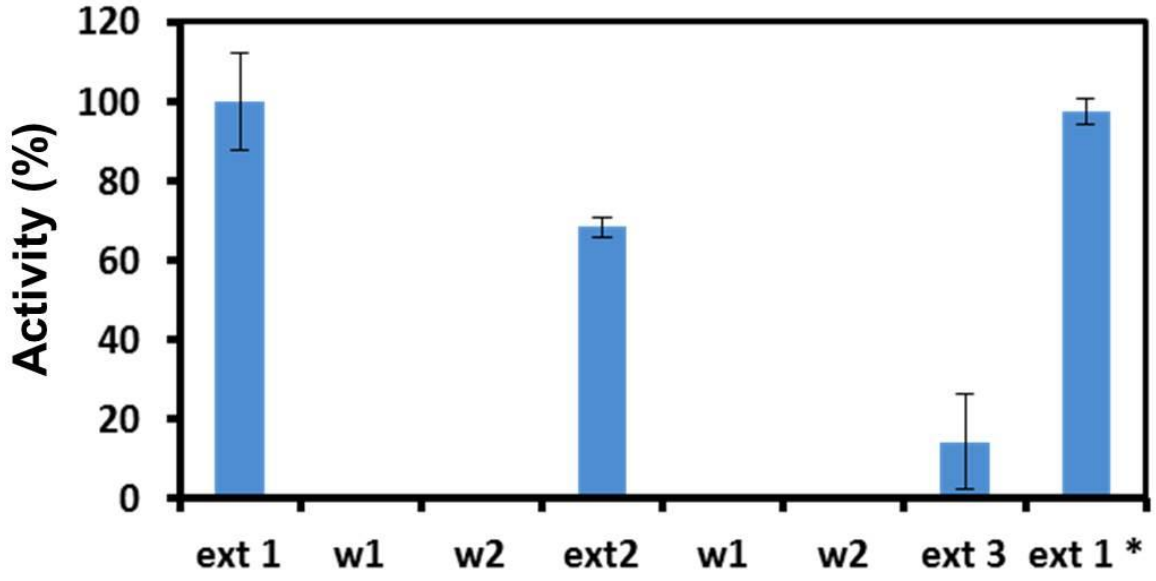

Figure 3-6: Digital droplet TRAP (ddTRAP) assay on samples from the multiple extensions on the tethered telomerase. Activity is going down after each extension. Ext 1* (far right) is the control sample that was allowed to sit at room temperature to test stability. The control sample, Ext 1*, was operated with the reaction mixture lacking nucleotides. The experimental sample Ext 3 reactions was operated in parallel The high level of activity in Ext 1* demonstrates the high stability of the telomerase in the experimental duration of time.

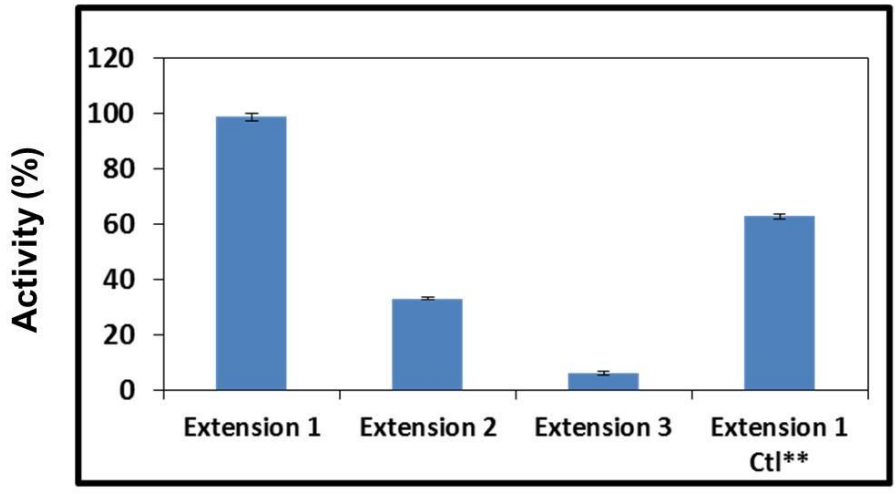

Figure 3-7: Digital droplet TRAP assay on the endogenous tethered telomerase. Endogenous telomerase was tethered to protein A/G beads via an antibody that recognized exon 2 of hTERT. Experiment was conducted in a similar fashion to the recombinant telomerase (Figure 3-6) and yielded similar results.

Purified Telomerase Complex is Stable and Intact

Control experiments were carried out to determine if the telomerase enzymes tethered to the beads were in fact stable. Stability and integrity of a protein/enzyme is fundamental to every experiment. Western blots revealed the tethered hTERT subunit of our telomerase complex to be highly stable, with little to no degradation throughout the 3+ hours on the beads at room temperature (Figure 3-8). Furthermore, ddPCR was used to track hTR levels throughout the multiple rounds of extensions. Again, the results revealed an intact and stable complex. Telomerase RNA, hTR, did not dissociate nor was it degraded while the enzyme was tethered to the beads or while the enzyme was extending the substrates (Figure 3-9). Taken together, these results demonstrate that telomerase is losing activity even though it is highly stable.

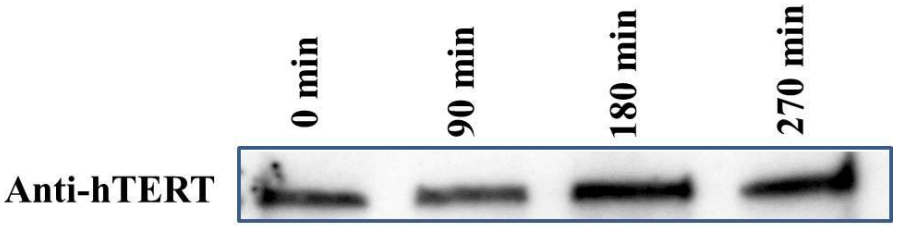

Figure 3-8: Western blot using anti-hTERT antibody reveals a very stable enzyme with little to no degradation of hTERT protein.

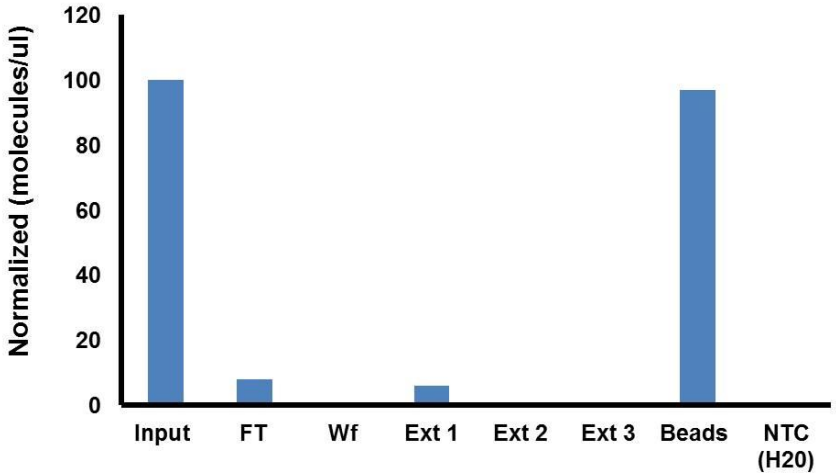

Figure 3-9: Digital droplet PCR was carried out on samples from the multiple rounds of extension on tethered recombinant telomerase to detect levels of hTR in the sample. Instead of assaying the extension supernatant for telomerase extension products we instead assayed for hTR (using Universal Probe Library probe #4). If there was dissociation from the complex, then hTR should be present in the supernatant collected. However, the data shows almost no dissociation of hTR from the telomerase complex (Ext 1, Ext 2 and Ext 3). Telomerase integrity is maintained. Almost all of the input hTR is captured and retained on the beads.

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Discussion

A conventional enzyme, if stably active, is limited by the number of substrates available for its catalytic formation of products. A single-turnover enzyme (single pass is a similar term for telomerase), however, is self-destructive and thus limited in its product production. After one round of catalysis the enzyme becomes catalytically exhausted and is rendered inactive. Although rare, single-turnover enzymes do exist. Most recently, it has been shown to be the case for the endonuclease, Cas9 (Sternberg et al 2014). Cas9 is the enzymatic subunit of the CRISPR/Cas9 complex. More interestingly, Cas9 is also a ribonucleoprotein like telomerase. Kinetic data for single-turnover enzymes reveal an activity-time course similar to most enzymes, but with one major difference. The single-turnover enzyme may become permanently inactive or may be inactivated because of other factors. The conventional enzyme (not single pass enzymes) could reach saturation because substrate has been exhausted or because the product inhibits its activity. If one were to input more substrate into the reaction and wash away the products, the

curve would spike until the newly added substrate was exhausted or the new products became inhibitory. The single pass enzyme however, will reach a plateau due to its self-imposed inactivation. Even if one were to add more substrate to the reaction mixture and wash away the products, the curve would still be flat. The novel data on telomerase obtained support the idea that telomerase is a single pass enzyme.

The data presented in this chapter demonstrate the telomerase enzyme loses its activity after catalysis. After the double affinity pulldown, telomerase was rendered inactive. One interpretation of such a result would be that telomerase was lost or degraded. However, by tracking the RNA component of telomerase, hTR, we were able to detect telomerase in our sample. These results, although indirect, led us to propose the hypothesis that telomerase be a single pass enzyme. To further explore this hypothesis, we tethered our engineered recombinant enzyme to the surface of streptavidin-coated beads via its biotin tag. This system allowed us to carry out consecutive extension reactions by removing the products of the extension reaction without having to remove telomerase. Removal of the products from the reaction also allowed us to eliminate the possibility of product-induced inhibition of telomerase. Furthermore, this system gave us the ability to add fresh substrates and nucleotides to the reaction in excess and in turn eliminated the possibility of substrate deficiency. The data collected displayed a substantial decrease in activity for the tethered recombinant enzyme regardless of the addition of excess substrate in each extension reaction. Moreover, similar results were seen for the endogenous telomerase, providing confidence that the phenomenon observed was not an artifact of the recombinant telomerase.

In order to properly interpret such data, enzyme stability must be taken into account. Control experiments were designed to show the tethered telomerase was stable through the course of the experiment. The time delayed control sample was able to account for >90% of the total activity after 270 min at room temperature. Furthermore, western blots on the tethered hTERT revealed a stable protein and ddPCR quantification of hTR molecules showed an intact telomerase complex throughout the multiple rounds of extensions. Taken together, these results provide robust support for the hypothesis that

the telomerase RNP is behaving as a single-pass enzyme. This novel form of telomerase activity regulation emphasizes the complex network of regulation for telomerase and telomere maintenance.

Chapter 4: Kinetic and Thermodynamic Studies on Human Telomerase

Introduction

The droplet digital TRAP (ddTRAP) assay for the first time allows researchers in the telomere/telomerase field to obtain more much reliable quantification of telomerase activity within a sample. Previously, researchers have relied on assays such as telo-spot, gel based-TRAP and primer extension assays. These assays require the quantifications of the relative intensity of the bands or spots in order to assign values to the data obtained. However, the quantification that follows lacks the sensitivity to distinguish samples with less than 2 or sometimes even 10 fold differences. Furthermore, the assays lack the high-throughput capability in order to assay samples in duplicate or triplicates for statistical evaluation. With the advent of the ddTRAP assay, we were finally able to overcome these obstacles.

In this study, we exploited the highly quantitative and sensitive ddTRAP assay to analyze several kinetic and thermodynamic properties of human telomerase. The most striking discovery is that the telomerase holoenzyme exhibits two kinetically distinct active sites, one of which behaves faster with respect to its catalytic activity than the other one. More interestingly, the two sites shut off after their catalytic cycle and display different affinities for a common substrate. Using biophysical modeling, we compare three different catalytic schemes, providing some favorable support for a sequential model with a delay between two steps. Finally, we develop a tethered telomerase assay to test a prediction from our favored model for telomerase catalysis.

Results

Kinetic Analysis of both Endogenous and Recombinant Enzymes.

Endogenous and recombinant telomerase was partially purified in a continuous glycerol gradient. The partially purified telomerase was then added to extension reaction mixtures containing necessary components for telomerase to fulfill its catalytic addition of telomeric repeats to the substrates. The reactions were allowed to proceed at 27 °C for a specific amount of time, varying from 1 to 300 minutes, before they were terminated with heat inactivation. The reaction products from each specimen were then

analyzed using ddTRAP. The readouts from specimens collected at specific times points were plotted as a function of time (Figure 4-2). These plots reflect the kinetic behavior of the enzyme in time domain.

In the presence of saturating concentration of substrates, a live enzyme would continuously act on its substrate and product a linear monotonic increase of product over time unless there is inactivation of the enzyme or inhibition of the enzyme by its reaction products, which would lead to a plateau in the kinetic curve as what was observed for telomerase. We tested the stability of the telomerase over time and found that it retains more than 95% of its activity when preserved at RT or 27 C for a period of 4-5 hours, suggesting that the enzyme is stable. We also examined and found that the products from the reaction did not inhibit the fresh enzyme added to the reaction mixture or when the reaction products were added to the reaction mixture for fresh enzymes. These controls led to the possible turnoff of the enzyme after its catalysis, similar to the single-turn enzymes reported before. Because of the processive nature of the telomerase activity, we will designate the telomerase as a single-pass enzyme.

Because the kinetic curves in Figure 4-2 show at least two kinetic components, we fitted the data with double exponentials, either in parallel or in sequential fashion, using a function of

$$P(t) = A_1 * \exp(-k_1 t) + A_2 * \exp(-k_2 t).$$

Both the endogenous and the recombinant telomerase enzymes shared similar time constants (Table 4-2). More importantly, the time courses revealed what we had predicted for a single pass enzyme. Both plots reached a plateau after about 5 hours (Figure 4-2) regardless of the excess in substrates and nucleotides. It is important to note that a single exponential model also fit the data very well. However, the time course seemed to show two components of telomerase activity (we further validated this in later chapters as well as in figure 4-4). The first component, with a time constant of ~0.4 min^{-1}, was rapid in its activity and accounted for 10-40 % of the total activity in only 10% of the total time (less than 5 minutes). The second component was much slower with a time constant of ~0.009 min^{-1}, and accounted for the rest of the activity in a much longer time frame of a couple of hundred minutes.

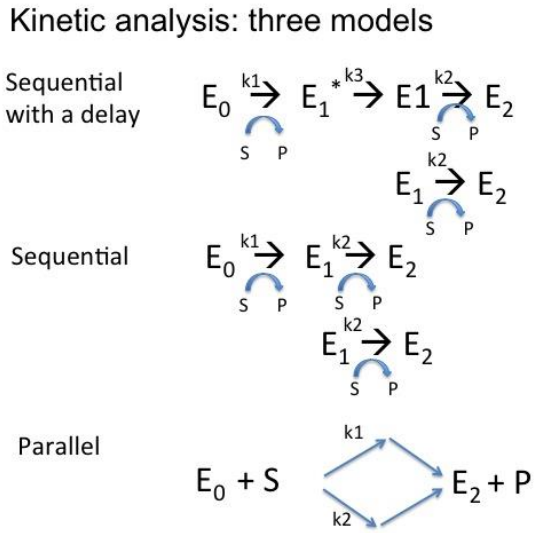

Figure 4-1: Three different models to explain the kinetic behavior of the telomerase.

We assume that there are four different forms of the enzyme for the sequential model with a delay, three forms in the sequential model and two in the parallel model with probably one or two hidden forms (Figure 4-1). The substrate (S) can be converted into products (P) in specific transitions. Based on the data, k1 >> k3 > k2 is likely to be true. Kinetic different equations were generated. Analytical solutions for these equations (see the appendix) were derived and used in MATLAB for Least Squares-based fitting to different datasets. The sequential and parallel models gave rise to two exponential equations as we used above, and the sequential model with a delay has three exponential equations. Among the 14 different data sets we analyzed, only two favored the sequential model with a delay, meaning better fitting by more than 15% less residual error. The remaining were fitting equally well by three models. The kinetic modeling of these three models is not sufficient to distinguish these three models, even though the sequential model with a delay is slightly favored.

Kinetic and Thermodynamic Properties Reveal Heterogeneous Populations of Telomerase Enzyme

We therefore sought to use experimental data to find if we can separate the two forms. We set out to characterize the dimeric complex by investing the similarities / differences between the thermodynamic and kinetic properties of the subunits. In order to achieve this, we needed to separate the telomerase

subpopulations: pristine enzyme (E_0), once-used enzyme (E_1), and twice-used enzyme (E_2; catalytically inactive) (Figure 4-4). The one-step affinity purification, described in chapter 2, was used to purify the telomerase complex. The partially purified complexes contained likely all three forms of the enzyme. Among these three forms only E_0 and E_1 were able to bind to the substrates. The rationale now is to convert the "pristine" dimer (E_0) to the once-used dimer (E1) during the elution from the oligo substrate as well as convert the once-used dimer (E1) to the catalytically exhausted twice-used dimer (E2). The input and elution samples were assayed with ddTRAP in order to explore their thermodynamic and kinetic properties. Time courses for both samples show a plateau in activity corresponding with the previous data that telomerase is behaving as a single-pass enzyme (Figure 4-2). Although both samples (input and elution) show a plateau after 5 hours, there is a clear difference in the activity leading up to that eventual catalytic brake. The time course for the input sample containing both pristine enzymes as well as once-used enzymes can be broken into a fast-acting and a slow-acting component (Figure 4-4). This is not the case for the elution sample containing the once-used (E1) enzyme. The time course for this sample (E1) does not contain the fast component and seems to be only comprised of the slow acting component. This clear difference was also displayed in the *Km* values between the two samples. The *Km* for the input sample is ~3 fold greater than that of the elution sample (Figure 4-4). This 3-fold difference is an underestimation because as noted earlier the input sample contains two populations. Without the hindering of the once-used enzyme in the mix, it is entirely possible that the *Km* for only pristine enzymes would be well below 10 nM. There data suggest that after one-round of catalysis, all the enzymes became slow-acting.

The elution from the substrate-anchored beads was performed within ~15 minutes by catalyzing the movement of the enzyme from G position to A/T position. We then quickly separated the enzymes from the substrate beads using a strong magnet. The lack of dGTP not only made it impossible for the catalysis to happen but also made it difficult for the released enzymes to bind again to the R3-substrates on the beads, thus not able to exhaust all fast-acting components if the two components were acting in

parallel to the slow-acting enzymes. Our observations support the idea that the two components were acting in sequential order such that the fast-acting component first binds to the substrate. The one-round of catalysis releases the enzyme molecules from the anchored substrate, which then turns off the fast-acting sites. We therefore propose that the catalysis by human telomerase follows a sequential model.

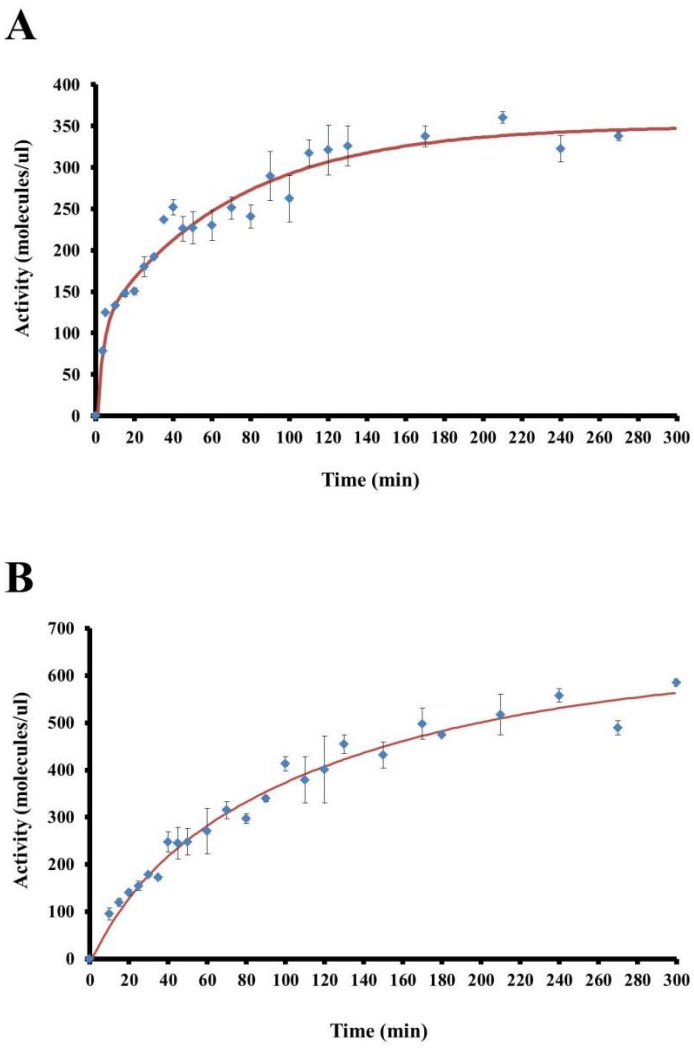

Figure 4-2: Kinetic comparison of the native and recombinant telomerase holoenzyme. *(A)* Time course for endogenous telomerase partially purified by glycerol gradient. Samples were mixed together with extension reaction for the allotted time. Samples were heat killed and assayed with ddTRAP. *(B)* Time course for recombinant telomerase partially purified by glycerol gradient. Both samples show eventual plateau at around 200 min. After fitting with a double exponential association model, we were able to calculate the k1 and k2 constants shown in table 4-1. Both plots show a huge spike in activity within the first 50 min (the endogenous more so than the recombinant) followed by a slow steady increase until the eventual activity plateau.

Sample	K1 (min ^-1)	K2 (min ^-1)
Native	0.400	0.0144
Recombinant	0.416	0.00915

Table 4-1: Table indicating calculated constants from time course fitting. Both endogenous and recombinant telomerase have similar time constants.

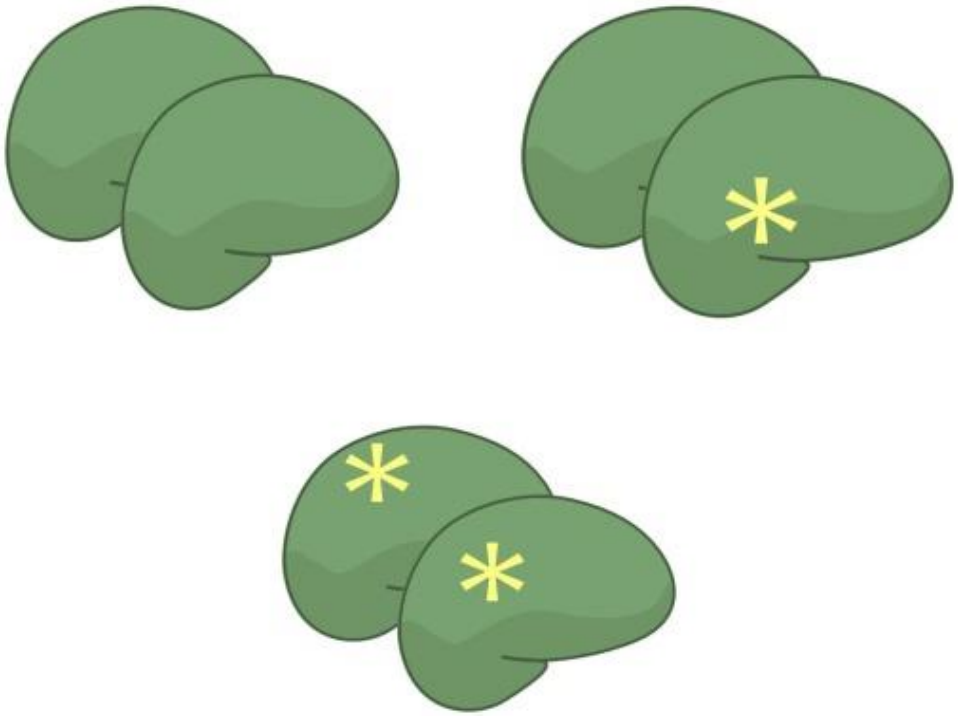

Figure 4-3: Schematic representations of the hypothesized three subpopulations of telomerase dimer enzymes in the cell. The dimeric enzyme with no yellow stars indicated the unused pristine enzyme (E_0) that has not seen extended any substrates. The enzyme with one yellow star is the once-used enzyme (E_1). We hypothesize this complex to have one free available site. The last enzyme has two yellow stars (E_2). This enzyme has had both sites extend a substrate and is not catalytically exhausted.

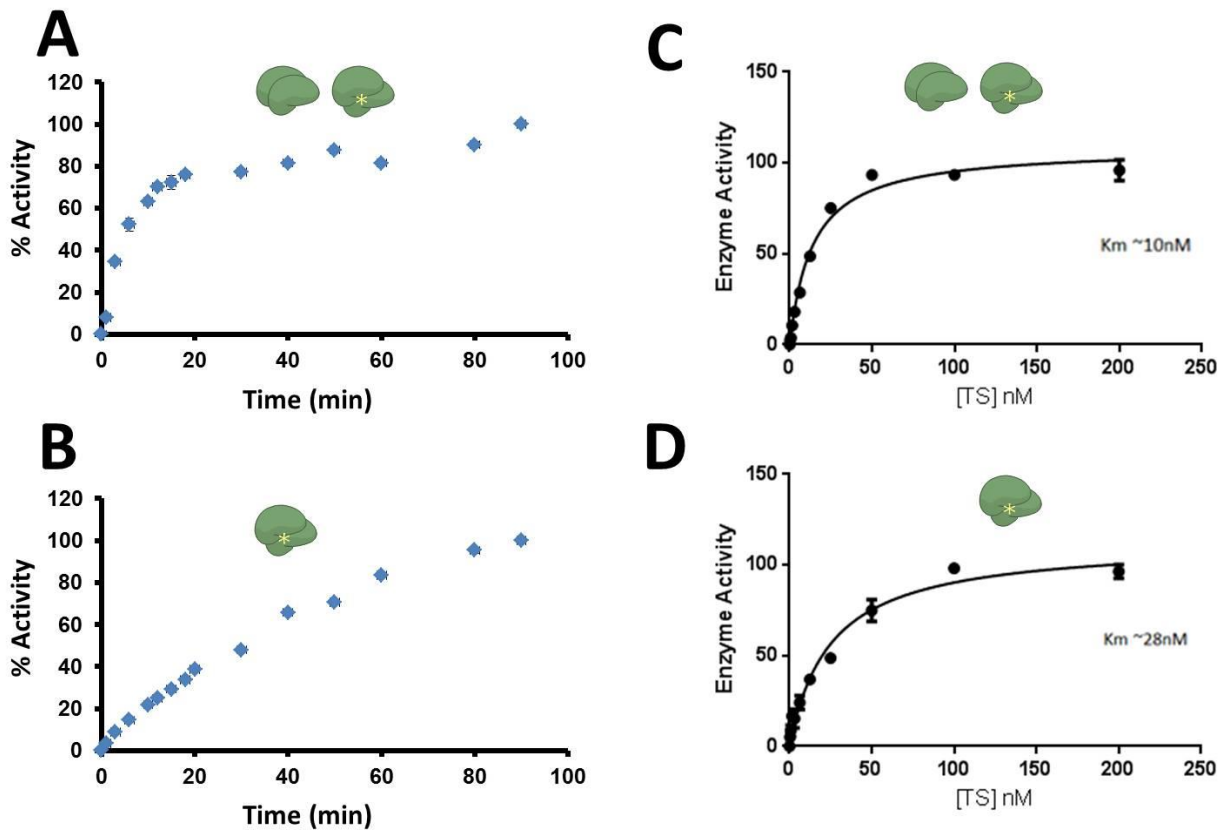

Figure 4-4: Endogenous telomerase complex was bound and eluted from the substrate oligo by taking advantage of the stable interaction with the TTAGGG repeats as well as its catalytic ability to extend the oligo in-vitro with dATP and dTTP. *(A and B)* Here the data shows the time course for the complexes before binding the substrate oligo and after elution from the substrate oligo. In order for the enzyme to elute, it must catalytically add nucleotides to the 3' end of the substrate oligo. This allows us to compare the "pristine/unused enzyme" to the "once-used" enzyme. *(C and D).* Michaelis-Menten plot for the enzyme complexes before and after elution from the substrate oligo. Using ddTRAP to check activity, we were able to detect almost a 3 fold difference in Km between the two samples. The hypothesized subpopulations are indicated above each plot. We believe those samples to make up of those subpopulations.

Sequential Model for Human Telomerase Activity

Combining the data we have had so far, we would propose to use the sequential model with a delay as a good system to represent the kinetic property of human telomerase and will further test the model against experimental data. We will employ the tethered telomerase prep to tightly control when and for how long the enzyme can interact with the substrate to further distinguish the kinetic models. By tightly controlling these two parameters, we should be able to get a better understanding of whether telomerase dimer is behaving in a parallel or sequential manner (Figure 4-5).

The first question is whether the two sites of the telomerase bind to the substrate at the same time. In the tethered enzymes, we controlled the duration of time when the enzyme was able to interact with the substrate (TS primer). We first tethered the recombinant enzymes to the streptavidin-coated beads and allowed for a first round of extension (Figure 4-5). This extension was carried out at room temperature for 2 hours with excess substrate. The sample was then split into two groups and the second and third round of extension were carried out either in the presence of the substrate (control; +TS) or without (-TS) (Figure 4-5). If a tethered telomerase dimer bound two substrates at the same time, then regardless of whether the substrate is present in the mix (extension mix includes dNTPs and buffer) for the second round of extension, the measured products from the second round of reactions (+TS vs. –TS) should be comparable. However our experiments showed the opposite. The tethered enzyme with no substrate in the second extension exhibited a significant drop in activity compared to the control sample (Figure 4-6). The residual amount of activity for the no substrate reaction [2(-TS)] likely came from the substrates on which the enzyme did not finish the reaction within 2 hours because of the slow-acting enzymes (E_1). As expected the third round of extension for both sets of tethered enzymes displayed little to no activity (Figure 4-6). The telomerase reactions in the tethered enzyme preps contain an excess amount of DNA substrates (TS primer) as well as excess nucleotides. The sequestered binding capability of one of the subunits of the dimeric complex presumably, as alluded to earlier, is due to a type of negative cooperativity such that occupation of the first binding site suppresses the binding of the substrate into the

second binding site. When the first subunit is in the middle of the first extension, the second subunit sits in a dormant state and is prevented from binding the substrate. This also implies that after the first round of reaction is finished, the second subunit needs to switch from the dormant to the active state, which might be represented by the (E_1* → E_1) transition in the sequential model with a delay.

The results from figures 4-6 and 4-4 have lent strong support for a sequential model. In order to further cement this new concept, we tested the behavior of the enzymes based on the model by varying the duration for the first telomerase extension on the tethered enzymes. For this experiment, tethered telomerase complex were allowed to extend the substrates for 30, 90, or 120 min in the first extension. Following the first round of varying extension times, the enzyme was then subjected to equal extensions times for both the second and third rounds (120 min for all samples). This variation in extension times could probe the sequential model by revealing a more strict time-dependent nature of the dimeric complex. As the fast-acting components were quickly exhausted within the first 30 minutes of reaction, largely the E1 enzymes would be left for the second and third reactions. The sequential model predicts that the reaction products would be accumulative and after ~300 minutes all the enzymes should be used up. The 30 min extension one sample (blue) as expected had the lowest activity in the first round, but showed significantly greater activity in both the second and third rounds (Figure 4-7) as compared to the 90 min and 120 min extension one samples. The 120 min extension one sample behaved as expected with its second round showing a significant decrease in activity culminating with little to no activity in the third round (single pass nature of telomerase). The 90 min extension one sample consistently had the second most activity in every round of extensions.

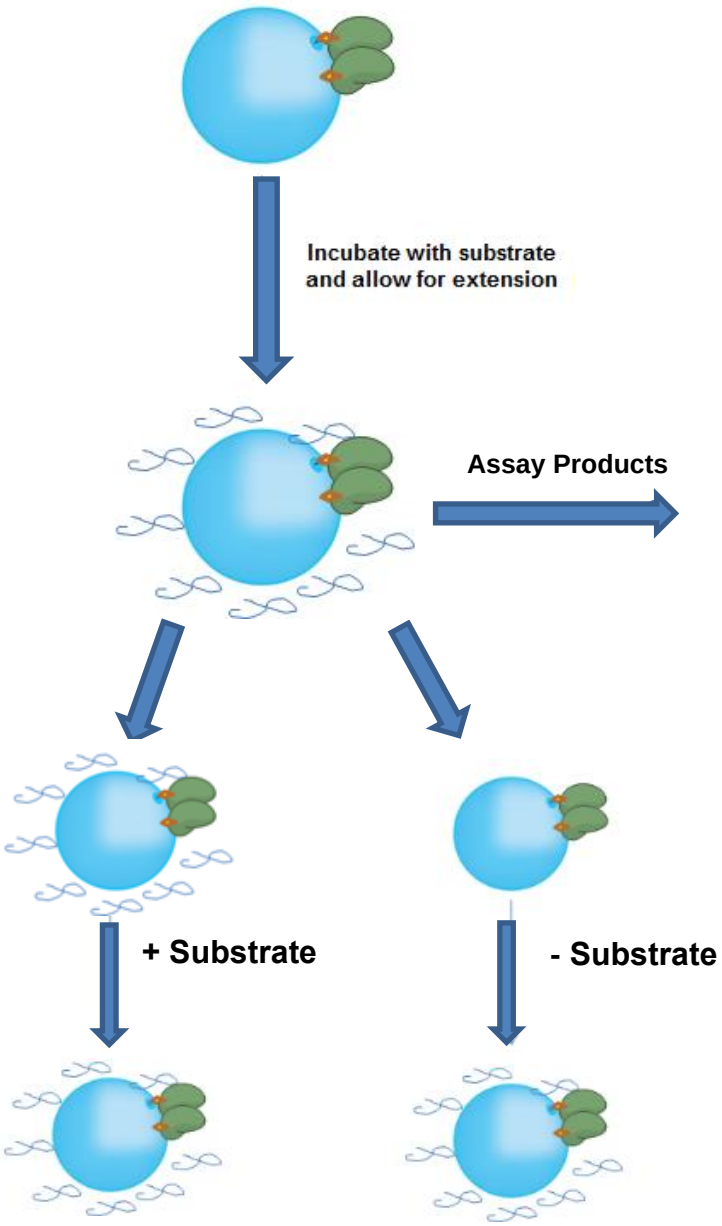

Figure 4-5: Schematic representation of experimental design/setup.

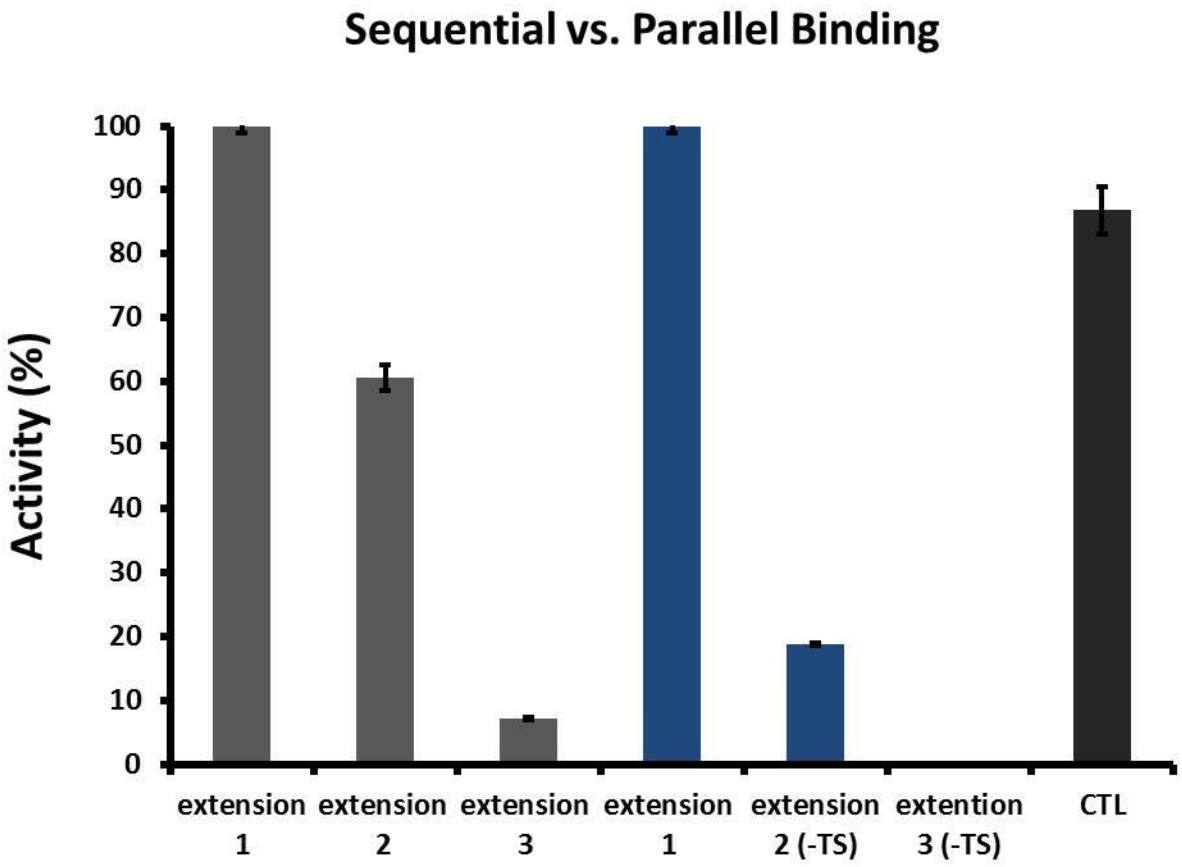

Figure 4-6: In this experiment, tethered telomerase enzymes were allowed to extend the substrate and undergo one round of extensions. The samples were then split into two groups. The control group was allowed to incubate with excess substrate for the next two rounds of extension while the test group was not going to have any more substrate for the following two round of extensions. If both subunits of telomerase dimer were interacting with the substrate at the same time, then we should see similar levels of activity in extension 2. The data here indicate that both subunits of the dimer complex are not binding the substrate at the same time and that only one complex is able to bind and act on the substrate. The sharp drop-off in activity between the "extension 2 ctl" and "extension 2 (-TS)" indicate this conclusion.

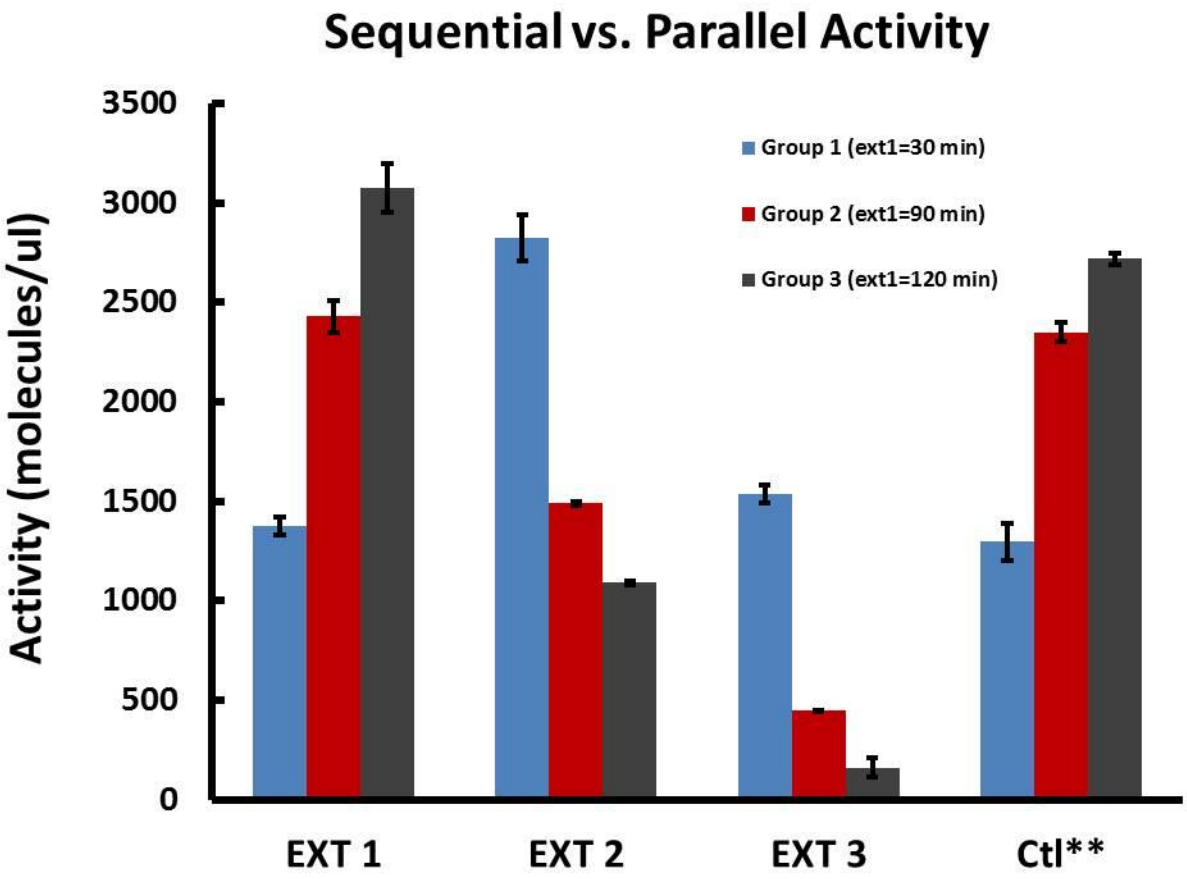

Figure 4-7: Data showing the strict time dependent nature of telomerase activity. The extension times were varied for the first extension. We chose a short (30 min), medium (90 min) and long time interval (120 min) for telomerase to extend. However, the second and third extensions were kept the same at 120 min for each group. The model predicts an accumulative time-dependent consumption of the active enzyme.

Discussion

Considerable evidence in literature supports the notion that human telomerase is a dimer (Cohen et al. 2007) (Wenz et al. 2001) (Beattie et al. 2001) (Gardano et al. 2012). The biochemical data we presented in chapter 2 are also in line with the published literature. By using the ddTRAP and the biochemical purification of recombinant telomerase, we were able to explore and characterize the kinetic and thermodynamic properties of the human telomerase complex. The time courses for both endogenous and recombinant telomerase further solidified the hypothesis that telomerase is behaving as a single-pass enzyme. The activities plateaued despite the excess substrate and nucleotides available to the enzyme. After further analysis, the time courses were consistent with the hypothesis that telomerase is composed of two components, a fast and slow acting component.

Our working hypothesis assumes that there are at least three subpopulations of the telomerase enzyme obtained from the cell: unused pristine enzyme (E_0), once-used enzyme (E_1) and twice used (E_2) catalytically inactivated enzyme. Two of the three populations (E_0 and E_1) are active and can contribute to the activity assay by catalyzing the conversion of substrates to products. Using the one-step affinity purification of endogenous enzymes, we were able to separate the subpopulations of telomerase (E1 plus inactive E2) to characterize them. The time course data as well as the Michaelis-Menten plots for Km suggest that the dimeric complex contains one fast acting site with a $Km < 10$ nM and one slow acting site with a Km ~30 nM. These observations are consistent with the hypothesis that there is a type of negative cooperativity between the two sites in the telomerase dimer such that the catalytic action in the first site suppresses the binding of the substrate to the second site and the inactive state of the first site renders the second site to show low affinity towards the substrate.

To further investigate this dimeric complex, we proposed a model for its interaction with its DNA substrate. In our comparison of the three models, the parallel model for telomerase would indicate a dimer that could bind two substrates at the same time and extend both of them in parallel. In contrast, a sequential model would allow the enzymes to interact with one substrate at a time and suggest that only

after the binding, extension, and dissociation of the first substrate would the enzyme bind and repeat the process with another substrate. We have two key pieces of data to support the sequential model (Fig. 4-3 and Fig. 4-5). First, the telomerase bound and eluted from the substrate beads displayed only the slow acting component in the time course profile. The lack of a fast acting component reveals the telomerase dimer is binding in a sequential fashion. If telomerase were binding in a parallel model, then both fast and slow acting sites would be bound to the substrate in a random distribution. After catalytic extension and elution from the beads, we would expect a mix of fast and slow acting components to contribute to the time course in figure 4-3B. However, this was not shown to be the case. Second, we took advantage of the tethered telomerase assay to explore the binding and activity of telomerase to the substrate. The data revealed that only one of the subunits of the dimer can interact/bind to the substrate at a time. This supports the ideas that some form of negative cooperativity regulates the accessibility of the substrate to the second site. Furthermore, the strict time-dependent nature of the activity of telomerase further suggests that the telomerase dimer behaves in an accumulative manner as we expected for a single-pass enzyme with its two sites acting in a sequential manner.

Chapter 5: Identification and Purification of Novel Telomerase Activating Factors

Introduction

Cells have evolved over billions of years to reach that pinnacle of efficiency we witness today. For a cell to invest the energy to build a large RNP complex such as telomerase to only have it become catalytically exhausted after one cycle of extension is counterintuitive. It is known that telomerase needs to be regulated as telomerase activation is one of the hallmarks and possibly a significant hurdle a cancer cell must overcome to become malignant. However, cancer cells are not ideal models as they are littered with all that can go wrong inside. This leads us to hypothesize that there is potentially a telomerase factor that can activate/re-activate the inactivated telomerase and allow normal cells (that express telomerase) to use telomerase repeatedly to maintain its telomeres.

In this study, we set out to accomplish the following: test for telomerase reactivation, propose a mechanism for the activation and identify/purify the factor. This ambitious undertaking relied heavily on time-consuming classical biochemistry; biochemistry that was made even more difficult because we lacked the knowledge for what to look for in the first place. However, we combined the biochemistry with the newly developed ddTRAP assay as well as modern mass spectrometry in order to achieve the aims mentioned earlier. First, we identified from a crude lysate the ability of the telomerase to be activated/re-activated. We then proposed a kinetic and thermodynamic mechanism for how this factor activates telomerase. Finally, we used classical biochemical purification strategies to separate and identify this factor with mass spectrometry. Although incomplete, we have made significant progress on this new and exciting avenue of telomerase research.

Results

Telomerase Activation/Re-activation with BJ Human Fibroblast Cells

In order to test our hypothesis, tethered telomerase enzymes were mixed with human BJ fibroblast cells after catalytic exhaustion (Figure 5-1). Telomerase was exhausted with three rounds of

extensions as we disused in chapter 4 (Figure 5-1). We then proceeded to do a fourth round of extension, one without BJ cell lysate and one with. As we predicted, the control sample, telomerase without the lysate, had little to no activity. However, the sample with the lysate saw a 7-fold spike in activity (Figure 5-2). This was the first indication that telomerase was being activated/re-activated. We proceeded to test this phenomena on a variety of cell lines including normal telomerase-negative human cells (BJ cells), normal telomerase positive human cells (keratinocytes and lymphocytes), cancer cells (MCF-7, A549, H1299, HEK293, HCT-116 and HeLa) and telomerase negative cancer cells (ALT-SAOS, ALT-SW13, ALT-MDA0487 and ALT-VA13). All of the cells we tested were able to activate/reactivate telomerase (data not shown).

Telomerase Kinetic and Thermodynamic Studies With and Without Lysate Containing Factor

Kinetic and thermodynamic studies were carried out on telomerase with and without the cell lysate that contains the putative activating factor. Telomerase was purified and tethered to the beads. The beads were split into two groups, one with ALT-SW13 lysate and one without. We compared the time course plots and Michaelis-Menten plots to propose a mechanism for how telomerase is being activated by the lysate. The samples varied drastically in their kinetic and thermodynamic properties as displayed by their Kfast (from time course) and Km (from M&M plot) (Figure 5-2). The samples with the cell lysate had a Km of 0.36 nM, which is 14-fold higher than the control samples, Km of 5.29 nM. The Kfast for telomerase plus lysate was 1x10^7 fold higher than the Kfast for telomerase only. The time courses were strikingly different (Figure 5-3). Telomerase with the activating factor was able to continue to extend well past 100 min. The second "slow component" was able to contribute much more to the total activity of the enzyme compared to the control sample which displayed the quick jump followed by the slow increase in activity.

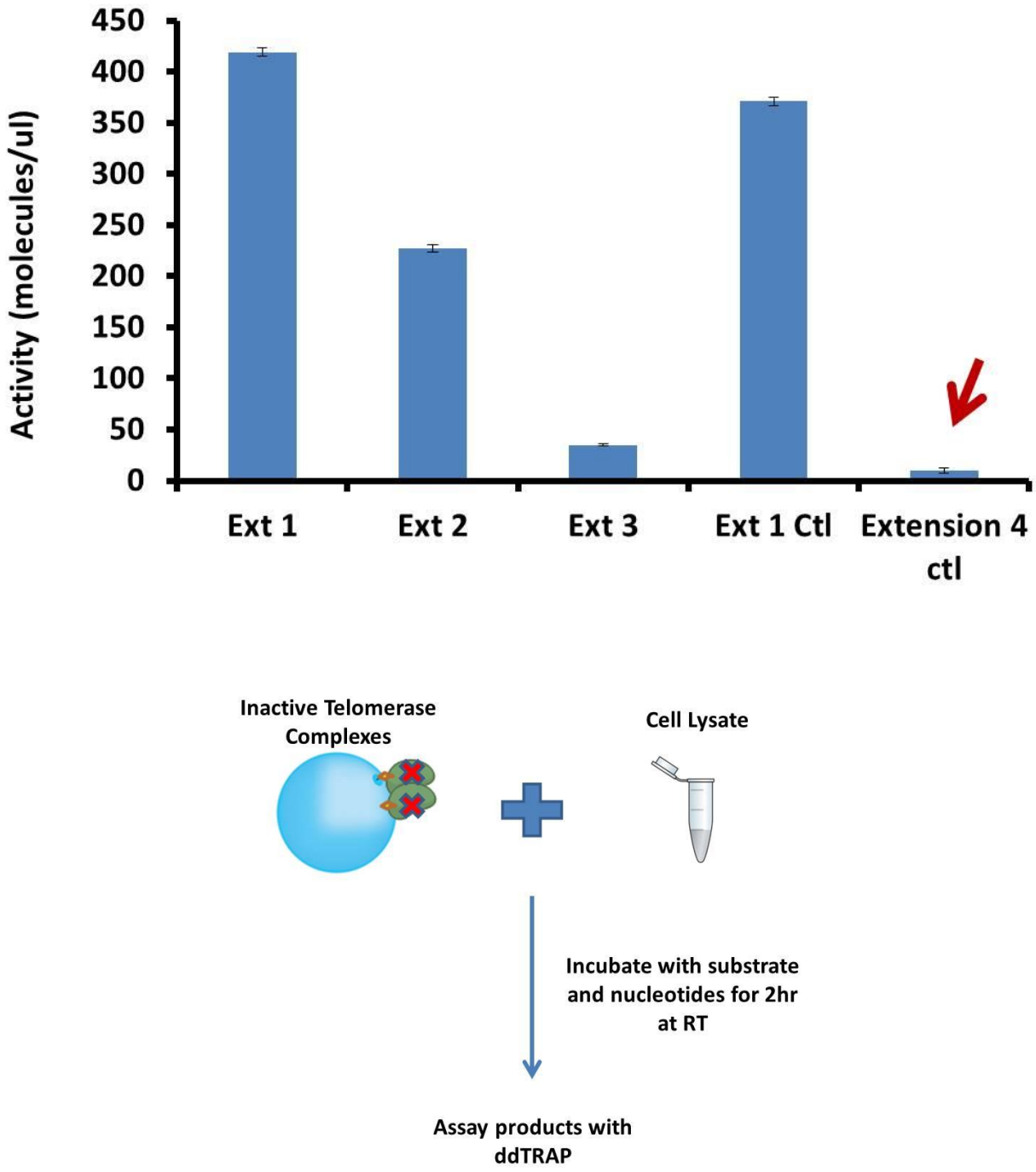

Figure 5-1: *(Top Panel)* Tethered telomerase enzymes were allowed to extend the substrates for three rounds of extension. Telomerase activity goes down as shown previously in chapter 3. There is little no activity after a fourth round of extension as indicated by the red arrow. *(Bottom Panel)* Schematic representation of experimental design/setup. Catalytically exhausted telomerase complexes were mixed with cell lysates and allowed to extend substrates for a fourth round of extension.

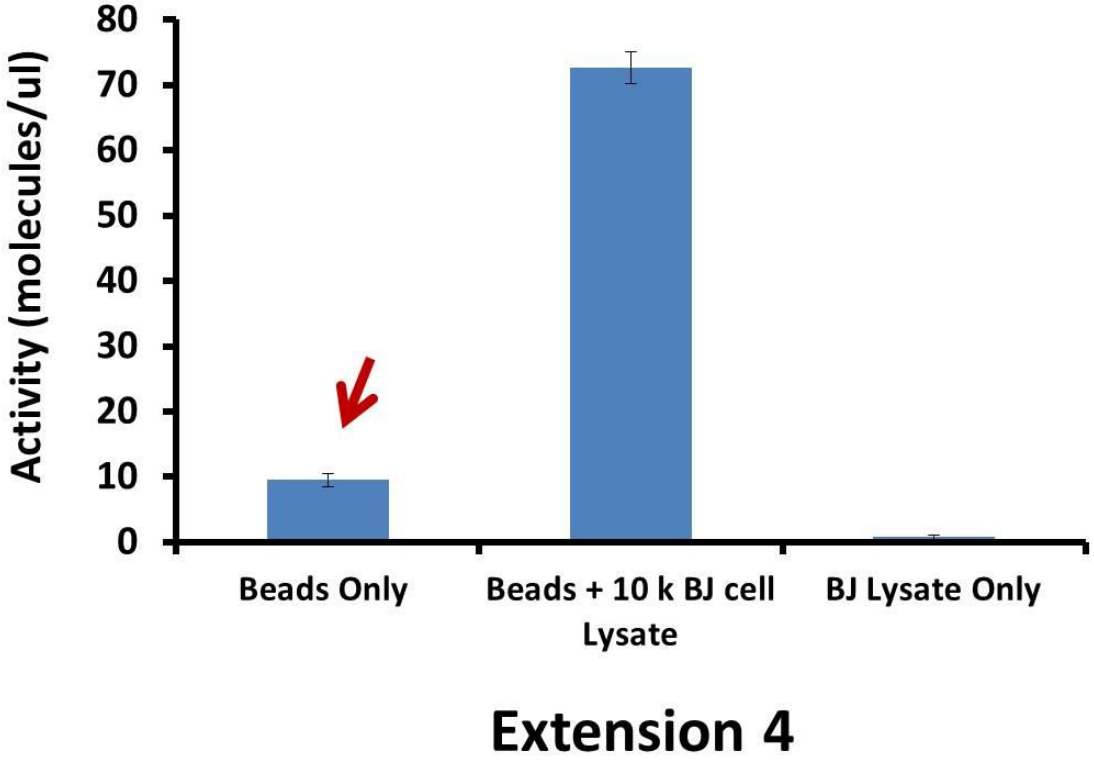

Figure 5-2: Telomerase was mixed with human BJ fibroblast cell lysates before being allowed to extend the substrate for a fourth round of extension. The sample with the cell lysate had a 7-fold increase in activity compared to the sample without the lysate. The control sample indicated by the red arrow is the same sample from the previous figure (top panel from figure 5-1). As expected, telomerase negative BJ cells had no detectable activity via ddTRAP.

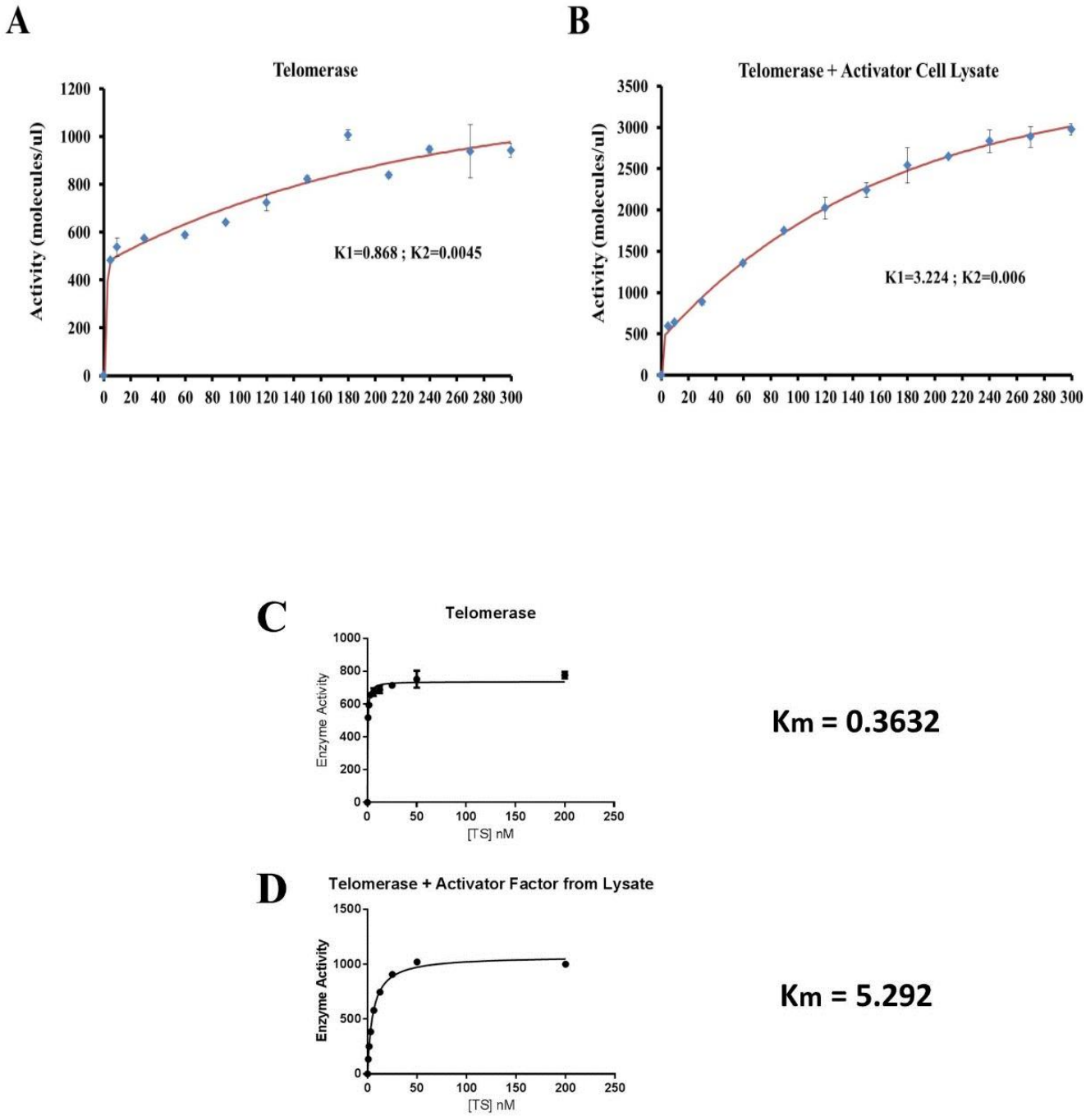

Figure 5-3: The top two plots are the time courses for telomerase enzymes with and without the lysate containing the activation factor. The bottom two plots are the Michaelis-Menten plots for telomerase with and without activation factor. Both K1 and Km values are drastically different between the two samples.

Using Tethered Telomerase to Pull Down the Intracellular Activating Factor from Cell Lysates

The next aim was to determine whether or not there is a direct interaction between telomerase and the unknown factor(s) from the cell lysate. Telomerase was tethered to streptavidin coated magnetic beads and was split into 8 groups. These groups were then used for a pulldown of the activating factor from the lysate with various conditions (Figure 5-4). Telomerase beads were either incubated with the lysate in the same tube where the extension reaction was performed or incubated with the lysate, then pulled down and washed multiple times before being assayed for the telomeric extension reaction. All samples were assayed with ddTRAP for telomerase activity. As shown in Figure 5-5, we were able to pull down the intracellular factor from the lysate of ALT-SW13 cells. The total activity from the ddTRAP matched the total from the positive control sample. Interestingly, we were able to abolish the activation of this factor on telomerase activity simply by heating the cell lysate at 95 C for 5 min (Figure 5-5). This result suggests that the factor is more likely a protein. As negative control, we were not able to achieve the 4-5-fold activation using the rabbit reticulocyte lysate (RRL). This was the first known example we had of a cell line that did not contain the unknown factor. As mentioned earlier, RRL was used in the successful assembly of active in vitro translated human telomerase in the 1990s by the Shay/Wright Lab (Holt et al. 1998). This suggests that it should contain the necessary components for producing active telomerase from newly translated hTERT, hTR and other factors, but not the factor to (re)activate the inactive telomerase. This further suggests that this is a novel factor involved in regulating telomerase activity.

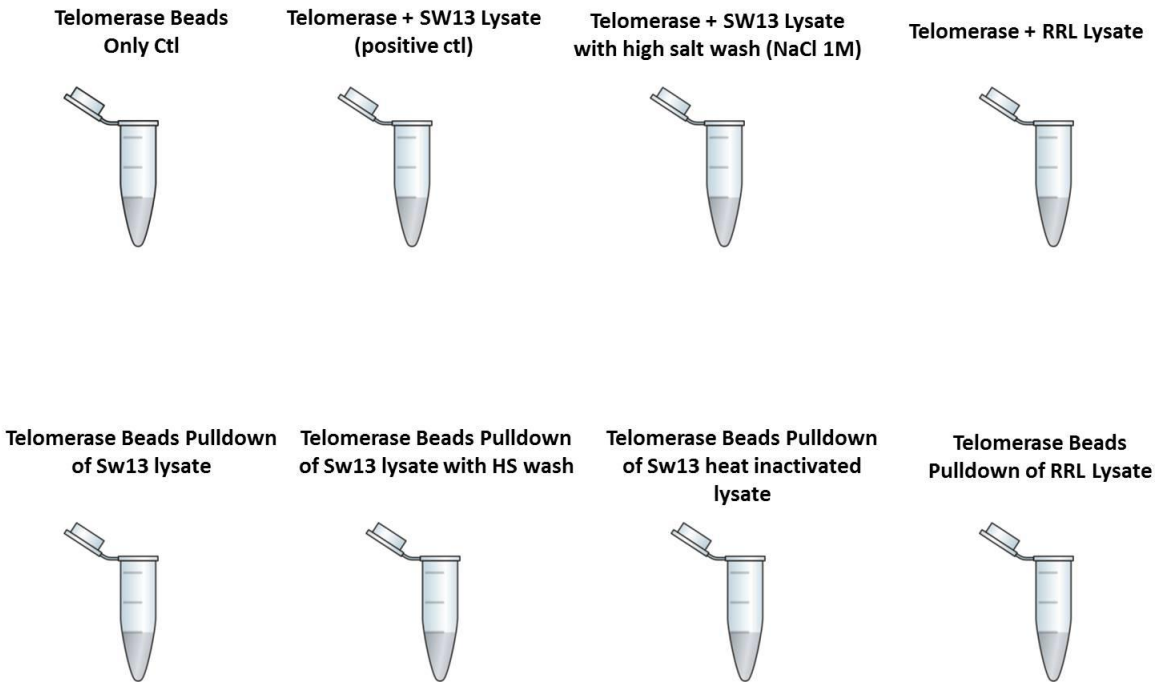

Figure 5-4: Schematic diagram of sample groups used for the telomerase pulldown experiment in figure 5-5.

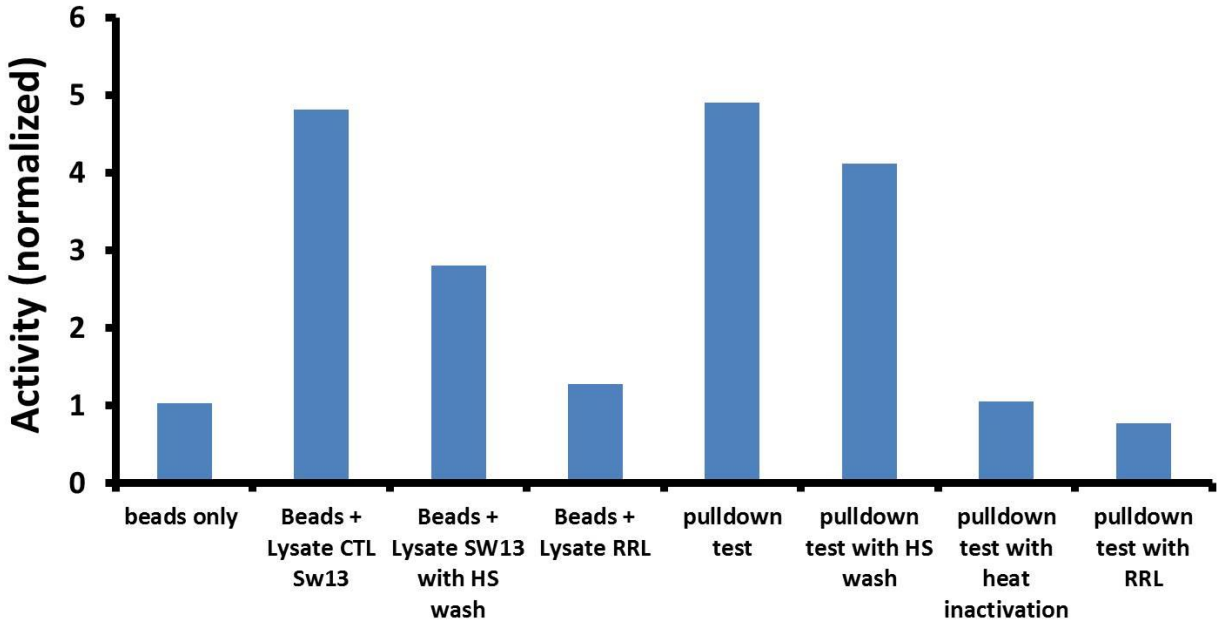

Figure 5-5: Tethered telomerase pulldown of activation factor from ALT-SW13 and rabbit reticulocyte lysates. The "pulldown test" sample shows a successful pulldown of the factor from the ALT-Sw13 lysate. The ddTRAP shows an equal amount of activity between the positive control (Beads + lysate CTL SW13) and "pulldown test". Also the rabbit reticulocyte sample was shown to not contain the activation factor as indicated by the two samples "Beads +Lysate RRL" and "pulldown test with RRL". Furthermore, heat inactivation of the ALT-SW13 lysate (pulldown test with heath inactivation) prior to incubation with the tethered telomerase restores activity to the baseline levels (beads only).

Purification and Identification of Novel Telomerase Activating Factor

The results from the pulldown of the activating factor indicated that this novel factor is most likely a protein. Unlike RNA or DNA, most proteins unfold at 95 C. Therefore we set out to identify this protein using a combination of classical biochemistry, ddTRAP and mass spec. The biochemical purifications summarized in this section involve three main strategies: separation by size, separation by charge and separation by subcellular localization. We used the tethered telomerase beads to locate and track the factor by measuring the enhancement of telomerase's activity in the ddTRAP assay. The pulldown from the previous section revealed that rabbit reticulocyte lysates were not able to activate telomerase. RRLs are essentially lysates from red blood cells and red blood cells have no nucleus. This led us to hypothesize that telomerase activating factor is most likely a nuclear protein. In collaboration with Dr. Gaya Yadav (a Postdoctoral Fellow in Jiang Lab), we developed a strategy to enrich this nuclear factor using various chromatographic methods for protein purification. The first step of the purification involved separating nuclear proteins from cytoplasmic proteins (Figure 5-6). Continuous glycerol gradients (Figure 5-7) and size exclusion chromatography (5-8) were employed to separate the factor based on its size. Finally, we used Mono Q (Figure 5-9) and Mono S (not shown) columns to separate based on the charges of the factor. After the purifications we ran the final fractions on a silver stain and Coomassie-stained gel (Figure 5-10) and then sent the proteins isolated in gels out to mass spec identification. Using the proteome software MASCOT and Scaffold, we were able to identify ~300 possible candidates. The selection criteria for these candidates were as follows: established a greater than 95.0% probability, contained at least 2 identified peptides and contained no common peptides seen in the negative control samples.

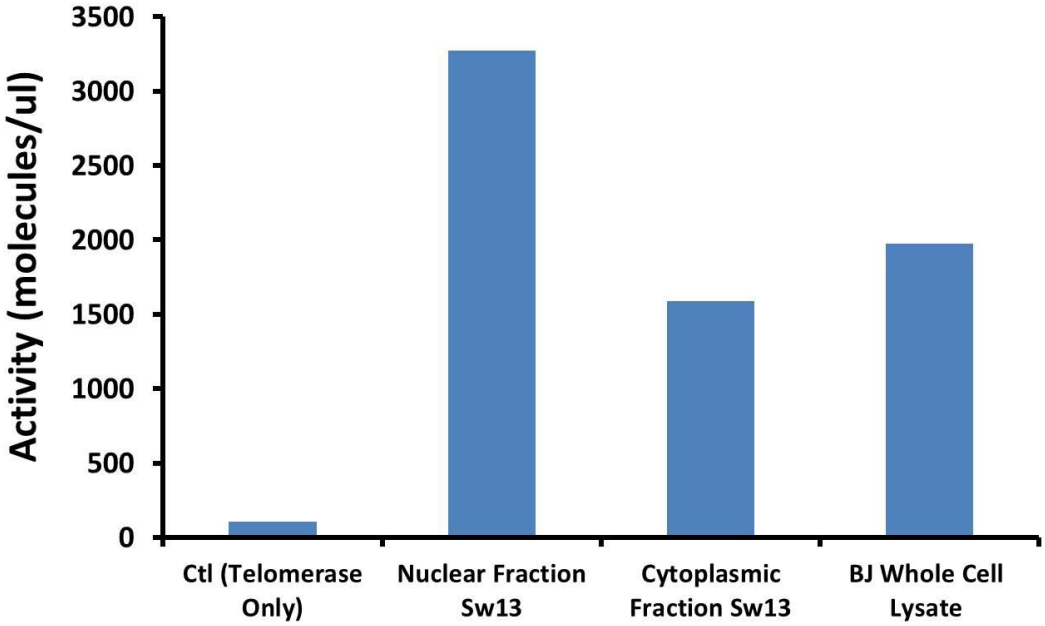

Figure 5-6: Fractionation of the cell into nuclear and cytoplasmic fractions reveals that the activating factor is mainly in nucleus. Cytoplasmic fractions also showed higher activity compared to the control telomerase only sample but this is most likely due to cross-contamination during the fractionation of the ALT-SW13 cells. More importantly the nuclear fractions contain much fewer contaminating proteins compared to the cytoplasm and thus will increase the chances of separating this factor from the rest of the proteins.

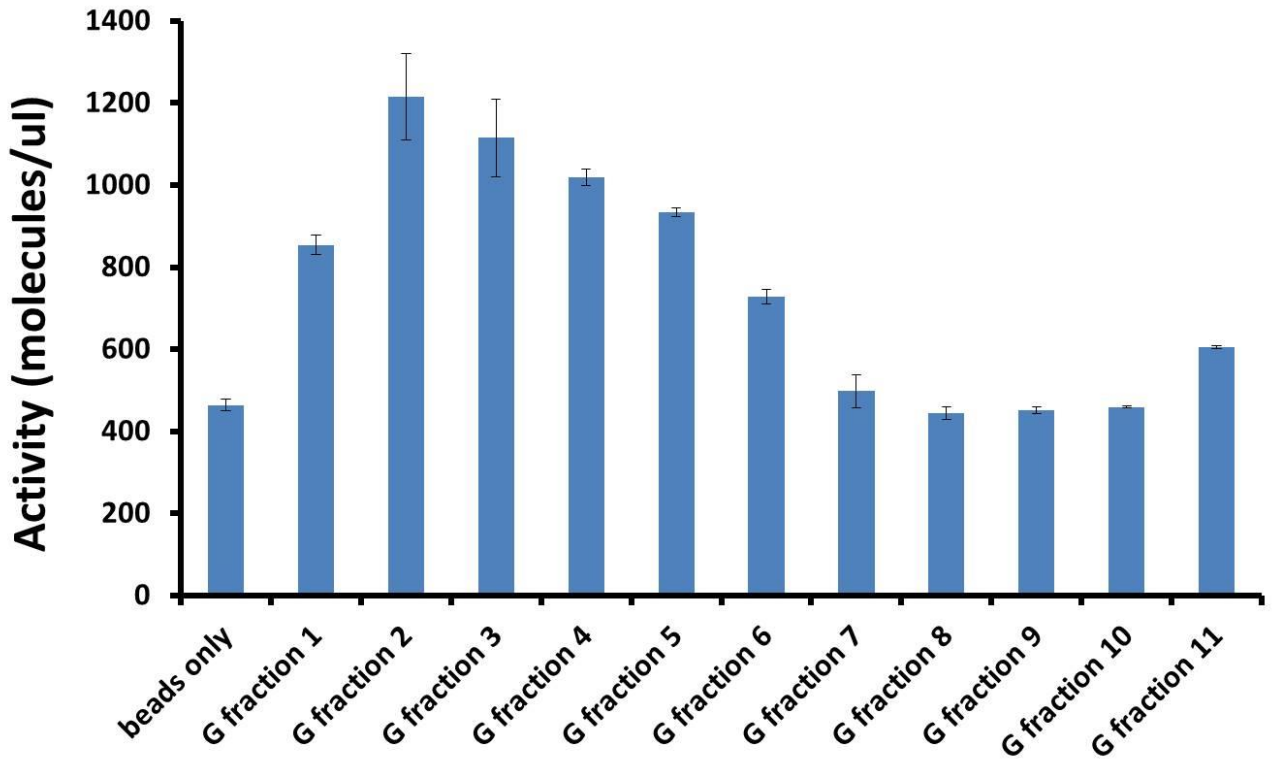

Figure 5-7: DDTRAP assay on glycerol gradient fractionated BJ human fibroblast cells. BJ nuclear lysates were loaded to a 10 ml continuous (10-30%) glycerol gradient and then ultra-centrifuged overnight. The activating factor was highly enriched in fractions 2 and 3 as shown by the ddTRAP results. Fractions 2 and 3 were pooled and used for the next step in the purification.

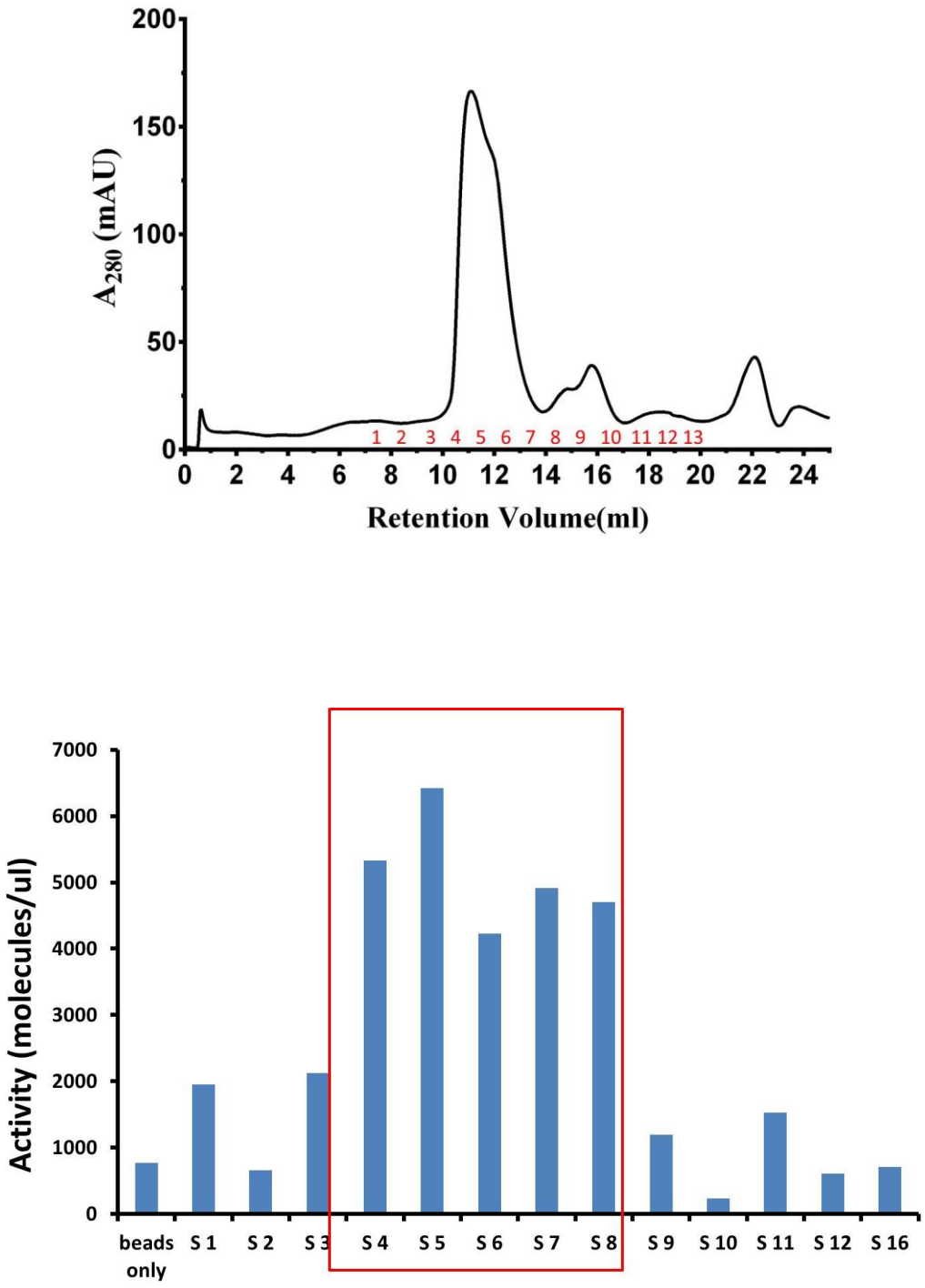

Figure 5-8: *(Top Panel)* Superdex 200 elution profile. Samples were eluted in 1 ml fractions and then assayed with ddTRAP to test for the activation of inactive telomerase. *(Bottom Panel)* Telomerase activating factor was isolated in S4-S8 as shown by the ddTRAP activity assay. These samples were pooled and used for the next step in the purification.

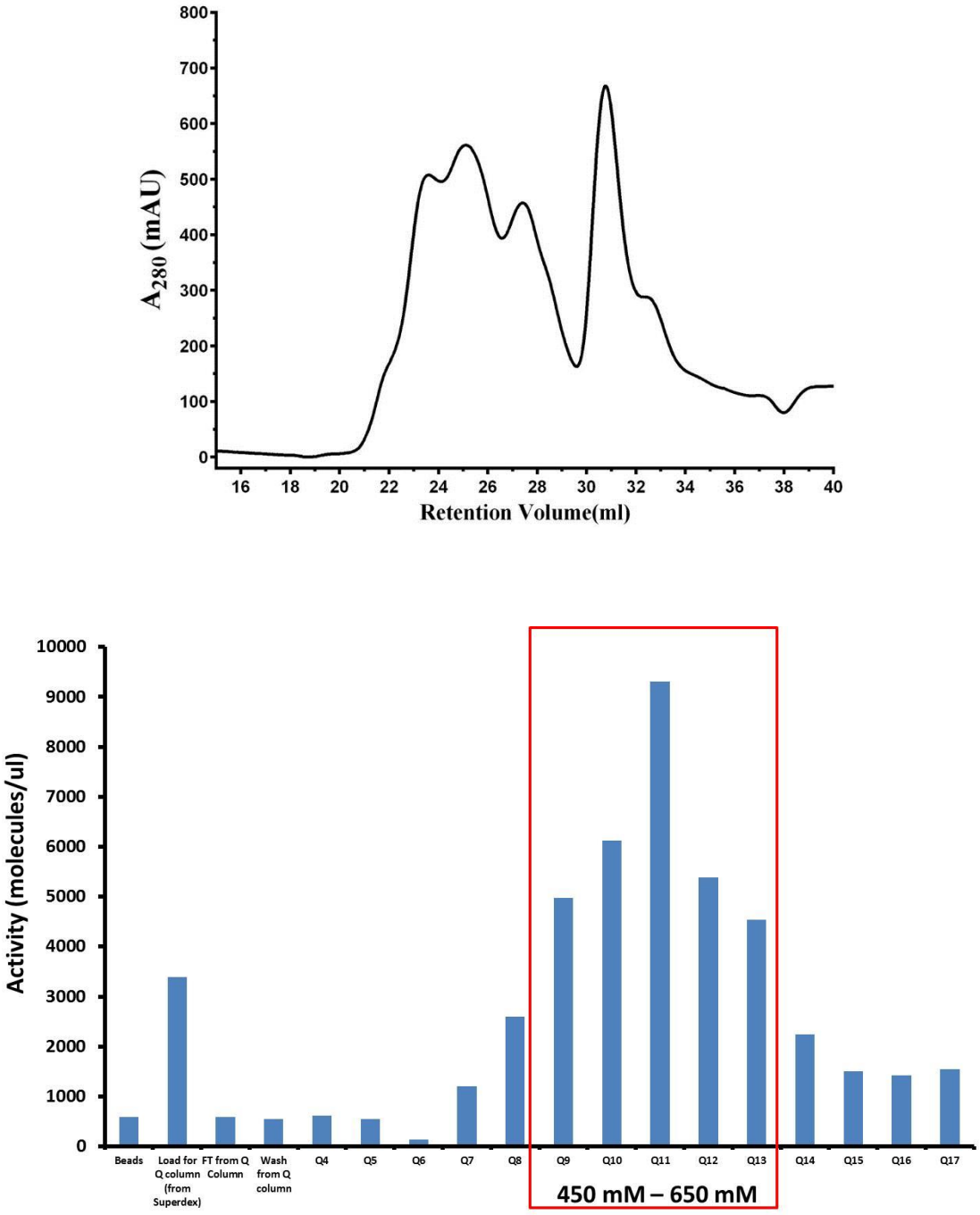

Figure 5-9: *(Top Panel)* Elution profile from MonoQ column. Samples were eluted from the column with a salt gradient (NaCl). Each sample is ~1 ml. *(Bottom Panel)* Elution samples were then incubated with telomerase and tested for activation. DDTRAP data shows the activation factor is located in Q9-Q13.

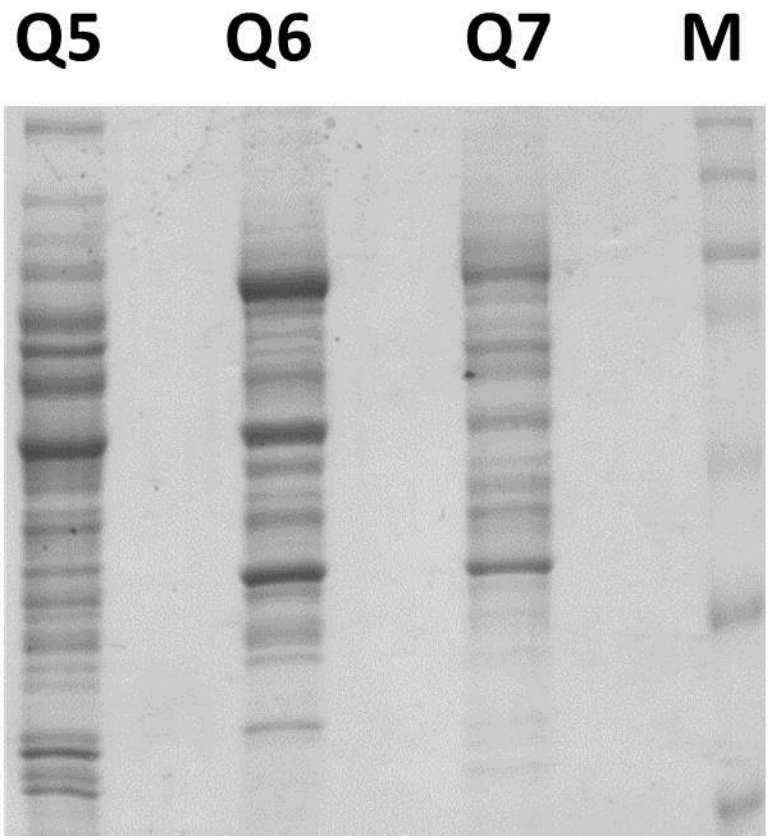

Figure 5-10: Silver stained gel showing proteins in the final concentrated sample from the second attempt at purifying the activation factor from H1299 cells. The lane labeled Q6 was sent for mass spec identification of the activation factor along with a negative control sample (also an elution from the Q column) that was shown to not activate telomerase (indicated by ddTRAP). The protein candidates were cross-referenced against each other and we ended up with a list of 300 possible candidates.

Discussion

Previous studies have shown telomerase to be highly regulated at every level in the cell. Along with the cellular regulation of telomerase, we have shown that telomerase is behaving as a single pass enzyme, hence adding a new level of kinetic regulation to the enzyme. We hypothesized the existence of a cellular factor that would allow normal cells and possibly even cancer cells to re-activate /activate telomerase after catalytic exhaustion. In this chapter we were able to successfully demonstrate the ability of cell lysates from almost all cell types tested to activate purified telomerase enzymes. These cells include cancerous as well as normal human cells. This phenomenon is not telomerase-dependent as both telomerase negative and telomerase positive cells were able to activate telomerase. Furthermore, we established that this factor was altering the affinity as well as the time constants of telomerase's fast and slow components. This was displayed by the time courses and Michaelis-Menten plots comparing telomerase with and without the activation factor. This proposed mechanism indicated that there must be direct interaction of some sort with telomerase. This led us to use the tethered telomerase enzyme to pulldown any possible interacting factors from the lysates that were able to activate telomerase. Using telomerase as the bait, we were able to pulldown the factor or factors from the lysate and achieve a similar level of activation as the positive control sample. Another control experiment we conducted was to heat-denature the cell lysate prior to the pulldown. When the heat-inactivated lysate was used in the extension reactions, we were not able to detect any activation of the inactive telomerase. This led to the current notion that the activation factor is probably a protein. This was critical in establishing the biochemical purification procedure we developed in order to purify and identify this protein or proteins. Using subcellular separation, size and charge, we were able to partially purify this factor.

The first round of mass spec data left us with approximately 300 candidates. Recently, we have performed another round of purification and narrowed down the list to ~39 candidates. Although we have not identified this factor, none of the known telomerase interacting proteins or accessory factors reported in literature was present in the mass spec list of possible candidates. This casts a promise that a novel

telomerase-activating factor is our target. We are currently searching for a cell line that will not activate telomerase. This cell line would in turn serve as the negative control for the purification and assist with the identification of the novel factor via mass spectrometry. Along with searching for a negative control, we are further refining the purification of this factor in order to narrow down the list of hits from mass spec results.

Chapter 6: Conclusions and Future Directions

Understanding the regulation of the human telomerase enzyme will allow researchers and clinicians to make significant progress towards the eventual treatment of a variety of human diseases. In the study presented, we proposed a novel model for telomerase regulation, one that is not associated with hTERT mRNA transcription, hTERT mRNA splicing, hTERT and hTR biogenesis, telomerase localization or telomerase post-translational modifications. Rather, the model proposed shows telomerase behaving as a single-pass enzyme. Active telomerase complexes are rendered inactive after recognizing, binding and catalytically extending the substrate. Furthermore, the dimeric telomerase enzyme is composed of a fast acting and slow acting site as shown by their time course and affinity constants. The two sites were shown to bind and act on the substrate in a sequential manner as opposed to a parallel model where both sites would be available to bind and extend substrates at the same time. Finally, we were able to reactivate/activate the enzymes after catalytic exhaustion of both units of the telomerase dimer by simply mixing the inactive enzyme with various cell lysates. We have made progress towards discovering that a nuclear protein in the lysate is responsible for activating telomerase. This nuclear protein was shown to directly interact with telomerase and change the kinetic and thermodynamic properties of the enzyme.

In order to carry out the in-vitro studies discussed in previous chapters, several obstacles associated with telomerase needed to be addressed. We overexpressed both hTERT and hTR in H1299 cells and generated a stable cell line. This allowed us to overcome the low abundance of telomerase in cells. The low abundance of telomerase is only made worse by telomerase being a notoriously difficult enzyme to work with. Telomerase is a very large ribonucleoprotein complex making it highly sensitive to degradation and difficult to purify. To overcome this, we engineered a recombinant hTERT that was biotinylated in-vivo. Using a 3-step telomerase purification protocol we were able to purify the recombinant hTERT. The highly purified samples were quite stable at both room temperature and 4 C, allowing us to assay the enzyme at longer time intervals without compromising the samples integrity.

Finally, we developed a digital version of the TRAP assay. This assay, ddTRAP, overcame the limitations of the traditional gel-based TRAP assay. The assay allowed us to reliably quantify telomerase activity. More importantly, the assay gave us the ability (for the first time) to conduct kinetic and thermodynamic studies on the enzyme with absolute quantification. The absolute quantification freed us from using relative intensity measurements of radioactive/ fluorescent signals from gel spots or bands that were known to be highly unreliable.

Despite the significant progress we have made towards a better understanding of the human telomerase enzyme, there are still questions that remain unsolved. Firstly, a clear mechanism to explain telomerase's single pass behavior has not been established. Telomerase is behaving as a single pass enzyme, but how? Is the enzyme undergoing a conformational change? It is possible that the overall conformation between hTERT and hTR has been altered to prevent telomerase from binding and extending a new substrate. It is also possible that telomerase may be losing an accessory factor (known or unknown) after or during its extension of the substrate rendering it incapable of repeating the catalysis cycle. Secondly, the data we have displayed can be interpreted to suggest some form of negative cooperativity between the two subunits of the telomerase dimer. However, we have not yet directly shown this using classical biochemistry and enzymology. The low levels of enzyme, substrate and product hinder us from calculating the Hill coefficient and establishing cooperativity between the subunits. Lastly and most importantly, we have yet to identify the activation factor from the lysate. The mass spec data has left us with many possible candidates. The best approach moving forward would require a better negative control to reduce the number of candidates to a manageable amount. From there one could envision a siRNA screen to knockdown the various candidates and look for a loss or decrease of the activation phenotype. The tethered telomerase assay in conjunction with the ddTRAP assay would make this approach feasible. Although the loss in activation of telomerase from a siRNA knockdown would strongly suggest that the candidate was the activation factor, it would still require further validation such as protein interaction assays or pulldown experiments.

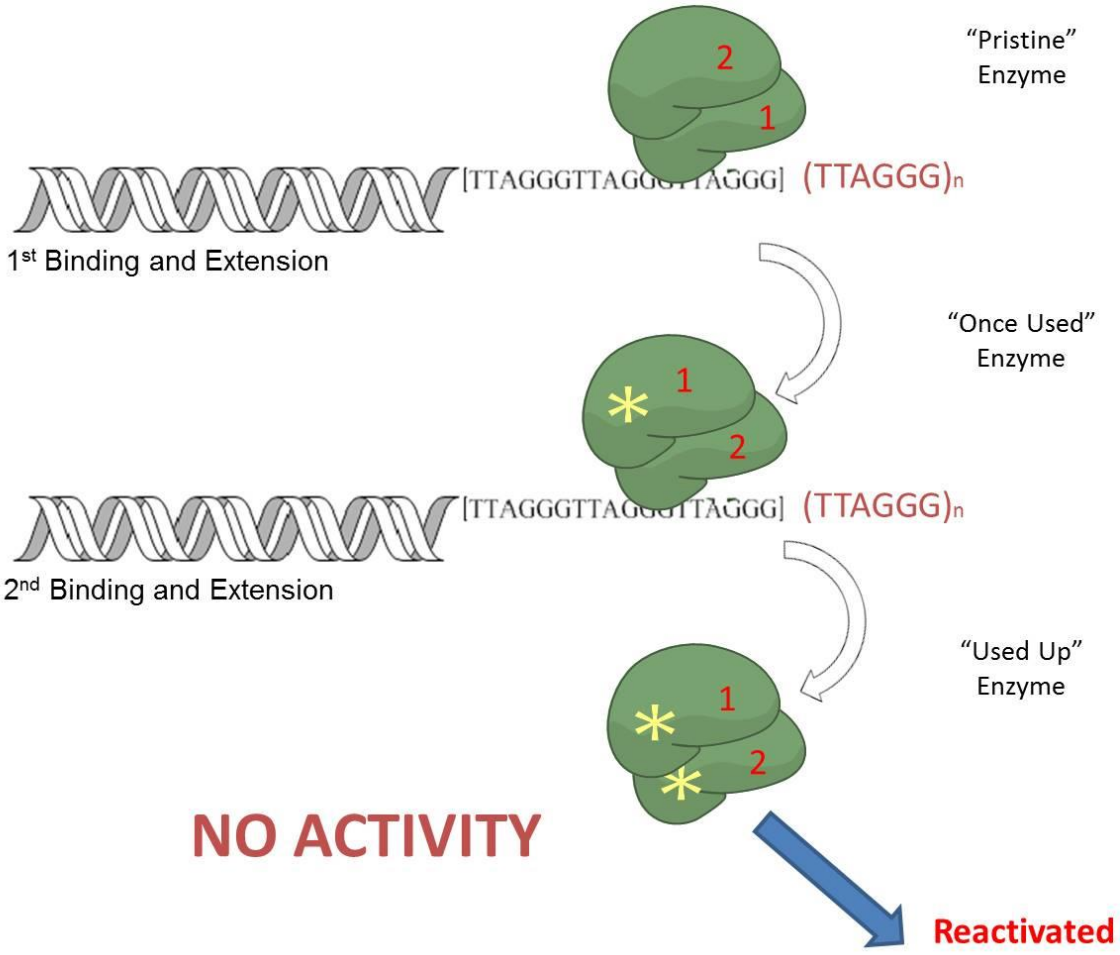

Figure 6-1: Proposed model for single pass human dimeric telomerase enzyme**.**

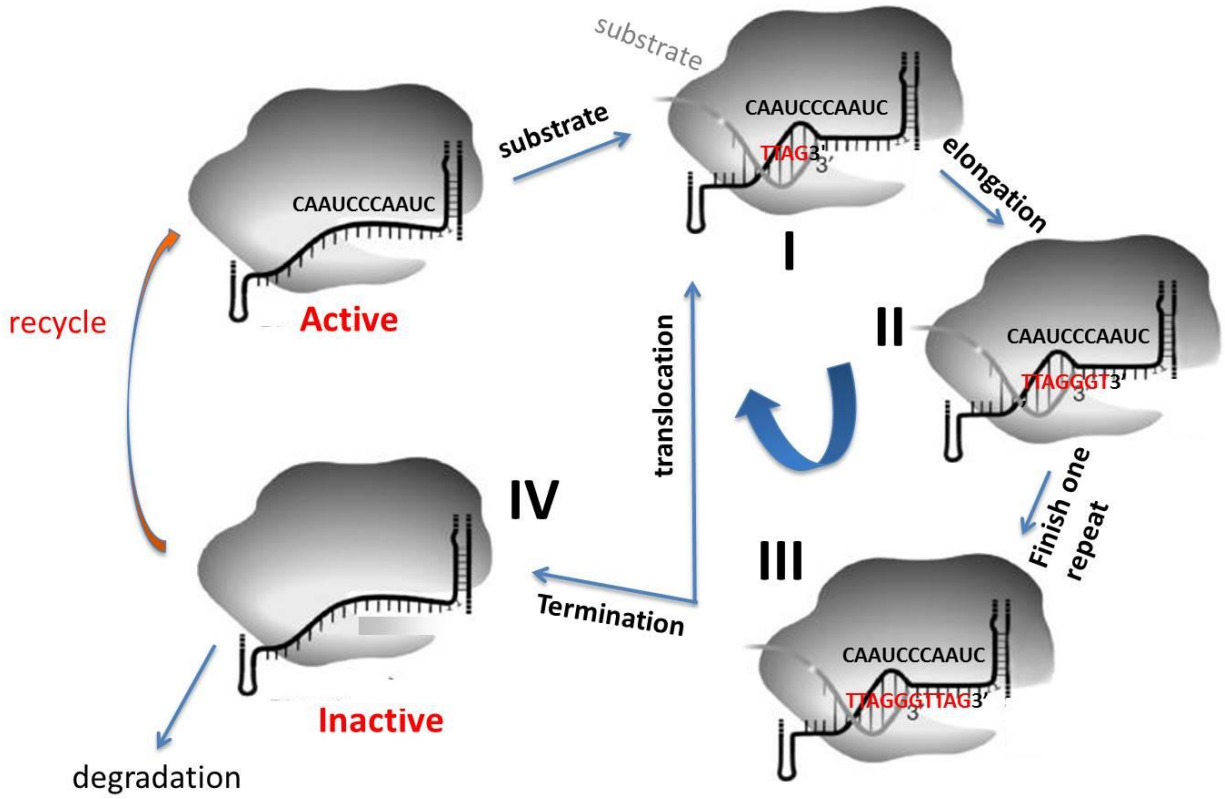

Figure 6-2: In-depth look into telomerase's catalytic cycle.

Chapter 7: Methods

Cell Culture

H1299 lung adenocarcinoma, Super H1299, Hela cervical carcinoma, BJ human fibroblast and ALT-SW13 fibroblast cells were cultured in 5% CO_2, 4:1 DMEM:Medium 199, 10% calf serum (HyColne, Logan, UT) at 37ºC.

Generation of Stable Super H1299 Cell Line Containing Overexpressed Recombinant hTERT and hTR

Biotin tagged hTERT carried in pBabe-hygro retroviral vector was firstly used to transfect into the transient packaging line PhoenixE. The virus supernatant was then used to infect the stable amphotropic packaging line PA317. The PA317 were then selected with hygromycin and produced stable virus which were used to infect expressing cell line H1299. The infected H1299 cells were selected with hygromycin.

For hTR, pSSI 7661 lentiviral vector carried together with two helper plasmids, psPAX2 and pMD2G were used to transfect 293 packaging cells. The virus supernatant was used to infect H1299 which already had the overexpressing biotin tagged hTERT. The infected H1299 cells were further selected with blasticidin.

RT-qPCR of Human Telomerase RNA Component (hTR)

50 ng of purified total RNA was used for each RT reaction (iScript Select cDNA Synthesis Kit from BioRAD). Quantitative PCR was then carried out on the cDNA prodcuts using the Universal Probe Library for humans (Cat.04683633001) and TaqMan Master (Cat. 0435286001) from Roche following the manufacture's recommendations. The probe used for hTR detection was UPL #4 and the forward and reverse primers were the following: forward-cgaggttcaggcctttca and reverse-ccacagctcagggaatcg.

Digital Droplet PCR Amplification of Human Telomerase RNA Component (hTR)

The droplet-digital PCR for hTR assay was performed as described in (Ludlow et al. 2014).

Digital Droplet Telomeric Repeat Amplification Protocol (ddTRAP)

The droplet-digital TRAP (ddTRAP) assay was performed as described in (Ludlow et al. 2014).

Western Blot for Telomerase Protein Subunit hTERT

We performed western blots on protein samples as opposed to whole cells. Samples were diluted in Laemmeli SDS buffer (50mM Tris-HCL pH6.8, 2%SDS and 10% glycerol) and boiled for 5 min at 95º C. Protein concentrations were measured with nandrop absorbance (A280) and then equal protein concentrations were loaded unto 4-15% gradient PAGE gels. The gels were run at 120V for 1 hour and then w proceeded to transfer them to nitrocellulose membranes for western blotting. Western blotting was performed with BIORAD Trans-Blot® Turbo™ Transfer System according to manufacturer's manual. Blots were imaged using Syngene G-Box imaging system and software.

Purification of Recombinant Telomerase from Super H1299 (3-Step)

100-200 million frozen cell pellets of super H1299 cells were lysed in a 1.5% CHAPS lysis buffer (10%glycerol, 1mM EGTA pH8.0, 0.1mM $MgCl_2$, 10mM Tris-HCL, 0.01 mM PMSF, 1u Ribolock RNAse inhibitor and 1u PI cocktail) for 30 min rotating end over end at 4ºC. Cells were then centrifuged at 17,500 *g* for 1 hr at 4ºC. Supernatants were collected and placed in clean tubes. A 10 ml continuous glycerol gradient (10-30%) was generated with gradient buffer (glycerol, 20mM Hepes pH7.5, 300mM KCl, 0.1mM $MgCl_2$, 0.1% Triton X-100 and 1mM EGTA). Sample was loaded onto gradient and ultra-centrifuged at 126,000 *g* for 19 hrs at 4ºC (SW41 Beckman rotor). Gradient was fractionated into 11 fractions (1 mL each). The bottom 5 fractions contained telomerase activity. These 5 fractions were

pooled together and then incubated with monomeric Avidin beads (Peirce). Telomerase was incubated with beads for 2 hrs at 4ºC. After incubation, beads were placed on a micro bio spin chromatography columns (BIORAD). Flow-thru was collected and beads were then washed 2 times with 150 mM Sodium Phosphate buffer pH 7.0 and 100 mM NaCl. Telomerase was then eluted with 400mM NaCl, 150 mM Sodium Phosphate buffer pH7.0 and 4 mM D-biotin (Pierce). Telomerase was eluted into 3 elutions of 1 ml each. These elutions were then pooled together and inbucated with the final column, Sepharose Fast Flow (SPFF). SPFF columns were equilibrated in 50 mM Sodium Phosphate Buffer pH7.0 and 50 mM NaCl prior to inbubation with telomerase. Telomerase was incubated with SPFF beads for 2 hr at 4ºC. After incubation, beads were loaded to micro bio spin columns. Flow-thru was collected and beads were washed 2 times with Sodium Phosphate Buffer pH7.0 and 50 mM NaCl. Telomerase was then eluted in a NaCl salt gradient (200mM-1M). This was done in 9 separate elutions (250ul). Elutions from 300mM, 400mM and 500mM contained most of the telomerase activity. These elutions (E2, E3 and E4) were pooled together and used for subsequent assays and experiments.

Affinity Purification of Endogenous Human Telomerase from H1299 Cells (1-Step)

The one step affinity purification of endogenous human telomerase was performed as described in (Cohen et al. 2007). However, we did not use the antibody pre-clearing of the lysate to pulldown telomerase. Instead we used all telomerase molecules form the lysate.

Tethered Telomerase Assay

Purified recombinant telomerase were incubated with Myone T1 Streptavidin coated magnetic Dynabeads (Invitrogen) for 2 hours at 4º C. Beads were pre-treated for RNAse and DNAse contamination using manufacture's manual before incubation with telomerase. After 2 hrs, supernatant was removed and beads were washed 3 times with 50 mM Sodium Phosphate buffer pH7.0 and 50 mM NaCl. All of this was performed under the hood using proper sterile techniques. Telomerase was now tethered and ready for subsequent downstream assays and experiments.

Purification of Telomerase Activation Factor from Factor Containing Cell Lysates

800 million – 1 billion frozen cell pellets of H1299 cells were lysed in a 1.5% CHAPS lysis buffer (10%glycerol, 1mM EGTA pH8.0, 0.1mM $MgCl_2$, 10mM Tris-HCL, 0.01 mM PMSF, 1u Ribolock RNAse inhibitor and 1u PI cocktail) for 30 min rotating end over end at 4ºC. Cells were then centrifuged at 17,500 *g* for 1 hr at 4ºC. Supernatants were collected and placed in clean tubes. Multiple 10 ml continuous glycerol gradients (10-30%) were generated with gradient buffer (glycerol, 20mM Hepes pH7.5, 300mM KCl, 0.1mM $MgCl_2$, 0.1% Triton X-100 and 1mM EGTA). Samples were loaded onto each gradient and ultra-centrifuged at 126,000 *g* for 19 hrs at 4ºC (SW41 Beckman rotor). Each gradient was fractionated into 11 fractions (1 mL each). Fractions 2 and 3 were collected from each gradient and pooled together.

Pooled fractions that contained activation factor were then concentrated to ~ 1ml using a 30 kDa cutoff column (Millipore). Sample was then loaded unto Superdex 200 10/30 GL column (Pharmacia Biotech) at a flow rate of 0.5 ml/min using AKTA pure FPLC system (GE Biosciences). Sample from column were eluted into 1 ml fractions (16 total). The buffer used for this was 20 mM Hepes pH7.5, 50 mM NaCl, 1mM EGTA pH8.0, 1mM $MgCl_2$ and 0.02 % Triton X-100.

Fractions were then tested for activation of telomerase using ddTRAP. Fractions containing activation factor was then pooled and diluted 2 fold in the same buffer for Superdex 200 column. The sample was then loaded onto a MonoQ column (GE Healthcare Life Science)also at a flow rate of 0.5 ml/min and eluted with a 0-1M NaCl linear gradient. There were a total of 20 fractions (500mL each). Samples were then tested for activation of telomerase using ddTRAP.

Gel Based TRAP Assay

Gel based TRAP assay was performed as described in (Herbert et al. 2006)

Chemical Modification of Carbon Coated Grids for Telomerase Capture and Substrate Extension

Telomerase substrate was attached to carbon coated EM grids as described in (Llaguno et al. 2014). Primer extension assay using radioactive dGTP [alpa-32P] as described in (Cohen et al. 2008).

Mass Spectrometry Analysis

Tandem mass spectra were extracted by [unknown] version [unknown]. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1). Mascot was set up to search the UniprotHuman_20160202 database (unknown version, 147981 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Gln->pyro-Glu of the n-terminus, deamidated of asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications.

Scaffold (version Scaffold_4.5.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (Keller, A et al Anal. Chem. 2002;74(20):5383-92) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Al et al Anal. Chem. 2003;75(17):4646-58). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters

APPENDIX

MATLAB programs for comparing three different models in Fig. 4-1

The m-function for comparing three models:

```
% this is to compare the same data
% TimeCourse01 contains the data, which can be read from a spreadsheet by
% xlsread
% SequentialModelwithDelay(para, t) ; all five terms of para are used.
% ParallelModel(para,t) ; only the first four terms of para are used
% SequentialModel01(para,t); only the first four terms of para are used
%
%timecourse01=xlsread('TImeCourse01.xlsx');
%each data pair can be compared separately

% read in the data from a spreadsheet
timecourse06=xlsread('TimeCourse08.xlsx');

timecourse01=timecourse06(:,1:4);

timecourse01(:,1)=timecourse06(:,1);
timecourse01(:,4)=timecourse06(:,4);


para_ini=[300,2000,.9,.002,0.5]
figure(1);

plot(timecourse01(:,1), timecourse01(:,4),'*');

fseqdelay = @(para) norm(SequentialModelwithDelay(para,timecourse01(:,1)) - timecourse01(:,4))^2

[Xval_seqdelay Fval_seqdelay]=fminsearch(@(para) fseqdelay(para), para_ini)

fitp=SequentialModelwithDelay(Xval_seqdelay,timecourse01(:,1));
plot(timecourse01(:,1), timecourse01(:,4),'*'); hold on;
plot(timecourse01(:,1), fitp,'-'); hold off;

f2=@(para) norm(ParallelModel(para,timecourse01(:,1)) - timecourse01(:,4))^2

[Xvalpara Fvalpara]=fminsearch(@(para) f2(para), [120 150 0.25 0.005 0.01])

figure(2); plot(timecourse01(:,1), timecourse01(:,4),'*'); hold on;
plot(timecourse01(:,1), ParallelModel(Xvalpara,timecourse01(:,1)),'-');hold off;

%%% now the sequential model without delay
f3=@(para) norm(SequentialModel01(para,timecourse01(:,1)) - timecourse01(:,4))^2;
```

```
[Xvalseq Fvalseq]= fminsearch(@(para) f3(para), [120 150 0.25, 0.004 0])

figure(3); plot(timecourse01(:,1), timecourse01(:,4),'*'); hold on;
plot(timecourse01(:,1), SequentialModel01(Xvalseq,timecourse01(:,1)),'-');hold off;
```

The m-function for the sequential model with a delay.

```
%% minimization of scheme 2, sequential model with a delay after the first step
%%  T0 -> T1' + p -> T1 (delay) -> T2 +p
%% we assume that the reactions are forward-preferred and there is no product-related inhibition.
%% the purpose of the process is the develop a mechnical model for the catalysis
%% the delay period from T1 to T1' is assumed to be statistical or a Poisson process (irreversible switch).
%% T0(t) = [T0]o exp(-k1*t)
%% T1' = k1(k3-k1)*[T0]o*exp(-k1*t) +(k1/(k1-k3))*[T0]o *exp(-k3*t)
%% T1= k1*k3*[T0]o*((1/((k2-k1)*(k3-k1))*exp(-k1*t)) + (1/((k1-k2)*(k3-k2))*exp(-k2*t)) + (1/((k1-
k3)*(k2-k3)) *exp(-k3*t))]

%% old one: (k3/(k1-k2))*[T0]o exp(-k1*t) + ([T1]o - (k1*k3/(k1*k2-k2*k2))*[T0]o)exp(-k2*t) +
(k3/k2)[T0]o
%% product:
%% P(t)= [T0]o *(1-exp(-k1*t)) + [T1]o *(1-exp(-k2*t))+k1*k2*k3*[T0]o*[(1-exp(-k1*t))/(k1*(k2-
k1)*(k3-k1)) + (1-exp(-k2*t))/(k2*(k1-k2)*(k3-k2)) + (1-exp(-k3*t))/(k3*(k1-k3)*(k2-k3))]
%% OLDone: p2 = [T0]o(1+(k2*k3)/(k1*(k1-k2))*(1-exp(-k1*t)) + ([T1]o -(k1*k3)/(k2(k1-k2)) *
[T0]o)(1 - exp(-k2*t)) + k3*[T0]o *t

function p2=SequentialModelwithDelay(para,t)

T00=para(1);
T10=para(2);
k1=para(3);
k2=para(4);
k3=para(5);

if(k1 == k2)
        print('k1 and k2 can not be equal');
        p2=0.*t;
else
   imT1=(k1*k2*k3)*((1-exp(-k1.*t))/(k1*(k2-k1)*(k3-k1)) + (1-exp(-k2.*t))/(k2*(k1-k2)*(k3-k2)) + (1-
exp(-k3*t))/(k3*(k1-k3)*(k2-k3))) ;
   p2=T00*(1-exp(-k1.*t)) + T10*(1-exp(-k2.*t)) + T00 * imT1  ;

   %%%T00 *(/(k1*k1-k1*k2))*(1-exp(-k1.*t)) + (T10 -(k1*k3)/(k2*k1-k2*k2)*T00)*(1-exp(-k2.*t)) +
k3*T00*t ;

end
%error=norm(p2-y)^2;
```

```
return
```

The m-function for the sequential model without a delay.

```
%% minimization of scheme 1
%%  T0 -> T1 -> T2

%% at time 0 , [T0, T1] are at certain level, T2 can not recover due to the separation of the reactivting
factor
%%  the other two parameters are rates K1, and K2
%%
%% Substrate +  T0 -> T1 + p
%% substracte + T1 -> T2 + P
%% dT0 / dt = -k1 [T0][Substrate]
%% dT1/dt = + k1[T0][substrate] - k2[T1][substrate]
%% d[p]/dt = -d[T0]/dt - d[T1]/dt = -2k1[T0][sub] + k2[T1][sub]
%% at end [p] = 2[T0]o + [T1]o (the initial concentrations of T0 and T1)

%% T0=[T0]o exp(-k1[substrate]t)
%% K2 and K1 are not equal.
%%% T1=(k1/(k2-k1))[T0]o exp(-k1*t) + ([T1]o - (K1/(k2-k1))[T0]o )  exp(-k2*t)
%%% product p(t) = p1 exp(-k1 *t) + p2 exp(-k2*t)
%%% p1 = ([T0]o (1 + k2/(k2-k1))   ;  p2 = ([T1]o - k1/(k2-k1) * [T0]o)
function p1=SequentialModel01(para,t)

T00=para(1);
T10=para(2);
k1=para(3);
k2=para(4);
k3=0;
p1= 2*T00+T10 - (T00 *(1+ (k2/(k2-k1))) * exp(-k1 .* t) + (T10 - (k1/(k2-k1)) * T00) * exp(-k2 .* t));

return
```

The m-function for the parallel model.

```
%% minimization of scheme 3
%%      T2 + p <- T0 -> T1 + p
%%
%%   T1(t) = T1[

function p3=ParallelModel(para, t)
```

```
T00=para(1);
T10=para(2);
k1=para(3);
k2=para(4);
k3=0;

p3= 2*T00 + T10 - T00 * exp(-k1.*t) - (T10+T00) * exp(-k2.*t);

return
```

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