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Kathryn Anne Carroll Date

TELOMERE DYSFUNCTION IN HUMAN DISEASES: FUNCTIONAL CHARACTERIZATION OF TELOMERASE GENE MUTATIONS AND OF TELOMERE-ASSOCIATED GENE EXPRESSION REGULATION

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By

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B.A., New York University, 2006

Advisor: Hinh Ly, Ph.D.

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ABSTRACT

TELOMERE DYSFUNCTION IN HUMAN DISEASES: FUNCTIONAL CHARACTERIZATION OF TELOMERASE GENE MUTATIONS AND OF TELOMERE-ASSOCIATED GENE EXPRESSION REGULATION

By Kathryn Anne Carroll

It has been over one hundred years since the first reported case of dyskeratosis congenita (DC) and over twenty since the discovery of telomerase, an enzyme that adds telomeric DNA repeats to chromosome ends. Emerging evidence suggests that telomere dysfunction plays an important role in the pathogenesis of DC and other human disorders involving tissues that require rapid repair and renewal capacities. However, we still do not fully understand how mutations in telomere maintenance genes contribute to disease development in affected individuals. We have identified and characterized a number of telomerase mutations found in patients with DC, aplastic anemia (AA), myelodysplastic syndromes (MDS), idiopathic pulmonary fibrosis (IPF), and acute myeloid leukemia (AML). These studies have revealed important domains/residues of the telomerase reverse transcriptase hTERT protein and of its intrinsic hTERC RNA template required for optimal enzymatic function. We have also investigated the effects of patient-associated sequence changes in the proximal promoter region of the *hTERC* gene and explored the possible regulatory mechanism of expression of the telomere-associated protein TIN2. Collectively, these studies offer important insights into the molecular mechanisms of telomere maintenance under normal and pathological conditions in humans.

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CHAPTER 1: INTRODUCTION [1]

1.1 Human Telomerase Basics

Telomerase is a ribonucleoprotein complex whose main function is to add nucleotide repeats onto the ends of chromosomes utilizing its reverse transcriptase (hTERT) and its intrinsic RNA template (hTERC), as well as the associated proteins dyskerin, NOP10, NHP2, and GAR1 (Figure 1.1). This DNA elongation is necessary to overcome the "end-replication problem" whereby the conventional DNA polymerases cannot fully replicate linear chromosomes [2,3]. This phenomenon, coupled with oxidative damage, and other exogenous or endogenous effects, causes our telomeres to be shortened by approximately 50-100bp per cell division. Telomere erosion limits the replicative capacity of the majority of somatic cells which do not express active telomerase [4,5]. Cells whose telomeres shorten to a "critical length" enter a stage termed replicative senescence whereby cell division is prevented [6,7]. Stem cells, germ cells, and certain types of somatic cells circumvent this barrier by expressing the telomerase enzyme, allowing them to maintain their telomere length and escape senescence.

The human telomerase reverse transcriptase (hTERT) has been extensively studied and hence several of its functional domains have been mapped [8]. The protein is defined by the catalytic domain, which contains seven reverse transcriptase motifs essential for enzymatic activity. The C terminus is short and highly divergent among different species, and its exact function is not completely clear at this point. However, one clear region has emerged, termed the C-DAT for the C-terminal region that dissociates the activities of telomerase. Mutations in this domain generate enzymes

which are catalytically active *in vitro* but biologically inert. In contrast, the N-terminus contains several evolutionarily conserved regions important for hTERT's cellular localization, RNA interaction, protein-protein multimerization, and enzymatic function. Functionally important regions have also been defined in the human RNA template (hTERC) (Figure 1.1) [9]. Most obviously, the template region is absolutely required for the hTERT protein to reverse transcribe it into telomeric DNA repeats. In addition, the pseudoknot domain is required for telomerase activity, hTERT binding, and hTERC RNA dimerization, and the Box H/ACA domain is important for hTERC RNA processing and stability. Despite extensive work to map the aforementioned motifs, it is still relatively unclear which particular residues are absolutely required for the activity of either of the telomerase core components.

1.2 Mammalian Telomere Structure and the Shelterin Complex

The tips of mammalian chromosome ends consist of a long strand overhang composed of the G-rich strand (TTAGGG; as opposed to the C-rich strand CCCTAA). In order to avoid being recognized as a double-strand break and corrected by homologous recombination, a fate quite detrimental to the cell, the single-stranded region folds back upon itself and tucks into the adjacent double-stranded telomeric region, forming a telomeric loop (t-loop) (Figure 1.2B; [10]). This structure is formed and protected by a collection of six proteins, termed the shelterin complex, which with the telomeric DNA repeats compose the entire nucleoprotein structure commonly referred to as telomeres. The shelterin complex is formed by the double-stranded DNA binding proteins, TRF1 and TRF2; a binding partner of TRF2, Rap1; a single-stranded DNA binding protein

POT1; and the two bridging proteins, TIN2 and TPP1 (Figure 1.2A; [10]). Not only do these proteins function in protecting the chromosome end, they also function in telomere length regulation. Telomere length is maintained within a strict range throughout cell division, suggesting a negative feedback loop involving the shelterin complex. Due to the exquisite specificity of these DNA binding proteins, the amount of shelterin protein bound to telomeres is roughly proportional to their length (Figure 1.2C; [10]). Thus, a long telomere would have a greater ability to inhibit telomerase activity, while a short telomere, with less bound protein, would be more accessible to telomerase elongation. In addition to their telomeric roles, it has recently been shown that several shelterin proteins, namely TRF1, TRF2, and Rap1, may play a role in regulating gene expression through their ability to bind to interstitial telomeric sequences proximal to gene loci[11,12].

1.3 Telomere Maintenance in Other Organisms [13]

Ciliates

Many important discoveries in telomere biology have been made in ciliated protozoa owing to their unusual genomic organization, including sequencing of the first telomeres [14], isolation of the first telomere-binding protein [15,16], and discovery of the telomere-elongating enzyme telomerase [17]. The reason ciliates are uniquely suited for studying telomere maintenance lies in their two functionally distinct nuclei, resulting in each cell containing millions of telomeres. The germ-line micronucleus is transcriptionally silent and serves as a repository of genetic information during sexual development [18,19]. The micronucleus is diploid, divides by mitosis, and contains from 5-120 large chromosomes. In contrast, the somatic macronucleus, which is

transcriptionally active and used to support vegetative growth, is highly polyploidy and undergoes amitotic division. The complex process of macronucleus development involves a series of genomic rearrangements that result in small chromosomal units to which telomeres are added, generating anywhere from 40,000 to 48,000,000 telomeres in a single macronucleus [18]. The two nuclei have similar telomeric sequences, most commonly consisting of G_4T_2 or G_4T_4 repeats, but macronuclear telomeres are usually less than 1 kilobase in length. The extra length of the micronuclear telomeres allows them to fold into large terminal loops similar to t-loops found at mammalian and plant telomeres [20]. The telomere protection complexes present at *Oxytricha* and *Euplotes* macronuclear telomeres seem to be composed of a single telomere end-binding protein (TEBP) that binds specifically to the single-stranded G-strand overhang [21,22]. Although the sequences of G-overhang binding proteins are very divergent, the first oligonucleotide-binding (OB) fold of the α subunit of this heterodimeric protein is conserved among member of the POT1 family. Both macronuclear and micronuclear telomeres are maintained by telomerase [23]. In ciliates, the telomerase holoenzyme contains the catalytic subunit TERT, an RNA template, and one or more accessory proteins necessary for stability and processivity, similar to the mammalian telomerase enzyme.

Budding Yeast

Saccharomyces cerevisiae telomeres are composed of an irregular telomeric repeat array owing to degenerate copying of the yeast telomerase RNA by telomeric alignment in multiple registers along the 17-nucleotide template region

[24,25,26,27,28,29]. However, even these irregular telomere sequences retain a conserved core sequence of approximately 6 nucleotides, preserving a binding site for the essential duplex telomere-binding protein Rap1 [30], a role served by TRF1 and TRF2 in mammalian cells. While Rap1 was originally identified as a transcriptional regulator, it has been discovered that the number of Rap1 molecules bound to the telomeric end seems to constitute a telomere-length measuring mechanism in yeast [31,32,33]. The immediate effectors of this length regulation are Rif1 and Rif2 which both interact directly with the C-terminus of Rap1. The single-strand G-overhang in yeast is bound by Cdc13, where it contributes to both telomere capping and telomere length regulation. Despite these similarities to mammalian chromosome end structure, the absence of an obvious TRF2 ortholog in *S. cerevisiae* combined with the current hypothesis that the heterogeneity of yeast telomere sequences provides insufficient base-pairing between an invading single strand and the duplex telomeric tract argues against a role for functional t-loops in budding yeast. The search for genes involved in budding yeast telomere maintenance has been vastly widened by the generation of 4800 viable yeast deletion strains which carry alterations in telomere length [34]. This analysis has led to the identification of more than 150 nonessential genes, indicating that as much as 3% of the yeast genome contributes, either directly or indirectly, to telomere length regulation. The telomerase RNP in budding yeast is composed of two catalytically essentially components, the ever shorter telomeres 2 protein (the TERT subunit) and the TLC1 RNA, along with the regulatory subunits Est1 and Est3. The primary role for Est1 is to recruit telomerase to the telomeric end through its interactions with Cdc13, while the precise role for Est3 is not yet known. Strains deleted for any of these 4 components exhibit indistinguishable

senescence and telomere-shortening phenotypes and appear to be equally defective in an *in vivo* telomere addition assay [35,36,37,38].

Fission Yeast

Schizosaccharomyces pombe is nearly as distant evolutionarily from budding yeast as it is from humans, but fission yeast chromosome organization, dynamics, and telomere-binding proteins are remarkably similar to humans. Fission yeast telomeres on each of its three chromosomes are only about 300bp in length and are degenerate in sequence with TTACAGG as the most commonly occurring motif. Like chromosome ends in all eukaryotes, fission yeast telomeres are bound by protein complexes which serve to maintain and protect the telomeric end. The double-stranded region is bound by the only known ortholog of TRF1 and TRF2, Taz1 [39,40,41], which recruits Rap1 to the complex. Unlike the Rap1 protein in budding yeast, fission yeast Rap1 lacks the ability to bind DNA directly, requiring protein-protein interactions to localize to telomeres, analogous to hRap1. Fission yeast telomeres are also bound by Rif1, although its role in fission yeast has diverged from its budding yeast counterpart in that its interaction with telomeres is independent of Rap1 and instead depends on Taz1 [42]. These findings suggest that Rif1 and Rap1 may in fact compete for binding to Taz1. The single-stranded region is bound by Pot1, identified by the sequence homology of its OB-fold motif to those found in *Oxytricha nova* TEBP and budding yeast Cdc13 [43,44]. The catalytic component of telomerase Trt1 shares 30% identity with the human TERT protein within the seven RT domains and the T domain [45]. Fission yeast Est1 was found based on its sequence homology to budding yeast Est1 and is absolutely essential for telomerase

enzymatic activity *in vivo* [46]. The recently identified RNA component TER1 is similar in size to its budding yeast counterpart TLC1 and serves as a scaffold for the assembly of the protein subunits [47,48]. Interestingly, following any genetic manipulation that results in complete loss of telomeric DNA, two classes of survivors can be found [49]. The first class carries circularized chromosomes, possibly through end-joining between each of a single chromosome's ends. The second class, observed in *trt1*Δ cells, possesses telomeres which are highly heterogeneous in length, presumably via a recombination-based method similar to the ALT pathway.

Drosophila

Drosophila telomeres are dramatically different from those of other organisms. This species lacks telomerase and its telomeres contain arrays of two non-long terminal repeat (non-LTR) retrotransposons *HeT-A* and *TART* [50,51,52]. The most likely mechanism of telomere elongation is the addition of copies generated from RNA templates by the activity of a reverse transcriptase through the association of the 3' end of the retrotransposon with the chromosome end [53,54]. *Drosophila* can live for several generations with a terminal deletion lacking all the telomeric transposons as a normal telomere contains more than 20kb of transposon sequence and the estimated rate of loss is about 2.5kb per year [55,56]. Furthermore, recombination-based mechanisms also seem to be important for regulating telomere length, arguing against the requirement of telomere elongation in proliferating tissues during development [57,58]. In addition to its association with heterochromatic regions, heterochromatin protein 1 (HP1) is also a stable component of all telomeres in *Drosophila*, essential for telomere stability [59].

Plants

The field of telomere biology was pioneered in large part by the seminal studies of Barbara McClintock in maize, and plant telomere maintenance continues to be a highly curious area of study owing to the plasticity of plant development. The first telomere sequence cloned from a higher eukaryote, TTTAGGG, was obtained from the model organism in the plant family, *Arabidopsis thaliana* [60]. Telomere length in various species of plants varies widely from 0.5-160kb, suggesting that telomere regulatory mechanisms may differ among these organisms. However, data from *Arabidopsis* agree well with recent studies in yeast and argue that telomere length homeostasis is achieved through the preferential action of telomerase on the shortest telomeres in the population [61,62]. Telomerase activity is highly regulated in plants and limited to cells with high proliferation capacity such as embryos and the reproductive organs [63,64,65,66]. Very little biochemical information is available concerning the telomerase RNP from plants. The most well-characterized component is the catalytic TERT subunit from *Arabidopsis*, although an RNA subunit has not been identified yet [67,68]. *Arabidopsis* is unusual in that it harbors two Pot1-like genes, similar to the case in mice (see below) which can bind the single-stranded telomeric region [69,70]. However, *Arabidopsis* contains more than 100 genes related to the double-stranded DNA binding proteins TRF1 and TRF2. The most promising candidate for a double-strand telomere protein from plans is NgTRF1 from tobacco as it displays a cell-cycle regulated expression profile that is opposite that of telomerase [71,72]. No obvious homologs for any of the other shelterin components are evident.

1.4 Telomerase and Disease

Bone marrow failure syndromes (BMFS) represent a diverse group of diseases with similar presentations, including dyskeratosis congenita, aplastic anemia, myelodysplatic syndromes, and others [73]. Overlapping symptoms and lack of concrete disease characterization make early diagnosis extremely difficult, especially when the few definitive phenotypes do not usually manifest until later in the disease progression. The prognosis for affected individuals can be bleak as the most prominent treatment is bone marrow transplant and frequently matching bone marrow donors are difficult to find. The fact that patients with BMFS have shortened telomeres led us and other researchers to screen these patients for mutations in telomerase and some protein components of the shelterin complex. These efforts yielded mutations in hTERT (Figure 1.3; [74,75,76,77,78,79,80,81,82,83,84,85,86,87]), hTERC (Figure 1.4; [83,85,88,89,90,91,92,93,94,95,96,97,98,99,100,101,102,103,104,105,106]), the telomerase-associated proteins dyskerin [107,108,109,110,111,112,113,114,115,116,117,118,119,120], NOP10 [121], and NHP2 [122], and the shelterin components TRF1 [123], TRF2 [123], and TIN2 (Figure 1.5; [124,125,126,127]). These findings support the hypothesis that dysfunctional telomeres due to mutations in telomere maintenance genes lead to exhaustion of the stem cell compartment and hence to various defects in cell types with a high turnover rate such as the hematopoietic system. In addition to hematopoietic malignancies, mutations in these components can also contribute to idiopathic pulmonary, liver, and heart fibroses [75,76,77,82,104]. Why mutations in the same proteins can be found in diseases with similar yet different phenotypes is unclear. It is likely that other factors (exogenous

and/or endogenous) might be involved in the pathogenesis of these diseases, but the influence of telomere length regulation on cell proliferation cannot be discounted. Thus, a more thorough study of these molecules, their functions, and their regulation is necessary in order to fully understand them and to possibly allow more targeted therapies for these ailments.

1.5 Telomerase Mutations in Human Blood Disorders

Dyskeratosis Congenita (DC)

Dyskeratosis congenita is a rare inherited disorder of the mucocutaneous and hematopoietic systems associated with a wide variety of other somatic abnormalities. The diagnostic component consists of a triad of clinical symptoms: mucosal leukoplakia (particularly abnormal plaques on the tongue), nail dystrophy (abnormal nail growth or even complete nail loss), and abnormal skin pigmentation [128]. The majority of the cases (>80%) occur in children, who experience bone-marrow failure syndromes and the aforementioned physical anomalies generally by the age of 10. Other symptoms indicative of premature aging, including pulmonary diseases, dental abnormalities, esophagostenosis (narrowing of the esophagus), and alopecia (hair loss), are often associated with the disease in 15-25% of the cases. Solid tumors of the GI tract, nasopharynx and skin, and hematopoietic malignancies (e.g., MDS, Hodgkin's and acute myelogenous leukemias) have also been observed in some DC patients [129]. Since this disease affects rapidly renewing tissues, it has been speculated that DC is a telomerase disease in all three different patterns of inheritance: X-linked recessive, autosomal dominant, and autosomal recessive. In support of this theory, most DC patients have

short telomeres [130,131] and carry mutations in the three main components of the telomerase holoenzyme complex: dyskerin, hTERT (protein), and hTERC (RNA) [132].

The X-linked form of DC is the most severe and is caused by mutations in the *DKC1* gene on chromosome Xq28. DKC1 encodes dyskerin, a 514 amino acid, nucleolar protein in the H/ACA family, which is highly conserved throughout evolution. As is the case with other H/ACA proteins, dyskerin is predicted to function in ribosomal RNA processing, in addition to its predicted role in the biogenesis of the telomerase holoenzyme. Most *Dkc1* mutations are missense mutations and include a 3' deletion, suggesting that both frameshift and null mutations are incompatible with life [107,108,109,110,112,113,114,115,116,118,120]. Indeed, a DKC1-null mouse model is embryonic lethal [133]. In humans, one mutation (A353V) is seen quite frequently in both X-linked DC and a more severe form of the disease, the Hoyeraal-Hreidarsson (HH) syndrome (see below), and accounts for approximately 30% of all X-linked DC cases. A number of intronic mutations have also been found, in addition to a promoter mutation (-141C→G), which destroys an Sp1 binding site. Female carriers of DKC1 mutations show skewed X inactivation patterns due to the fact that cells expressing the normal allele have an inherent growth advantage. Yet, it remains to be seen the extent to which each of dyskerin's functions (rRNA processing or telomere maintenance) contributes to the X-linked DC phenotype.

Now considered a more severe allelic form of X-linked DC, the Hoyeraal-Hreidarsson (HH) syndrome is a multisystem disorder characterized by mental retardation, microcephaly, intrauterine growth retardation, and aplastic anemia [134]. More recently, progressive combined immune deficiency has come to be regarded as

another common symptom in this disease [135]. Missense mutations in dyskerin found in HH families segregate with the disease [111,117,119]. However, as of yet, there is no explanation for why different mutations in the same protein can cause such diverse phenotypes. The situation is further complicated by the identification of a female HH patient from a consanguineous marriage of asymptomatic parents who carries a homozygous mutation in hTERT [80]. However, it is quite possible that is in fact a very severe case of autosomal dominant DC due to the inheritance of moderately shortened telomeres from both parents.

The autosomal dominant form of DC (AD-DC) is much less severe and less common than the X-linked form. Like aplastic anemia (see below), mutations in hTERT and hTERC, as well as the telomere binding protein TIN2 have been associated with AD-DC (Figures 1.3, 1.4, and 1.5). As the vast majority of these mutations are heterozygous, it is possible that they could function as either haploinsufficient, with a single copy of the normal telomerase component being insufficient to maintain telomere length, or dominant-negative, with the mutant telomerase component negatively affecting the wild-type copy. To determine which of these is the case, researchers transfect cells with both a wild-type and a mutant copy of the given telomerase component and perform the telomere-repeats amplification assay (or TRAP) in order to detect for the effect of the mutated copy on normal telomerase enzymatic function. If the mechanism is haploinsufficiency, the TRAP activity of the doubly-transfected cells will either only be slightly reduced or be the same as the activity of cells transfected with a single wild-type copy. In contrast, cells transfected with a wild-type copy and a dominant-negative mutant would exhibit a significant reduction or complete abolishment of TRAP activity.

While most of the mutations have been found to exert their effect by a haploinsufficiency mechanism, 2 mutations in the RNA component located in the template region (Δ52-55 and A48G) seem to act as dominant-negatives [85]. It remains to be seen, however, whether this is truly the case *in vivo*. In fact, in a different system recently developed by Errington et al., these mutations do not show the same dominant-negative effect [136]. Interestingly, disease anticipation has been observed in families with AD-DC [101], a phenomenon whose mechanism has thus far always involved a genetic change, such as the expansion of triplet repeats in severe neurological disorders [137]. In AD-DC families, the genetic lesion does not change, yet the onset of disease features occurs, on average, 20 years earlier in the children than in their parents. Telomere length appears to play a role in this accelerated presentation as telomeres were significantly shorter in the second generation of affected families as compared to normal families. This trend is echoed in *Terc* knockout mice, where clinical features of telomere shortening and DC do not develop until the fourth generation, with sixth generation mice becoming infertile [138,139,140,141].

The causal gene for the autosomal recessive form of DC remains elusive. A recent study by Walne et al. aimed at uncovering the genetic basis for the disease concluded that there is no single locus responsible [121]. Nevertheless, a homozygous mutation in the NOP10 protein was found in all 3 affected members of a single family and is predicted to alter protein structure and may affect endogenous hTERC RNA levels as NOP10 is a telomerase-associated protein that is predicted to aid in hTERC processing and assembly. This mutation (R34W) in NOP10 appears to segregate with the disease as unaffected family members are heterozygous and both patients and unaffected carriers do

in fact have significantly shorter telomeres than controls. However, this mutation was not identified in any of the other 15 families screened, suggesting that it may be a very rare genetic risk factor of this form of the disease.

Aplastic Anemia (AA)

Aplastic anemia is a rare but serious bone marrow disorder, characterized by hypocellular bone marrow and low blood cell counts [142]. As patient leukocytes have significantly shorter telomeres than age-matched controls, we and other researchers have screened AA patients for mutations in telomerase components. Heterozygous mutations have been found in both the protein and RNA components of telomerase (Figures 1.3 and 1.4) as well as the telomere-binding proteins TRF1, TRF2 [123], and TIN2 (Figure 1.5; [125,126]). It appears that the AA-associated RNA mutations tend to cluster in the conserved pseudoknot region, which is required for telomerase enzymatic activity and hTERT binding. All hTERC mutations identified in AA patients that have been examined thus far function as haploinsufficient, as opposed to dominant-negative, at least *in vitro*. However, as patients with telomerase mutations present with highly varying symptoms, it remains to be seen if mutations at specific residues can explain the differing degrees of severity or if there are some other genetic or environmental factors at play. It has also been suggested that some cases of AA may be classified as cryptic and atypical form of DC as they develop slowly over time and do not show the characteristic triads of physical anomalies as frequently observed in X-linked cases. Recently, Calado et al. identified a mutation in the *SBDS* gene, the causative gene for another bone marrow

failure syndrome, Shwachman-Diamond Syndrome, in some AA patients [143]. The significance of this mutation in AA has yet to be determined.

Myelodysplastic Syndromes (MDS)

Myelodysplatic syndromes encompass a group of diseases caused by abnormal blood-forming cells, such that the bone marrow cannot effectively produce blood cells, resulting in low blood cell counts. MDS is a clonal disease, meaning the abnormal cell population arises from a single, abnormal cell. As such, some consider MDS a form of cancer, and, in fact, about 30% of MDS cases progress into acute myeloid leukemia (AML). Despite the bone marrow defects, mutations in telomerase components are extremely rare, with no mutations in hTERT or dyskerin having been identified to date. Only 4 isolated hTERC mutations have been reported (Figure 1.4; [92,94,96,106]), in addition to two promoter region mutations, one of which is located in a putative Sp1 binding site [96,105]. The significance of these mutations in the disease pathogenesis is unclear, however.

Acute Myelogenous Leukemia (AML)

Acute myeloid leukemia is a heterogeneous disorder of hematopoietic progenitor cells, causing abnormal proliferation and differentiation, and can evolve from acquired aplastic anemia (AA) and myelodysplastic syndromes (MDS) [144,145]. In addition, a predisposition to developing cancer, including AML, is a characteristic of DC patients [145]. As genomic instability has been shown to be important for the development of the disease, Calado et al. examined three cohorts of AML patients who show no physical

signs of DC for sequence variation in the *hTERT* and *hTERC* genes [87,144]. They identified three novel missense mutations in *hTERT* (Figure 1.3), and, while the V299M sequence change did not seem to affect telomerase enzymatic activity when tested by the TRAP assay, both the P65A and R522K mutations conferred dramatic defects. Surprisingly, they also identified three AML patients who are homozygous for sequence changes previously identified in a heterozygous state in AA patients and controls (A1062T and Δ441E) [86]. Thus, it appears that hTERT gene variants have low penetrance and are carried in patients with a wide variety of disorders. This phenomenon can be explained if short telomeres, as opposed to mutation status of telomerase, mediate disease pathogenesis, a hypothesis consistent with the fact that the median age at presentation for AML is 70 [144]. In corroboration with this, abnormal karyotypes were present in 18 of the 21 patients who were carriers of hTERT mutations, suggesting that these patients' excessively short telomeres have contributed to genomic instability and their development of AML. However, this correlation still needs to be validated *in vivo*.

Paroxysmal Nocturnal Haemoglobinuria (PNH)

Paroxysmal nocturnal haemoglobinuria is a clonal blood disorder arising from a defective blood cell lacking glycosylphosphatidylinositol (GPI) anchored proteins due to a mutation in the *PIGA* gene [146,147]. This disorder is commonly associated with aplastic anemia and as such, patients have been screened for mutations in telomerase components. While no mutations in dyskerin, hTERT, or hTERC have been found, a single mutation (-99C→G) within the Sp1 binding site in the promoter region of hTERC was isolated in a PNH patient [148]. Interestingly, this mutation was also found in

patients with MDS [96]. While the effect of disrupting this site has not yet been determined *in vivo*, its *in vitro* activity seems to vary depending on the exact promoter context used for the luciferase reporter assay. In the minimal promoter context (nucleotides -107 to +10), the -99C/G mutation results in an increase in luciferase activity, suggesting a repressive role for the Sp1 binding site. However, a similar experiment performed by the same group, using a longer hTERC promoter sequence (-107 to +69) and a double substitution in the same site (C-101A/C-100A), identified this site as a positive regulator of *hTERC* transcription. Furthermore, preliminary data from our lab suggests that the -99C/G sequence change may not confer a dramatic defect when considered in the context of a much larger promoter construct of 1457bp (unpublished data). As both of these mutations have been shown to disrupt Sp1/Sp3 binding, these results suggest that this site may act as both positive and negative regulatory element to control *hTERC* gene expression.

Essential Thrombocythemia (ET)

Essential thrombocythemia is a rare chronic myeloproliferative disorder (CMPD), usually characterized by the overproduction of platelets by megakaryocytes in the bone marrow which generally affects middle-aged to elderly individuals [149]. An ET patient was recently identified who carries an hTERC allele with a two-nucleotide deletion [92]. This mutation (Δ389-390) failed to reconstitute telomerase activity *in vitro*, suggesting that telomerase may play a role in the disease pathogenesis of some patients. Since this disease tends to have a later age of onset, progressive telomere shortening and resulting genomic instability could possibly contribute to the ET phenotype. We have undertaken

an effort to screen 90 patients who have been clinically diagnosed with CMPD, including 43 patients with ET, and found no mutations in the *hTERC* gene [89]. It is possible that other genetic factors may play a role in this group of diseases with highly diverse features. Indeed, recent studies have shown that a single acquired (somatic) mutation (V617F) in the tyrosine kinase JAK2 gene seems to be strongly associated with CMPDs, found in more than half of patients with either ET or chronic idiopathic myelofibrosis and in almost all patients with polycythemia vera [150,151,152]. The mutation leads to constitutive activation of JAK2, which promotes cytokine hypersensitivity [153]. It may cause constitutive activation of an erythropoietin receptor (EpoR) even in the absence of stimulation by its natural ligand erythropoietin, which has been shown to induce erythrocytosis in a mouse model.

1.6 Telomerase Mutations in Non-Hematological Disorders

Idiopathic Pulmonary Fibrosis (IPF)

Idiopathic pulmonary fibrosis is a progressive disorder with an autosomal dominant pattern of inheritance and variable degrees of penetrance and accounts for greater 70% of all cases of idiopathic interstitial pneumonias (IIPs). It is characterized by symptoms of chronic cough and shortness of breath, as well as diffuse interstitial fibrosis [154]. Approximately 20% of DC patients also have some form of pulmonary disease, which can sometimes lead to permanent scarring of the lungs. It has been hypothesized that since there is an inverse relationship between caveolin-1 and TGF- β1 expression and TGF-β1 negatively regulates telomerase activity, there may be a link between a genetic predisposition and the actual molecular signaling. Wang et al. has shown that patients

with IPF have reduced expression of caveolin-1, providing a possible mechanism by which a change in gene expression may lead to telomere shortening in certain lung tissue progenitor cells [155]. In addition, different groups have independently isolated heterozygous mutations in both hTERT and hTERC in patients with IPF, which appear to function via haploinsufficiency [75,77,82,104]. Both patients and carriers have shorter telomeres than age-matched controls. In fact, a recent paper by Alder et al. shows that telomere shortening, in the presence or absence of mutations in telomerase components, may contribute to disease risk in IIP patients who have no family history [104]. Although the mutations appear to impair telomerase activity to different extents in *in vitro* TRAP assays, they confer a dramatic increase in susceptibility to this adult-onset and fatal disease. The significance of telomerase mutations in the development of IPF still needs to be demonstrated *in vivo.*

Cri du Chat Syndrome (CdCS)

Cri du chat syndrome is a disease in infants, which is characterized by a distinct cat-like cry, in addition to other physical anomalies, including microcephaly, widely spaced eyes, low set ears, a low, broad nasal bridge, and palmar creases [156]. It results from loss of the distal portion of chromosome 5p, a region that encompasses the *hTERT* and several other genes. Indeed, FISH analysis on patient lymphocytes and fibroblasts showed only a single copy of *hTERT,* indicating that cells may be haploinsufficient for telomere maintenance [157]. The accelerated telomere shortening predicted by this hypothesis was confirmed in patient lymphocytes by a reduction in Q-FISH signal and shorter telomere restriction fragments (TRFs) as compared to those of age-matched

controls. While it has been shown that patient lymphocytes and fibroblasts have only one copy of *hTERT* and that dermal fibroblasts have an impaired replicative capacity, it is unclear how loss of the catalytic component of telomerase could cause all the symptoms associated with CdCS. It has been proposed that accelerated telomere shortening and subsequent progenitor cell death could adversely affect normal fetal development. However, since other genes are also deleted from chromosome 5p in this disease, it remains to be seen whether *hTERT* is truly the causal gene or just one of many genetic factors leading to CdCS.

1.7 Mouse Models of Dyskeratosis Congenita

X-Linked DC

Two approaches have been taken in order to generate a mouse model of X-linked dyskeratosis congenita: (1) targeted C-terminal deletion of the *Dkc1* gene utilizing the Cre-Lox system and (2) hypomorphic allele in which the wild-type *Dkc1* gene is expressed at reduced levels. While null dyskerin mutations are embryonic lethal, Gu, et al. have constructed a mouse which carries a dyskerin mutation designed to mimic a mutation found in a family with X-linked DC [118,133,158]. From studies on these mice and ES cells, they have shown that cells expressing wild-type dyskerin have a growth advantage over those expressing a truncated version, a phenomenon that is telomerase-, but not telomere length, dependent. In addition, mutant ES cells exhibit an enhanced DNA damage response via the classical p53/ATM pathway and the damage foci colocalize with telomeres [158]. Interestingly, this model does not show any alterations in ribosome biogenesis or any characteristic phenotypes of DC. On the contrary, mice

expressing a hypomorphic allele of *Dkc1* exhibit several phenotypes observed in DC patients, including bone marrow failure, dyskeratosis of the skin, lung abnormalities, and an increased susceptibility to cancer development [159]. Moreover, these pathological features were observed in first- and second-generation mice, suggesting that they arose independent of telomere length. Telomere shortening was not observed in these mice until generation 4 (G4), accompanied by a decrease in telomerase activity caused by decreased mouse telomerase RNA (mTERC) stability. The X-DC phenotypes in these mice seem to be initiated by decreased ribosomal RNA (rRNA) processing and an impairment in internal ribosomal entry site (IRES)-mediated translation [160]. While each of these models sheds important light on dyskerin's various functions in the cell, it seems most likely that a combination of the observed defects contributes to X-DC pathogenesis in humans. In fact, mice carrying two different mutations identified in patients exhibit varying defects in mTERC and small nucleolar RNA (snoRNA) accumulation, telomerase activity, telomere length, and rRNA processing [161]. More careful mapping of specific domains and residues necessary for each of dyskerin's cellular activities should help to shed some light on a possible mechanism underlying this disease.

Autosomal Dominant DC

Knock-outs of mTERC and mTERT exhibit very similar phenotypes. Neither component, although absolutely essential for telomerase enzymatic activity, is essential for embryonic development, and disease states do not manifest until later generations when telomeres have significantly shortened [138,139,140,141,162,163,164,165]. In

confirmation of this finding, Hao et al. has shown that, even in the presence of telomerase activity, short telomeres can limit tissue renewal in the bone marrow, intestines, and testes [166]. In order to generate mice with sufficiently short telomeres, they backcrossed an *mTERC*$^{+/-}$ C57BL/6 mouse with a CAST/EiJ mouse, which is known to have very short telomeres, for five generations in order to generate a heterozygous generation 1 (HG1) mouse. These heterozygotes were intercrossed to obtain successive generations of *mTERC*$^{+/+}$ (wt*), *mTERC*$^{+/-}$ (HG), and *mTERC*$^{-/-}$ (KO) animals. Several tissue renewal defects were observed in the *mTERC* null mice, including small intestine atrophy, hematopoietic defects, and impaired wound healing, and were found to follow the disease anticipation phenomenon observed in humans, whereby disease phenotypes appear at an earlier age in later generations due to the inheritance of shortened telomeres. Interestingly, despite the presence of telomerase activity, late generation heterozygous (HG) and wt* mice also exhibit telomere shortening and signs of occult genetic disease. Despite these exciting findings, none of these mice exhibit the characteristic triad of features associated with dyskeratosis congenita [139,141].

A couple other intriguing results have been obtained through studies on *mTERT* knock-out mice. First, Rajaraman et al. conducted a study on telomere dysfunction-induced apoptosis in the intestinal crypts of late generation *mTERT*$^{-/-}$ mice [167]. In doing so, they found that gastrointestinal (GI) progenitor cells undergo apoptosis due to shortened telomeres shortly after S-phase, but before mitosis, suggesting that telomere uncapping in these cells occurs in late S-phase or in G_2. This timing is consistent with the timing of telomere replication and supports a mechanism whereby disruption of telomere end structure induces apoptosis directly without the need for a fusion-bridge

breaking cycle. Secondly, in addition to its roles in telomere maintenance, TERT has been proposed to perform other "extracurricular activities" in the cell (summarized in [168,169]). Consistent with this idea, conditional induction of mTERT in mouse skin epithelium causes rapid proliferation of hair follicle stem cells independent of hTERC, suggesting that TERT may directly support the processes of differentiation and proliferation [170].

In contrast with the *mTERC* and *mTERT* knock-outs, inactivation of the telomere binding protein TIN2 results in early embryonic lethality which is not rescued by telomerase deficiency [171]. Embryos die before day 7.5 of their embryonic development, suggesting that TIN2 serves telomerase-independent roles in the cell which are absolutely required for life. Unfortunately, the exact cause of death could not be analyzed due to the rapid death of *TIN2*$^{-/-}$ ES cells in culture. The embryonic lethality of this mouse model mirrors that of TRF1- and TRF2-deficient mice [172,173]. Further analysis of the *in vivo* functions of these proteins will require conditional or tissue-specific knock-outs.

By far the most successful genetically engineered model of dyskeratosis congenita is a telomere degradation mouse generated by Hockemeyer et al. and Wu et al. [174,175,176,177]. Interestingly, while human telomeres are protected by one single-stranded DNA binding protein POT1, mouse telomeres contain two POT1 paralogs, POT1a and POT1b [175,177]. Lack of POT1a results in embryonic lethality, activation of the DNA damage response, and aberrant homologous recombination. In contrast, POT1b knock-out mice are viable and fertile, but exhibit an increase in C-strand degradation. Despite the independent functions of POT1a and POT1b, repressing a DNA

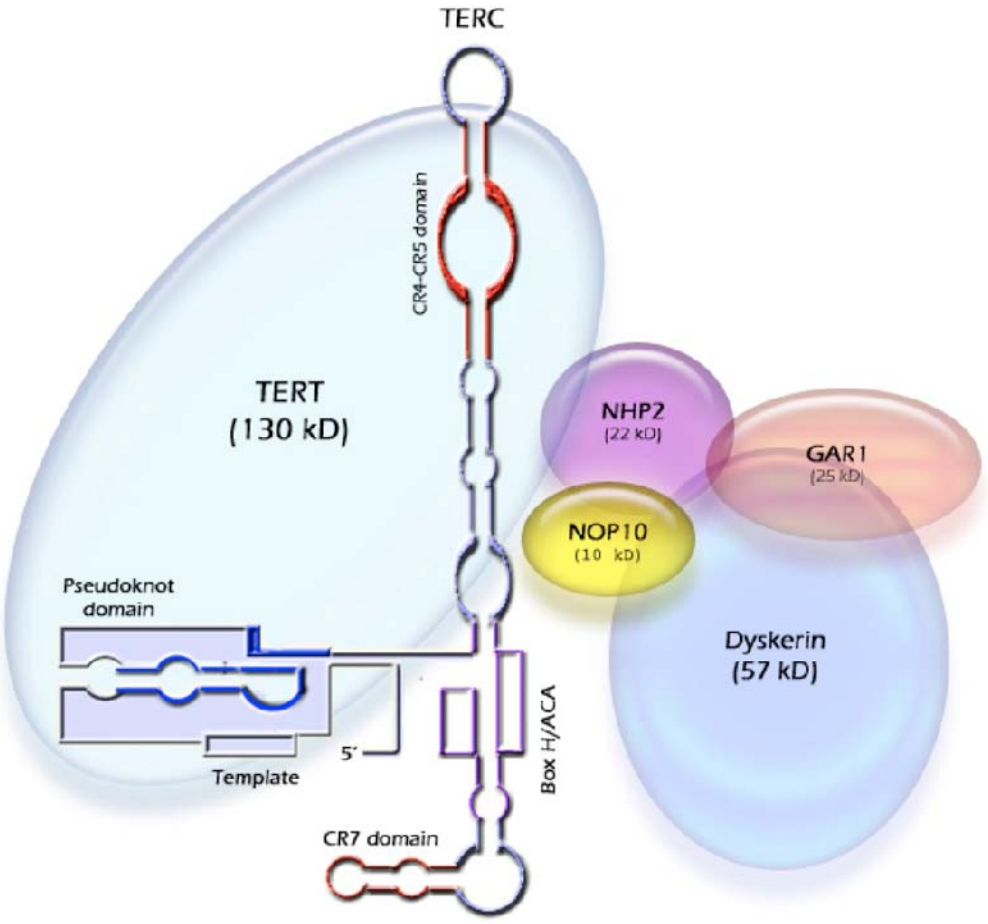
damage signal and regulating the structure of the telomere end, respectively, full protection of telomeres requires both factors. The most exciting finding is that POT1b-deficient mice display several distinctive features of DC patients: abnormal skin pigmentation, nail dystrophy, and bone marrow failure [174,176]. Furthermore, these phenotypes are exacerbated by haploinsufficiency for mTERC and double knock-outs for POT1b and mTERC are embryonic lethal. These symptoms arise in the background of normal telomerase activity, strengthening the argument that DC is due to dysfunctional telomeres. It is interesting to note that whereas mutations in the shelterin components TRF1, TRF2, and TIN2 have been identified in patients with bone marrow failure syndromes, mutations in POT1 have not yet been reported.

1.8 Conclusions

Although it has been over a century since dyskeratosis congenita was first described [178] and over two decades since the discovery of telomerase [17], this disease and its specific etiology as it relates to telomere dysfunction retain their mystery. *In vitro* studies have been helpful in dissecting the potential roles of telomere maintenance genes in disease pathogenesis, but much of this data still needs to be validated *in vivo*. Unfortunately, while it would be ideal to study patient tissues, this is difficult due to the limited availability of samples and the nature of the desired cells. It is potentially problematic to obtain sufficient bone marrow or blood cells from an already hematologically compromised patient. Thus, several mouse models have been developed, both genetic and chemical (reviewed in [179]), to study the physiological effects of deficiencies in the telomere maintenance pathway. Despite the large telomere

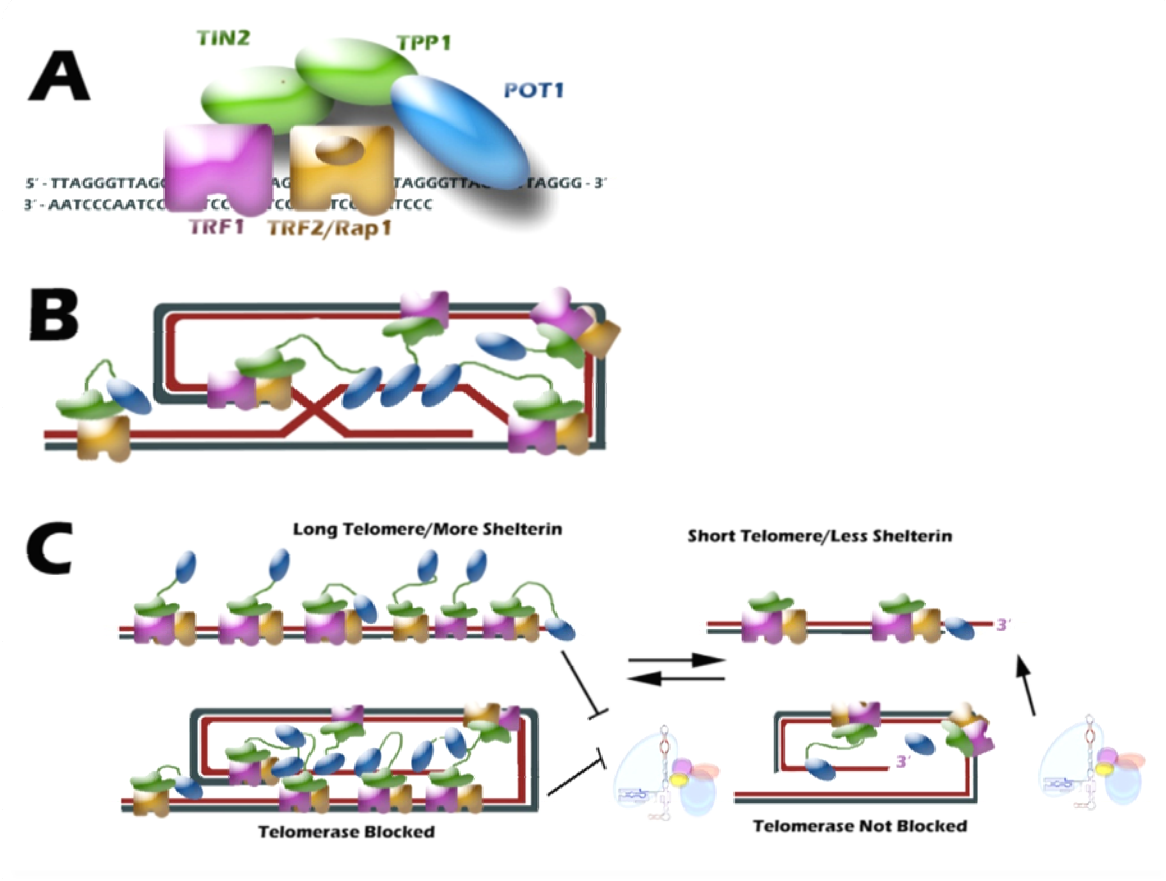
reserve of mice and the inherent differences in shelterin complex composition between mice and humans, mouse studies have not only strengthened the *in vitro* findings, but also shed light on some interesting new phenomena. Whether these conclusions will extend themselves to humans remains to be seen.

Figure 1.1 Telomerase Holoenzyme



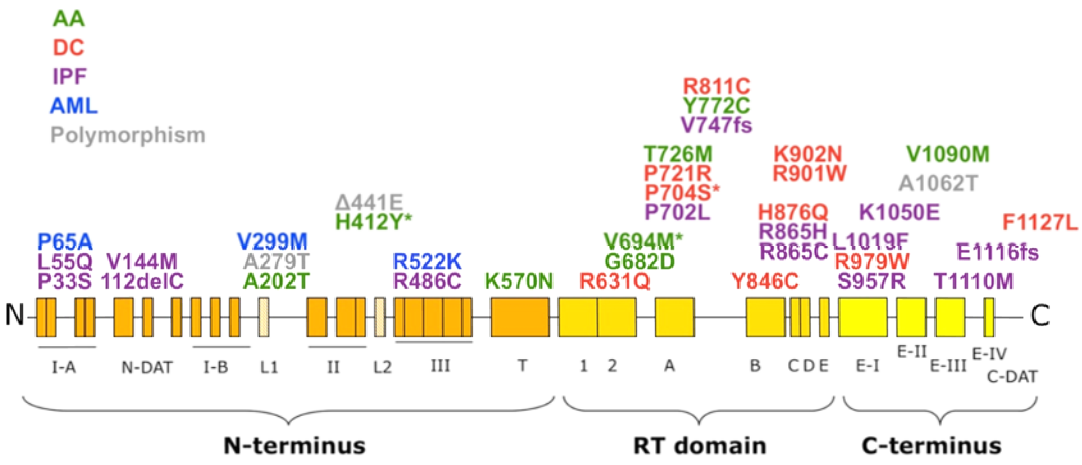
Simplified illustration of the telomerase holoenzyme showing its main components: hTERT, hTERC, Dyskerin, NOP10, NHP2, and GAR1. Functional regions of the hTERC RNA (template, pseudoknot, CR4-CR5, Box H/ACA, and CR7) are indicated.

Figure 1.2 Shelterin Complex



(A) Six components of the telomere-binding complex, shelterin, and their DNA and protein binding abilities. (B) A schematic of the shelterin complex bound to the telomere t-loop structure. (C) A proposed model for how shelterin can function to control telomere length in *cis*. Long telomeres allow for more shelterin binding, which can block access of telomerase to the telomere end. In contrast, a short telomere with less shelterin bound is preferentially elongated by telomerase.

Figure 1.3 Natural hTERT Mutations



All published exonic sequence changes identified patients and/or controls are shown. Mutations are color-coded according to the disease in which they were first identified. Those denoted with an asterisk (*) have been found in multiple telomere dysfunction disorders.

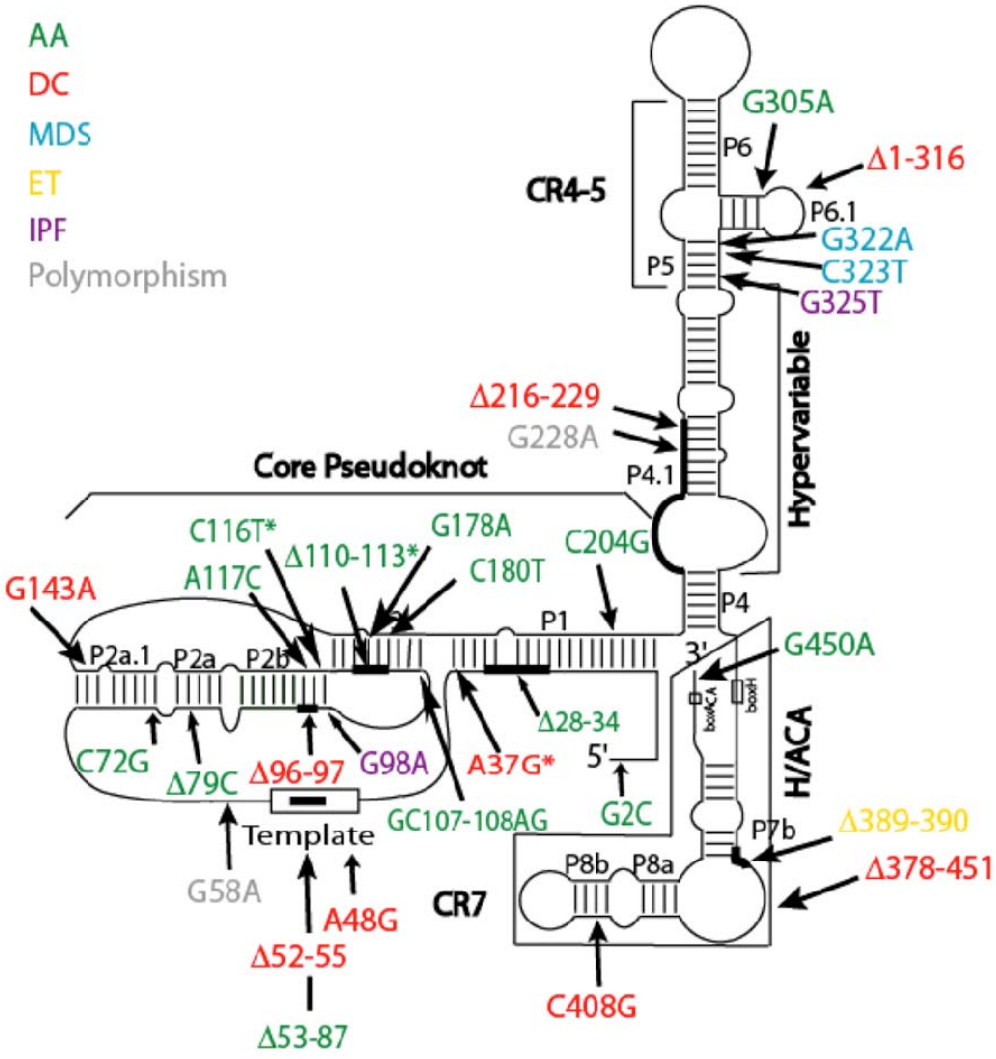
* H412Y: AA, AML

V694M: AA, IPF

P704S: DC, IPF

AA: Aplastic Anemia; DC: Dyskeratosis Congenita; IPF: Idiopathic Pulmonary Fibrosis; AML: Acute Myeloid Leukemia

Figure 1.4 Natural hTERC Mutations

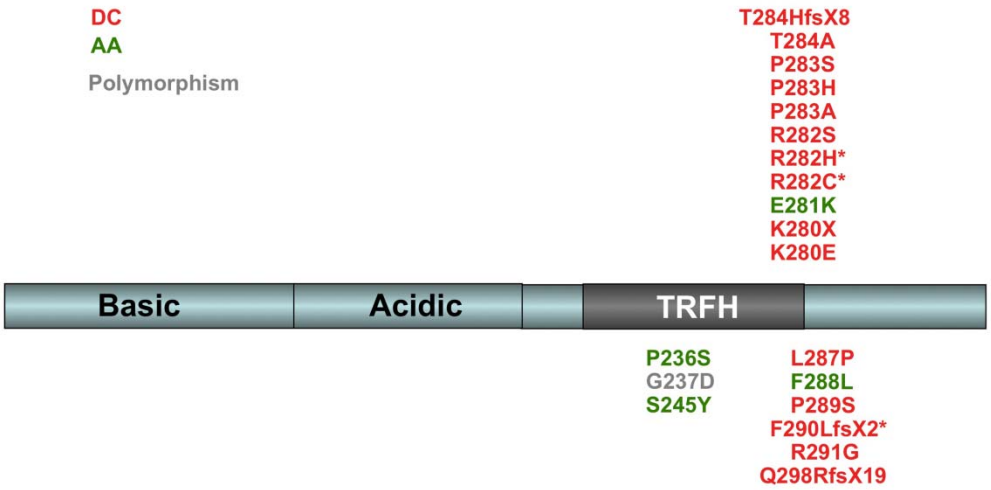


All naturally occurring sequence changes in the hTERC coding region are shown. Mutations are color-coded according to the disease in which they were first identified. Those denoted with an asterisk (*) have been found in multiple telomere dysfunction disorders.

* A37G: DC, IPF; Δ110-113: AA, MDS; C116T: AA, MDS

AA: Aplastic Anemia; DC: Dyskeratosis Congenita; MDS: Myelodysplastic Syndromes; ET: Essential Thrombocythemia; IPF: Idiopathic Pulmonary Fibrosis

Figure 1.5 Natural TIN2 Mutations



All known sequence changes in the TIN2 coding region are shown. Acidic and basic domains are denoted based on the amino acid content in these regions. The TRFH domain is the general region that has been shown to interact with TRF1. Mutations are color-coded according to the disease in which they were first identified. Those denoted with an asterisk (*) have been found in multiple telomere dysfunction disorders.

* R282C: DC, AA

R282H: DC, AA

F290LfsX2: DC, AA

DC: Dyskeratosis Congenita; AA: Aplastic Anemia

CHAPTER 2.1 [106]**:**

Identification and Functional Characterization of Novel Telomerase Variant Alleles in Japanese Patients with Bone-Marrow Failure Syndromes

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Abstract

As the incidence of bone-marrow failure syndromes (BMFS) is 2-3x higher in East Asia than in the West, we examined peripheral blood or marrow cells of 100 Japanese patients for possible pathogenic mutations in the two main components of the telomere-synthesizing enzyme telomerase (hTERC RNA and hTERT protein) that have recently been implicated in the disease pathogenesis. We analyzed samples collected from 34 patients with acquired aplastic anemia (AA), 66 patients with myelodysplastic syndromes (MDS), and 120 healthy controls. In addition to two polymorphic germ-line sequence changes (n-771A/G and n-714 C insertion) in the promoter region of *hTERC* and eleven *hTERT* polymorphisms that were identified in both patients and healthy individuals, we found a novel germ-line C323T mutation in the hTERC RNA in an MDS patient only. This heterozygous C323T mutation abolished telomerase enzymatic activity and functioned in a haploinsufficiency manner to modulate telomerase activity in cells. In summary, this study reports a novel telomerase natural variant that abolishes telomerase function, which may lead to telomere shortening and marrow hypocellularity in patients with BMFS. This study also highlights the rarity of genetic alterations in BMFS patients in Japan, which suggests that other factors may play a more prominent role in the disease pathogenesis in East Asia.

Keywords: telomeres, telomerase, aplastic anemia, myelodysplastic syndromes, bone-marrow failure syndromes, hTERC, hTER, hTR, hTERT

Introduction

Dyskeratosis Congenita (DKC) is an inherited bone marrow failure syndrome (BMFS) typified by reticulated skin pigmentation, nails dystrophy and mucosal leukoplakia [128]. About 35% of the cases are X-linked recessive, 5% are autosomal dominant and the rest of the cases are with unidentifiable pattern of inheritance [132]. Whereas the gene responsible for X-linked recessive cases is *DKC1* [120], those responsible for autosomal dominant cases are the telomerase *hTERC* and *hTERT* [74,83,84,100].

Telomerase is a ribonucleoprotein (RNP) complex with two main components: a protein (hTERT) with RNA-dependent DNA polymerase activity and an integral hTERC RNA, which provides a template to synthesize telomeric DNA repeats [180]. Telomeres are structural elements that seal and protect the ends of linear chromosomes from illegitimate recombination, end-to-end fusion, or being recognized as damaged DNA [180]. In human somatic cells, telomeres typically consist of more than 1000 simple repetitive DNA and associated proteins [180]. These repeats are gradually lost with cellular replication and aging, owing to the inability of DNA polymerase to fully replicate the 3' end of DNA. Telomere attrition eventually leads to critically short telomeres, inducing cellular proliferative senescence and/or apoptosis possibly due to genomic instability [180]. It is thought that telomeres are shortened as a result of pathogenic mutations in *DKC1* or telomerase gene components that lead to an impairment in the proliferative capacity of hematopoietic stem cells in patients with BMFS [83,100,181]. Furthermore, an association has been established between the degree of telomere shortening and that of disease severity and the age of onset [83,181].

The existence of possibly cryptic DKC in patients who develop the disease later in life [90] and many cases of aplastic anemia (AA) with significantly shortened telomeres can also be attributed to mutations in telomerase gene components [182,183,184]. Several groups, including our own, have recently reported that some patients with paroxysmal nocturnal hemoglobinuria (PNH), myelodysplasia (MDS), in addition to those with AA or DKC, carry heterozygous mutations in the telomerase *hTERC* or *hTERT* gene [9,79,84,86,99,103]. In vitro functional analyses of these mutations revealed that the mutations functioned either as dominant-negatives or haploinsufficiency to attenuate telomerase enzymatic activity, which could explain the short telomeres in patients [74,85,86,92,95]. The largest controlled epidemiologic studies reported that the incidence of AA in the West is 2 per million per year and is about 2- to 3-fold higher in Asia [185]. The subjects of previous studies have mostly been those with Caucasian, Black or Hispanic ancestry. Screening for telomerase mutations among Asian populations has rarely been done [79,186]. Therefore, we carried out an investigation to determine whether mutations in *hTERC* and *hTERT* genes are associated with the disease in our cohort of Japanese BMFS patients.

Materials and Methods

Patients and healthy controls

We examined mononuclear cells (MNC) of peripheral blood or bone marrow from 100 BMFS patients with acquired AA (n=34) or with MDS (RA) (n=66) diagnosed between 1993 and 2005 at the Nippon Medical School and its affiliated hospital. These patients were diagnosed with AA based on the blood count criteria of the International Study of

AA and agranulocytosis with severity determined by the criteria of Camitta et al [187]. We excluded AA patients who had achieved complete remission or good response to immunosuppressive therapy. Good response was defined as a resolution of all blood transfusion requirements and a more than 2 g/dL increase in hemoglobin as compared with pretreatment levels. Most AA patients (91.1%) received ATG and cyclosporine A combination therapy and showed either only partial or no response to treatment (Table 2.1.1). For a diagnosis of MDS, patients were sub-classified according to the French-American-British (FAB) nomenclature [188]. MDS (RA) classification might include several heterogeneous BMF or other hematologic diseases. However, we excluded patients who had developed MDS (RAEB) or MDS leukemia for more than 3 years from original diagnosis. Most of selected patients with MDS (RA) were treated with blood transfusion or anabolic steroid. As normal controls, we analyzed blood samples from 120 healthy individuals. Our volunteers provided informed consent prior to genetic testing as approved by our institutional review board.

Mutational analysis

Mononuclear cells (MNC) from bone marrow or peripheral blood were isolated by density gradient centrifugation using lymphocyte separation medium (Organon, Durham, NC). The genomic DNA of MNC was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) amplification of telomerase gene components (*hTERC* and *hTERT*) was carried out essentially as described previously [86,103]. The Advantage GC2 PCR amplification kit (BD Biosciences Clontech, CA, USA) and the TaKaRa Ex Taq DNA polymerase (Takara,

Shiga, Japan) were used to amplify the genes from genomic DNA. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced bidirectionally using the Big Dye Termination 3.1 kit and the ABI Prism 310 system (Perkin-Elmer Cetus, CA, USA). Specific sequences of primers used for sequencing are available upon request. To validate the sequencing results, PCR products were inserted into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, CA, USA). Recombinant plasmids isolated from 8 to 12 white colonies were sequenced.

In vivo reconstitution of telomerase enzymatic activity

Wild-type or mutant pcDNA3-hTERC DNAs (2 μg) were transfected into VA13+hTERT cells (at approximately 70% confluency) in 6-well polystyrene dishes using SuperFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In certain cases, two different versions of the *hTERC* gene, each on a separate vector and at 1 μg, were co-expressed simultaneously in the VA13+hTERT cells. To monitor transfection efficiency, a plasmid (peGFP-N1) (Strategene, CA, USA) expressing green fluorescent protein was transfected in a parallel transfection reaction. The eGFP protein expression level was examined under fluorescence microscope. Approximately 48 hours after transfection, cells were scraped from the dish in the presence of 1 mL cold phosphate-buffered saline. Cellular extracts were then prepared in 1X CHAPS lysis buffer as suggested by the manufacturer (Chemicon International, CA, USA). Telomerase activity of the cellular extract from $2x10^4$ cells was assayed using the TRAPeze Telomerase Detection Kit following the manufacturer's directions (Chemicon International, CA, USA), except that PCR was performed as follows: 95°C for 2 min; 25

cycles of 94°C for 10 s, 50°C for 30 s, 72°C for 30 s; and 72°C for 5 min. Products were analyzed on a 12% native polyacrylamide gel and examined by phosphor imaging (Molecular Dynamics, GE Healthcare Bio-Sciences Corp., NJ, USA).

Northern blotting analysis.

Wild-type or mutant pcDNA3-hTERC vector (2 μg) was transfected into VA13+hTERT cells (at approximately 70% confluency) in 6-well polystyrene dishes using SuperFect transfection reagent. Approximately 48 h after transfection, Trizol reagent was used to extract total cellular RNA as suggested by the manufacturer (Invitrogen, CA, USA). Northern blot analysis was performed essentially as described [93].

Immunoprecipitation-Northern blotting analysis.

FLAG-tagged hTERT protein was expressed in vitro from the pCR3-FLAG-hTERT vector using the TnT quick-coupled transcription-translation system (Promega) in the presence of 200 ng of in vitro-transcribed, gel-purified CR4-CR5 fragment of hTERC RNA spanning nucleotides 239 to 332 at 37°C for 2 h. The resulting telomerase complexes were affinity-enriched on anti-FLAG agarose beads (Sigma, St. Louis, MO). To detect hTERT-bound telomerase RNAs, Northern blotting was performed on the enriched telomerase preparations as described above.

Results

Telomerase mutational analysis

We selected 100 BMFS patients who were diagnosed with either AA, mainly those who showed either only partial or no response to immunosuppressive therapy, or with MDS RA (Table 2.1.1). Genomic DNA from peripheral blood cells or marrow stem cells was extracted in order to amplify the *hTERT* and *hTERC* genes for sequencing. Even though we did not find any pathogenic mutations in the *hTERT* gene, we identified eleven polymorphic sequence changes (Table 2.1.2). These sequence polymorphisms were identified in both intronic and exonic regions of the gene and did not result in amino acid substitutions in the corresponding protein. Two of the polymorphic sequence changes (IVS6 -93 G/A and codon837 CTC/CTG) have not been reported previously.

In regard to the *hTERC* gene, we identified two novel heterozygous sequence polymorphisms in its promoter (n-771A/G and n-714 C insertion) at about a similar frequency in both patients and healthy controls (Table 2.1.3 and Fig. 2.1.1). On the contrary, we identified a novel heterozygous germ-line mutation in the *hTERC* gene (C322T) in an MDS patient only (Table 2.1.3 and Fig. 2.1.1). This patient was a 72 year-old man, who was clinically diagnosed with MDS RA. His bone marrow was slightly hypocellular but showed no sign of dysplasia or chromosomal abnormality. He showed a good and sustained response to metenolone and did not have a family history for the disease. Since this is an archival case, primary specimen (blood) collected from this patient did not yield an adequate amount of genomic DNA for measuring telomere lengths. The patient has deceased from ischemic heart disease.

Functional analysis of the hTERC C323T mutation

The novel C323T mutation changes a cytosine to a uracil in the hTERC RNA and is located on one strand of the P5 stem of the predicted hTERC RNA secondary structure (Fig. 2.1.2A) [189]. As such, we hypothesize that it may disrupt the base pairing interaction of this stem structure, which may lead to defective telomerase enzymatic function. In order to test this hypothesis, we introduced this hTERC natural variant C323T [or C323T(rt)] into a mammalian expression plasmid encoding the full-length (451-base) hTERC sequence. Because this specific nucleotide change might disrupt the base pairing interaction of the predicted P5 stem structure (Fig. 2.1.2A), we also created additional mutants designed to test the importance of this base pair. We designed a mutant denoted as C323T(lt) in which a guanine nucleotide located at position 246, on the opposite strand from the natural mutation, was mutated to an adenine (Fig. 2.1.2A). We also created a compensatory mutant [denoted as C323T(comp)] in which the natural C323T(rt) base mutation was accompanied by a complementary mutation on the opposite strand [C323T(lt)], which would theoretically restore the predicted intramolecular base pairing interactions of the stem structure.

Biological activities of these hTERC variants were assessed by transient transfection of each vector into the VA13+hTERT cell line, a human lung-derived line that lacks endogenous hTERC but has been engineered to express stably the hTERT protein. These cells cannot ordinarily produce functional telomerase, but can assemble the active enzymatic complex when transfected transiently with a vector that expresses a functional hTERC copy. We prepared extracts of the cells approximately 48 h after transfection with the various hTERC constructs and estimated the steady-state levels of

exogenous hTERC expression by Northern blot analysis. We then tested the extracts for telomerase enzymatic activity by measuring their ability to add telomeric DNA repeats onto a synthetic DNA primer in vitro, using a semi-quantitative, PCR-based telomere repeat amplification protocol (TRAP).

As compared to cell lysates that carry either the wild-type hTERC RNA or the one with a known inconsequential G58A polymorphic sequence change [95,103] (Fig. 2.1.2B, lanes 1-3 and 13-15), we found that in each of the cases, the mutations located within the P5 stem drastically reduced telomerase enzymatic activity (lanes 4-12). More specifically, mutations located on the individual strands of the P5 stem [i.e., either the natural C323T(rt) mutation or the C323T(lt) mutation alone] abolished telomerase activity to about the same degree (Fig. 2.1.2B, lanes 4-9). Surprisingly, we found that the compensatory mutation C323T(comp) did not restore telomerase activity (lanes 10-12). Rather, this version of the RNA seemed to further reduce telomerase function in cells to an almost undetectable level. These results were not attributable to differences in hTERC RNA synthesis, processing, or stability, as Northern blotting verified that each construct produced comparable steady-state levels of hTERC expression in the transfected cells (Fig. 2.1.2C). As the disease-associated mutation is located within the highly conserved CR4-5 domain of hTERC that has been implicated as one of the hTERT-interacting sites, we asked whether our RNA mutants can also affect hTERT binding activity. Telomerase complexes were reconstituted in vitro using rabbit reticulocyte lysates to express a FLAG-tagged version of the hTERT protein in the presence of a synthetic hTERC RNA spanning nucleotides 239 to 332 of the CR4-5 domain. Anti-FLAG antibody was used to immunoprecipitate telomerase RNP complexes, which was used to probe for the CR4-5

RNA fragment. As shown in Fig. 2.1.2D, both the C323T(rt) and the C323T(comp) RNAs showed substantially impaired binding to hTERT protein. Taken together, these findings indicate that the disease-associated hTERC variant and its derivatives are functionally defective and that their defects may result from altering the conserved secondary structure and/or primary sequence of the RNA that abolishes its ability to interact with the hTERT catalytic protein component of telomerase.

hTERC C323T natural mutation functions as haploinsufficiency to modulate telomerase function

As the natural hTERC C323T variant was identified in an individual who is a heterozygous carrier for the gene, the altered allele might modulate normal telomerase function through either a haploinsufficiency or dominant-negative fashion. In order to address this, we performed TRAP assays on cell lysates prepared from VA13+hTERT cells that had been co-transfected with plasmids to express the wild-type hTERC sequence and either the disease-associated hTERC C323T variant or the polymorphic G58A variant. As shown in Fig 2.1.2B, little to no effects were observed between cells that carried only the wild-type hTERC vector and those that carried both the wild-type and the individual mutated hTERC copy (lanes 16-21), consistent with the idea that the natural variant functions in a haploinsufficiency manner to modulate wild-type telomerase function [84,95].

Discussion

This study shows that a novel variant telomerase RNA allele found in a Japanese patient with MDS is unable to support a normal level of telomerase enzymatic activity. This is consistent with the hypothesis that defects in telomerase function and telomere maintenance contribute to the pathogenesis of BMFS. This patient has deceased from ischemic heart disease. It has been documented that short telomeres may contribute to the pathophysiology of atherosclerosis and to the development of ischemic heart disease [190,191,192]. Some studies have implicated short telomere lengths to atherogenesis [193] and indicated that telomere lengths can serve as an effective marker for biological aging at a cellular level, such as aging cells of the vascular tissues [190,191,192].

The novel hTERC mutation reported in the current study is located on one side of the predicted P5 stem of the hTERC RNA secondary structure (Fig. 2.1.2A) that when altered drastically reduces telomerase enzymatic activity (Fig. 2.1.2B). More importantly, a combined substitution of both sides of this stem almost completely abolished telomerase function. It is noteworthy that the C323T variant allele described here is one of three known disease-associated alleles identified so far in this region (i.e., G322A, C323T, and G305A), and each seems to effectively abolish telomerase function [92]. Therefore, it is possible that this region may serve as one of the hotspots for the natural process of mutagenesis that can result in defective telomerase function and telomere shortening effect observed in patients with hematologic disorders.

Our study indicates that this P5 region of the hTERC structure contributes to optimal telomerase function, and this intricately base paired structure and/or its primary sequence serve as a critical feature for its biological activity. Indeed, it is striking that a

high proportion of the seemingly minor point mutations examined in our earlier study, which aimed at comprehensively analyzing the structure and function of the entire hTERC molecule, severely compromised telomerase function by perturbing RNA structural formation [194]. However, in those cases, we found that compensatory mutations could fully restore telomerase activity, highlighting the importance of the normal base pairing pattern of the RNA. In the current study, we describe for the first time a unique region (P5) of the telomerase RNA molecule that seems to be highly sensitive to sequence alteration. As this region is part of the conserved CR4-5 region (Fig. 2.1.2A) that has been shown to serve as one of the sites for the catalytic hTERT protein to assemble onto the RNA [92,195,196], we show here that indeed both the disease-associated mutant C323T(rt) and the compensatory C323T(comp) mutant substantially impair hTERT binding. Our data, therefore, support the idea that even a minute sequence change in this region can perturb this critical telomerase ribonucleoprotein interaction and hence abrogate its enzymatic activity.

Together with earlier reports [86,92,93,100,194], our data presented here are consistent with the idea that defects in telomerase function and telomere maintenance contribute to the pathogenesis of BMFS in a subset of these patients. As we found only one patient with a potential pathogenic mutation in the *hTERC* gene and none with pathogenic *hTERT* mutations among a cohort of 220 Japanese men and women, our study revealed that natural sequence variations in telomerase gene components rarely occurred in the Japanese population. This is consistent with a recent finding of no mutations in the *hTERC* gene in 35 Japanese MDS patients and 134 healthy volunteers [186]. A separate group examined 96 Japanese children with acquired AA and 76 healthy controls and

similarly found no mutations in the *hTERC* gene [92]. However, this study revealed two nonsynonymous mutations in the *hTERT* gene among several inconsequential polymorphic sequence changes in this gene. Collectively, these studies showed that mutational frequencies of telomere-synthesizing genes among Japanese BMFS patients were lower than what had been reported for other ethnic groups [83,84,86,103] and that this genetic difference could not explain the higher incidence of the disease in Asian populations.

Table 2.1.1

Patients' clinical background

		AA (n=34)	MDS (RA) (n=66)
Sex	Male	14	34
	Female	20	32
Age (range)		13–77	19–90
Family history	+	0	2
	–	34	64
Chromosome abnormality	Trisomy 8	0	4
	7q-	1	1
	del (20)	0	3
	Complex	0	2
Treatment			
Immunosuppressive therapy			
Partial response		17	5
No response		14	9
Others			
Blood transfusion only		0	27
Metenolone		2	22
Stem cell transplantation		1	3

Table 2.1.2

Polymorphisms of *hTERT*

Exon	Substitution	No (%)
2	Codon305 GCA/GCG(Ala/Ala)	25
3	IVS3+130C/T	24
4	IVS3-24C/T	14
5	Codon699 GCC/GCT(Ala/Ala)	3
7	IVS6-93G/A	2
	Codon837 CTC/CTG(Leu/Leu)	1
9	Codon840 CTG/CTA(Leu/Leu)	3
	IVS9+11C/T	3
13	IVS13+45C/T	5
14	Codon1013 CAC/CAT(His/His)	13
15	IVSE14-94C/T	2

Table 2.1.3

Polymorphisms and mutation of *hTERC*

	AA, MDS ($n=100$)	Control ($n=120$)
Polymorphisms		
n-771A/G	11 (11.0%)	18 (15.0%)
n-714C insertion	12 (12.0%)	20 (16.7%)
Mutation		
n323C/T	1 (1.0%)	0 (0%)

Figure 2.1.1

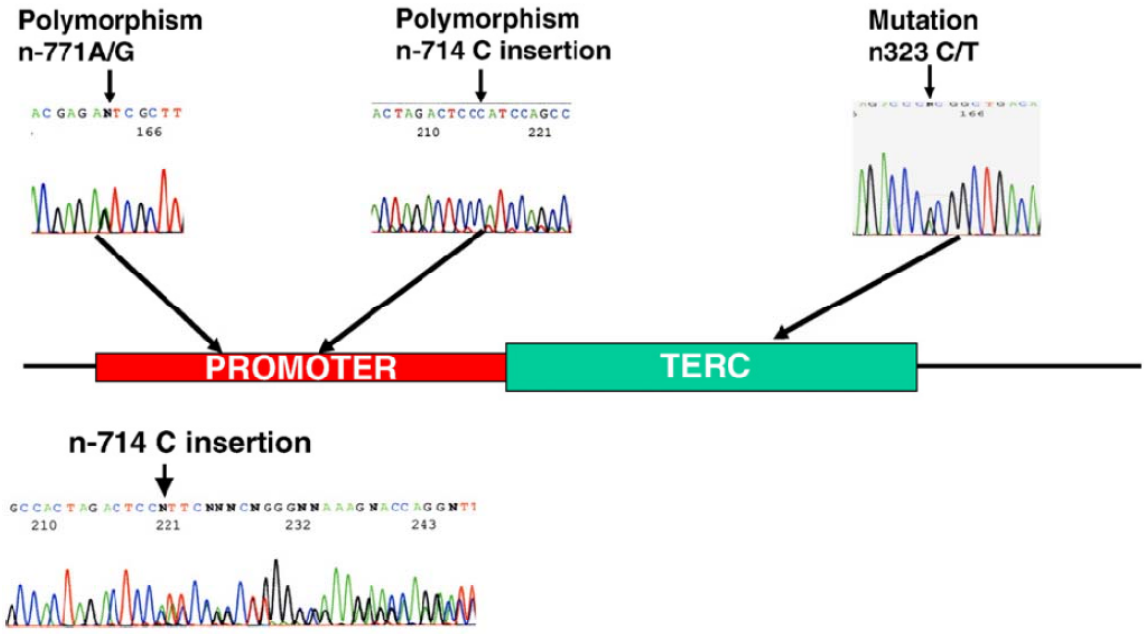

Polymorphism
n-771A/G
Polymorphism
n-714 C insertion
Mutation
n323 C/T
PROMOTER
TERC
n-714 C insertion

Figure 2.1.2

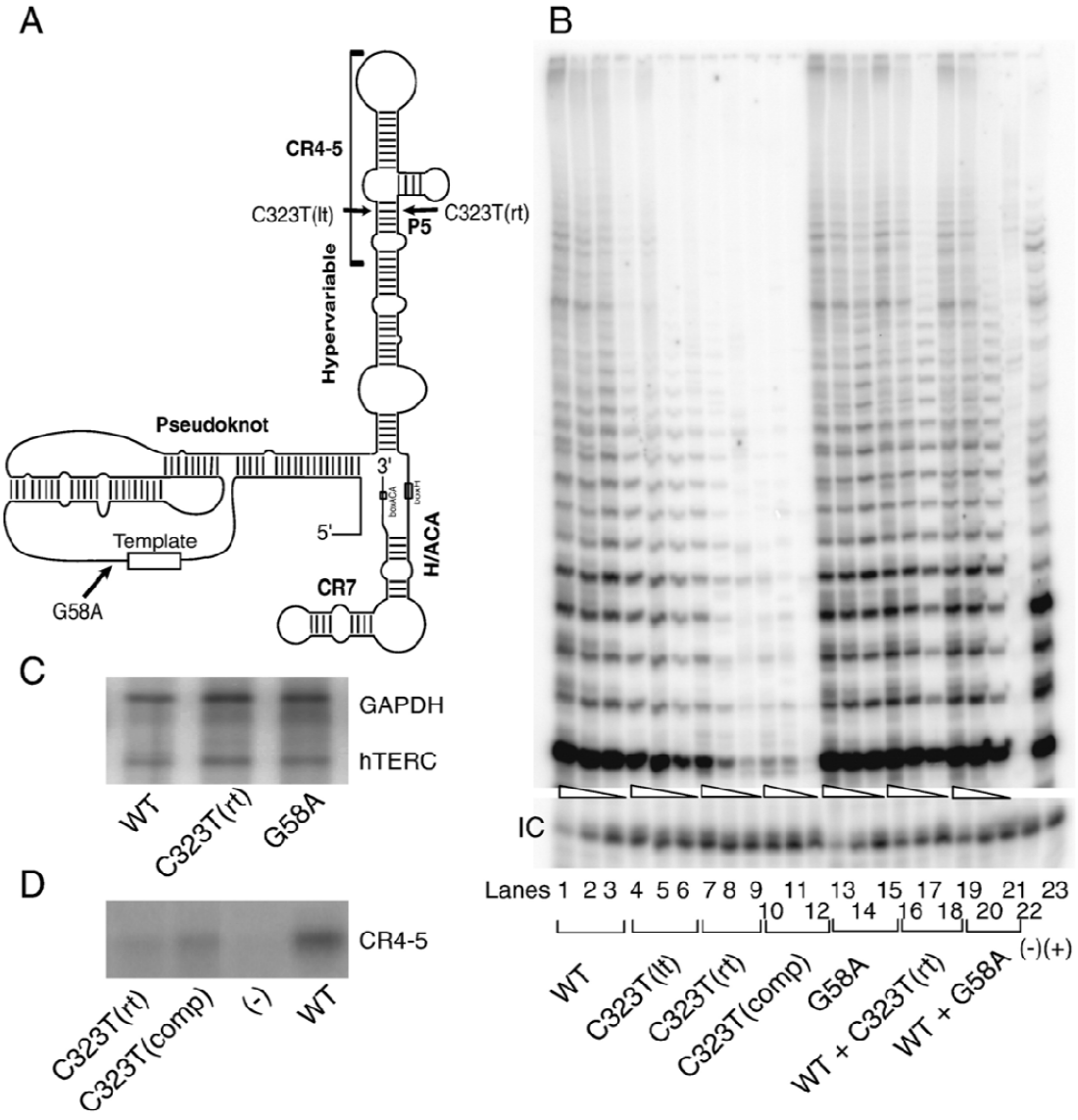

A
CR4-5
C323T(lt)
C323T(rt)
P5
Hypervariable
Pseudoknot
3'
5'
Template
G58A
CR7
H/ACA
B
IC
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
(-)(+)
WT
C323T(lt)
C323T(rt)
C323T(comp)
G58A
WT + C323T(rt)
WT + G58A
C
GAPDH
hTERC
WT
C323T(rt)
G58A
D
CR4-5
C323T(rt)
C323T(comp)
(-)
WT

Figure Legends

Figure 2.1.1: Schematic depiction of the *hTERC* gene with naturally occurring sequence variations that occur in its promoter and coding sequence. Electrophereograms showing the heterozygous nature of these sequence variations are also shown. In the case of the n-714 C insertion, hTERC PCR product was also subcloned into pCR2.1-TOPO expression vector before sequencing. Sequence analysis of the PCR product of this sequence variant showing the heterozygous nature of the mutation is also shown in the bottom

Figure 2.1.2: (A) Schematic depiction of the predicted secondary structure of hTERC as proposed by Chen et al. [189]. The 8-base template sequence (rectangle) and other structural features are indicated, including the pseudoknot, CR4-CR5, box H/ACA, and CR7 domains, as well as the hypervariable paired region. The inconsequential G58A polymorphism, disease-associated C323T mutation [aka C323T(rt)] and C323T(lt) engineered variant are shown. (B) Telomerase enzymatic activities as determined in VA13+hTERT cells for the naturally occurring hTERC mutation and its derivatives. A representative TRAP gel showing the relative telomerase enzymatic activities obtained from the substitution mutations and compensatory mutation. The compensatory mutation [C323T(comp)] was created in order to restore the P5 stem structure. Serial fivefold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lane 22 shows a negative control composed of wild-type cell lysate denatured at 95°C for 5 minutes prior to assay. Lane 23 shows PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit. "IC" indicates PCR products amplified from an unrelated DNA template,

which is included as an internal control for PCR amplification efficiency in each reaction. (C) Northern blot analysis of selected naturally occurring hTERC sequence variants and wild-type sequence expressed in transfected VA13+hTERT cells. Cellular GAPDH mRNA was assayed simultaneously. (D) Northern blot analysis of affinity-enriched telomerase complexes assembled using RNA fragments representing the CR4-5 domain of hTERC (Fig. 2.1.2A) with either the wild-type or mutated sequences and *in vitro* expressed hTERT catalytic protein. The negative control (lane 3) was a reaction that lacks the hTERT-expressing construct.

CHAPTER 2.2:

Functional Characterization of Telomerase Sequence Variants Identified in Patients with Short Telomeres

Introduction

Human telomeres at chromosome ends typically consist of more than 1000 simple repetitive DNA sequences and associated proteins [180]. In somatic cells, these repeats are gradually lost with cellular replication and aging, owing to the inability of DNA polymerase to fully replicate the 3' end of the chromosomes. Telomere attrition eventually leads to critically short telomeres, which induce cellular senescence and/or apoptosis possibly due to genomic instability [180]. In the germ line and stem cell compartments, however, telomere lengths are maintained by a specialized enzyme called telomerase. Telomerase is a ribonucleoprotein (RNP) complex with two main components: an RNA-dependent DNA polymerase (hTERT) and an integral hTERC RNA, which provides a template to synthesize DNA repeats onto chromosome ends [180]. It is thought that telomeres are abnormally shortened in patients with bone-marrow failure syndromes (BMFS) as a result of pathogenic mutations in telomerase gene components that lead to an impairment in the proliferative capacity of hematopoietic stem cells [83,100,181]. Furthermore, an association has been established between the degree of telomere shortening and that of disease severity and the age of onset [83,181].

Recently, the spectrum of diseases presenting with shortened telomeres and carrying mutations in the key telomerase components, hTERT and hTERC, has expanded to include those not only affecting the hematopoietic system, but other highly-proliferative tissues as well. Whether the gene mutations and telomere shortening are causative of the disease, as is believed to be the case for BMFS and idiopathic pulmonary fibrosis (IPF), or whether they are a result of cellular over-proliferation, a possibility for acute myeloid leukemia (AML) and IPF, remains to be seen.

Such investigations will require much more sophisticated testing methods and data sets than are currently available. The experiments currently used to test the effects of mutations in either hTERT or hTERC involve reconstituting a mutated version of the telomerase holoenzyme, either *in vitro* using purified components or a rabbit reticulocyte lysate (RLL) system, or *in vivo* by transfecting cell lines engineered to express either telomerase component (see "Materials and Methods"). From there, any effect the mutated enzyme may have on telomerase activity is assessed by incubating the enzyme with an artificial telomeric primer and observing the elongation products on a gel. However, these systems are quite artificial and do not test several potential telomerase defects, such as its recruitment to the shortest telomeres and enzyme processivity. Regardless of their shortcomings, these systems have shed some light on important domains and residues of both the hTERT protein and the hTERC RNA required for optimal telomerase enzymatic function. As such, I have investigated the effects on telomerase enzymatic activity of several hTERT and hTERC mutations, which either have been tested in *in vitro* but not *in vivo* systems, or have been newly identified in our cohort of patients with blood disorders.

Materials and Methods

Generation of point mutations

hTERC promoter mutants were created in both the pGL3-hTR plasmid, which carries 1457 bp of hTR promoter sequence upstream of the firefly luciferase gene, and the pLXSN plasmid, which carries the same 1457 bp of promoter sequence upstream of the hTERC coding region, using site-directed mutagenesis. Point mutations in the

hTERC coding region were generated in the pcDNA3-hTR backbone using site-directed mutagenesis. hTERT point mutants were generated in either the pCI-FLAG-hTERT or pCI-HA-hTERT backbone using site-directed mutagenesis. All mutations were confirmed by direct DNA sequencing, and their quantity and quality routinely checked by spectrophotometric analysis and agarose gel electrophoresis.

In vivo reconstitution of telomerase activity

Wild-type or mutant pcDNA3-hTERC DNAs (2 μg) were transfected into VA13+hTERT cells (at approximately 70% confluency) in 6-well polystyrene dishes using SuperFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Alternatively, wild-type or mutant pCI-FLAG-hTERT (or HA-hTERC) DNAs were transfected into VA13+hTR cells and transfected in a similar fashion. In certain cases, two different versions of the *hTERC* or *hTERT* gene, each on a separate vector and at 1 μg, were co-expressed simultaneously in VA13+hTERT or VA13+hTERC cells, respectively. To monitor transfection efficiency, a plasmid (peGFP-N1) (Strategene, CA, USA) expressing green fluorescent protein was transfected in a parallel transfection reaction. The eGFP protein expression level was examined under fluorescence microscope. Approximately 48 hours after transfection, cells were scraped from the dish in the presence of 1 mL cold phosphate-buffered saline. Cellular extracts were then prepared in 1X CHAPS lysis buffer as suggested by the manufacturer (Chemicon International, CA, USA). Telomerase activity of the cellular extract from $2x10^4$ cells was assayed using the TRAPeze Telomerase Detection Kit following the manufacturer's directions (Chemicon International, CA, USA), except that PCR was performed as follows: 95°C for 2 min; 25 cycles of 94°C for 10 s, 50°C for 30 s, 72°C for

30 s; and 72°C for 5 min. Products were analyzed on a 12% native polyacrylamide gel and examined by phosphor imaging (Molecular Dynamics, GE Healthcare Bio-Sciences Corp., NJ, USA).

Luciferase activity of hTERC promoter variants

293T cells were seeded at a density of 4×10^5 cells per well in 12-well plates 24 hours prior to transfection. The cells were transfected with 700 ng of the *hTERC* promoter-driven luciferase plasmid(s) and 100 ng of the pRL-CMV plasmid as an internal control of transfection efficiency, using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 hours after transfection and lysed in 200µl reporter lysis buffer (Promega). Luciferase activity was measured using the Luciferase Assay System (Promega). Luciferase activity (arbitrary units) was divided by the internal control in the same sample to normalize for transfection efficiency and expressed as relative luciferase activity. Transfection data represents at least three independent experiments, each performed in triplicate.

Results

Characterizing hTERC promoter sequence variants

We identified several sequence variants both in patients and healthy controls (some previously reported in [106]) in the proximal promoter region upstream of the *hTERC* gene. As they were identified in patients with shortened telomeres, we tested the hypothesis that these sequence changes would have an adverse effect on *hTERC* transcription. Plasmids carrying 1457bp of promoter sequence upstream of the full *hTERC* gene were transfected into VA13+hTERT cells, a human lung-derived line that

lacks endogenous hTERC but has been engineered to express stably the hTERT protein. These cells cannot ordinarily produce functional telomerase, but can assemble the active enzymatic complex when transfected transiently with a vector that expresses a functional hTERC copy. Upon harvesting the cell lysates approximately 48 hours post-transfection, we did not observe any significant decrease in telomerase enzymatic activity and hence, *hTERC* transcription, for any of the nucleotide changes as tested by the TRAP assay (Fig. 2.2.3). In order to validate these results, we created plasmid constructs carrying the same 1457bp of *hTERC* promoter sequence upstream of a firefly luciferase reporter gene and tested the ability of the sequence changes to modulate *hTERC* promoter-driven transcription. As shown in Figure 2.2.4, none of the polymorphisms caused a significant depreciation in luciferase reporter activity. Interestingly, the -99C/G sequence change was previously reported to significantly increase promoter activity [148]. However, that study evaluated the sequence change in the context of a short 100bp *hTERC* promoter sequence. Mapping putative transcription factor binding sites in the *hTERC* promoter may shed some light into this apparent discrepancy.

Mutations in the hTERC coding region significantly decrease enzymatic activity

A recent report has identified two large deletions in the hTERC coding region in some individuals with blood disorders (Fig. 2.2.1) [197]. The first mutation, Δ109-123, removes a large portion of the pseudoknot domain which is required for hTERT binding and telomerase activity. The proband suffers from moderate aplastic anemia and both her father and paternal grandfather died with liver cirrhosis. The second mutation, Δ341-360, deletes a portion of the hypervariable region adjacent to the Box H/ACA domain which is

required for RNA stability. The patient's symptoms include esophageal cancer, liver cirrhosis, aplastic anemia, and severely depleted bone marrow with a family history of liver and pulmonary disease. As shown in Figure 2.2.5, both of these mutations confer a significant decrease in telomerase enzymatic activity when transiently transfected into VA13+hTERT cells tested in our TRAP assay system (lanes 4-9). Since both of these mutations involve deleting a large portion of the hTERC molecule, it is possible that the impact they have on telomerase enzymatic function could be due to improper folding and thus instability of the RNA molecule, rather than the specific importance of the regions removed. A Northern blot to test for RNA stability would help to differentiate between these two possibilities. In addition, as cell lysates that have been transfected with both a wild-type and a mutant hTERC plasmid express normal levels of telomerase activity, these mutations do not appear to act via a dominant-negative mechanism, but rather assert their phenotypic effect via haploinsufficiency (Fig. 2.2.5, lanes 17-23).

hTERT coding sequence variants have varying effects on telomerase activity

In addition to our *in vivo* assembly method, there are other *in vitro* systems available to test the effects of mutations in the hTERT molecule on telomerase enzymatic activity. I have tested a battery of mutations in the hTERT molecule, some of which have been tested via these other systems and some of which are novel, indicated by asterisks in Fig. 2.2.2. Although many of the mutants do not appear to cause a significant decrease in telomerase enzymatic activity when transfected into VA13+hTERC cells, there are a few whose effects are worth mentioning. While our results on the V299M and the R522K mutation are in agreement with previously published results [87], the data on two

polymorphic sequence changes are confusing at best (Fig. 2.2.6). Nonsynonymous amino acid changes are typically classified as polymorphic if they occur at an equal prevalence in patients and controls, although it has been found that the relative frequency of some mutations may vary with the disease being investigated, such as an increased occurrence of the A1062T change in patients with acute myeloid leukemia (AML) [87]. However, although it is believed that these polymorphic sequence changes should have no effect on enzymatic activity as they are found in control samples who have normal telomere lengths, we found that the A1062T mutation causes a decrease in telomerase activity. This is in agreement with a recent study by Calado et al. [87], despite the fact that our data on the Δ441E sequence change do not agree. Western blots are needed to confirm that the changes we observe are due to mutating functionally important residues and not because of differences in protein expression level. Furthermore, all of the mutations appear to function via haploinsufficiency, in keeping with the hypothesis that half the normal amount of wild-type hTERT protein is simply insufficient to sustain high enough levels of telomerase activity to maintain telomere lengths (Fig. 2.2.7). Even though the R522K mutant appears to show some decrease in telomerase activity, observation of all 3 lanes suggests that the serial 10-fold dilutions may be inaccurate (Fig. 2.2.7, lanes 16-18).

Our second batch of mutations includes the novel mutation 112ΔC which was identified in a patient with idiopathic pulmonary fibrosis (IPF). This mutation appears to confer an approximately 50% decrease in telomerase activity (Fig. 2.2.8, lanes 10-12). The rest of the mutant data are in agreement with previously published results [75,80,82]. In addition, all of the tested mutations act via haploinsufficiency, as cell lysates from

cells transfected with both a wild-type and mutant hTERT protein express wild-type levels of telomerase activity (Fig. 2.2.9).

The final mutation set contains three novel mutations. The first R865P occurs in a highly conserved residue and was identified in a patient with aplastic anemia. As can be observed in Fig. 2.2.2, this residue resides in the catalytically active reverse transcriptase domain (RT domain) and is commonly mutated in patients with bone-marrow failure syndromes (Fig. 1.3). In keeping with the importance of this arginine residue, mutating it to a proline reduces telomerase enzymatic activity by at least 60% (Fig. 2.2.10, lanes 7-9). The second novel mutation, T903S, was also found in a patient with AA, and, as show in Fig. 2.2.10, also appears to reduce telomerase activity by approximately 60% (lanes 19-21). Such a dramatic decrease in telomerase activity resulting from a relatively conservative amino acid change suggests a particularly important role for this conserved threonine residue. The final AA patient-identified mutation creates a C-terminally truncated protein by changing an arginine residue to a premature stop codon (R889X). Interestingly, despite the fact that this mutation removes part of the RT domain and the entire C-terminus (Fig. 2.2.1), cells transfected with this form of the hTERT protein still maintain some telomerase enzymatic activity (Fig. 2.2.10, lanes 13-15). A Western blot is needed to confirm that the mutation does in fact create a truncated protein. While none of our novel variant proteins negatively impact the enzymatic activity of a co-transfected wild-type protein, the R901W mutation, identified in a patient with dyskeratosis congenital (DC), seems to show some slight dominant-negative effect (Fig. 2.2.11). Several repetitions of this assay are needed before the significance of this effect can be accurately assessed.

Discussion

Our study shows that the BMFS patient-identified *hTERC* promoter mutations do not seem to have an effect on either telomerase enzymatic activity or *hTERC* transcription, although it is possible that these sequence changes may play some role in special cellular contexts. Thus, it remains to be discovered why these patients carry abnormally shortened telomeres when these sequence changes do not appear to decrease promoter activity or telomerase enzymatic activity. In direct contrast, the two variants in the hTERC coding region cause a dramatic decrease in telomerase enzymatic activity as measured by TRAP assay. This is not surprising as both mutations involve deleting large portions of the RNA molecule which have important roles in telomerase activity and may also affect the stability of the RNA molecule itself. A Northern blot is needed to confirm which of these possibilities is correct.

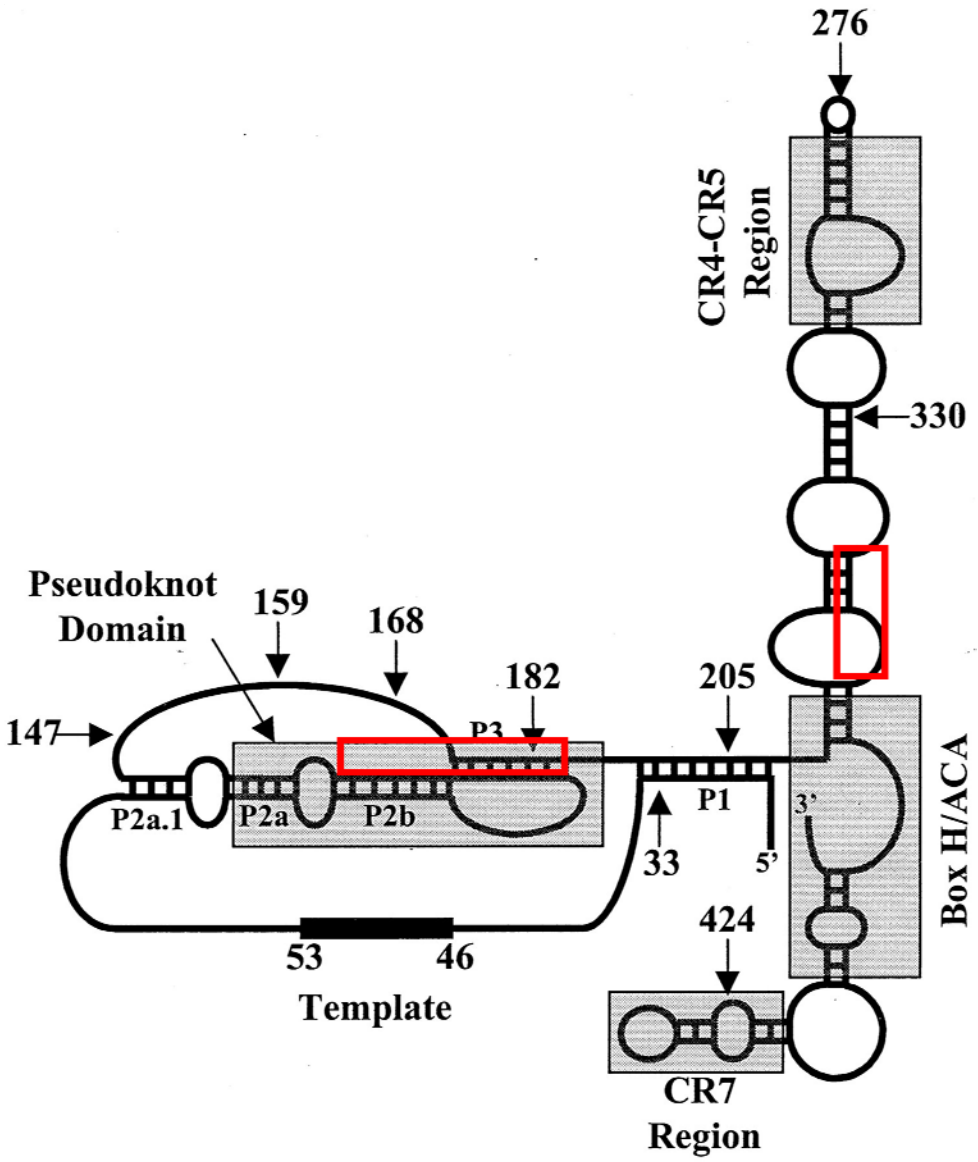
As all of the patients who carry hTERT mutations were diagnosed with shortened telomeres, we would anticipate that each mutation would have some deleterious effect on telomerase enzymatic that could explain the telomere phenotype. However, while there are some mutations that do support this hypothesis, there are others that appear to express wild-type levels of enzymatic activity, at least as tested in our assays. It is possible that these sequence changes cause defects in other areas not tested in our assay, such as telomerase enzymatic processivity and/or its recruitment to telomeres. Unfortunately, there are currently no technologies available which would allow ready assessment of these issues.

Table 2.2.1

Sequence Variant	Telomerase Activity*	Sequence Variant	Telomerase Activity*
hTERC Promoter			
-99C/G	+++		
-240C/T	+++		
-714+C	+++		
-771A/G	+++		
hTERC Coding Region			
Δ109-123	+		
Δ341-360	+		
hTERT Coding Region			
P33S	+++	R865C	++
L55Q	++	R865H	++
112ΔC	++	R865P	++
A279T	+++	H876Q	+++
V299M	+++	R889X	+
Δ441E	+++	R901W	+
R522K	++	T903S	++
Δ523S	+++	A1062T	++
V747fs	++	T1110M	+++
R811C	++	Δ3346-3352	+
Y846C	++		

***** The telomerase activity of each variant was determined in this study using reconstituted VA13 cells and is expressed in comparison to that of the wild-type. (+++, 50-100%; ++, 10-40%; +, under 10%)

Figure 2.2.1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CR4-CR5
Region
330
Pseudoknot
Domain
159
168
182
205
147
P3
P2a.1
P2a
P2b
P1
3'
33
5'
Box H/ACA
424
53
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Template
CR7
Region

Figure 2.2.2

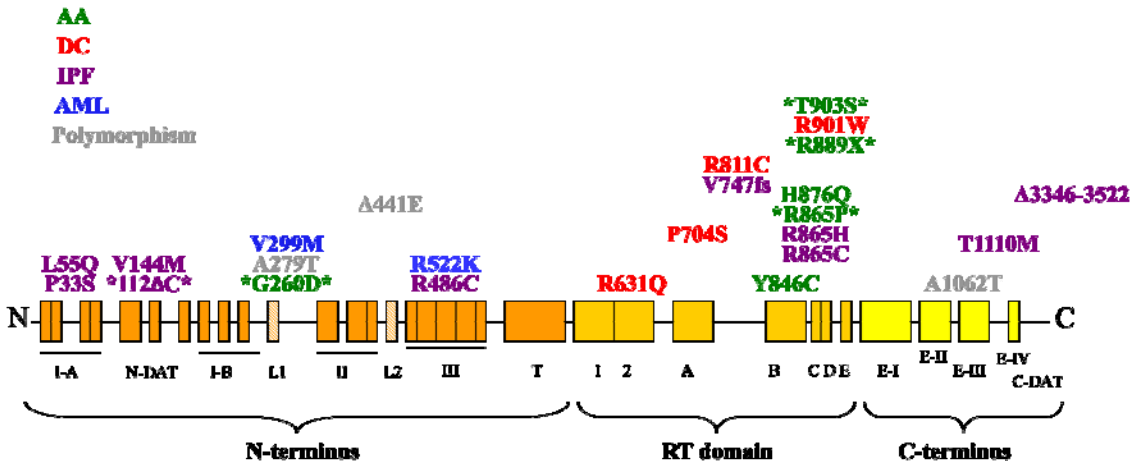

AA
DC
IPF
AML
Polymorphism
T903S
R901W
R889X
R811C
V747fs
H876Q
R865P
R865H
R865C
Δ441E
Δ3346-3522
P704S
T1110M
V299M
A279T
G260D
R522K
R486C
R631Q
Y846C
A1062T
L55Q
P33S
V144M
112ΔC
N
C
I-A
N-DAT
I-B
L1
II
L2
III
T
1
2
A
B
C D E
E-I
E-II
E-III
E-IV
C-DAT
N-terminus
RT domain
C-terminus

Figure 2.2.3

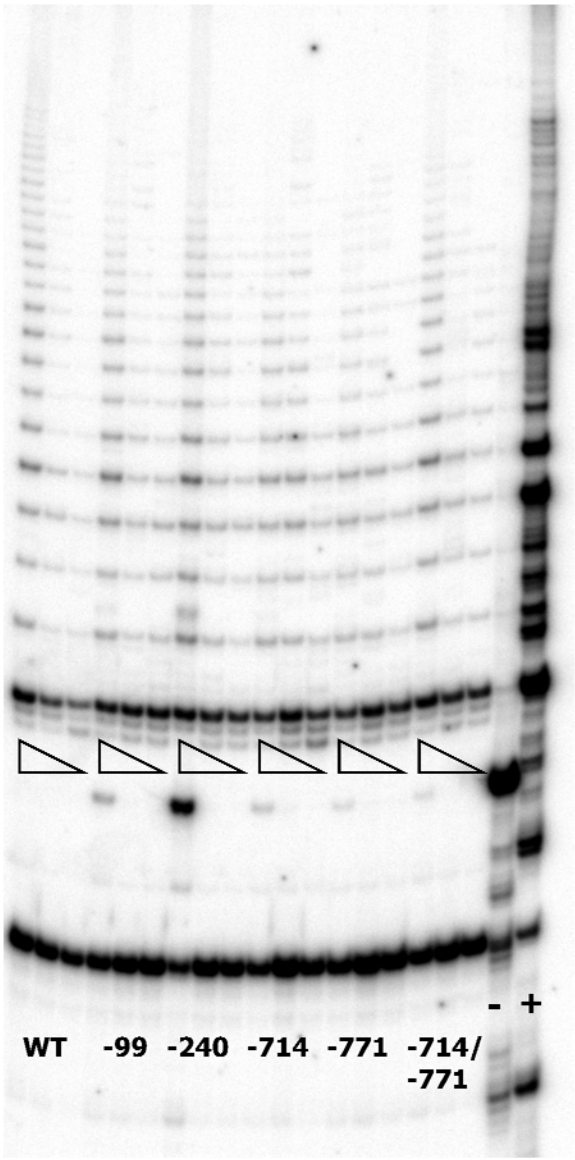


Figure 2.2.4

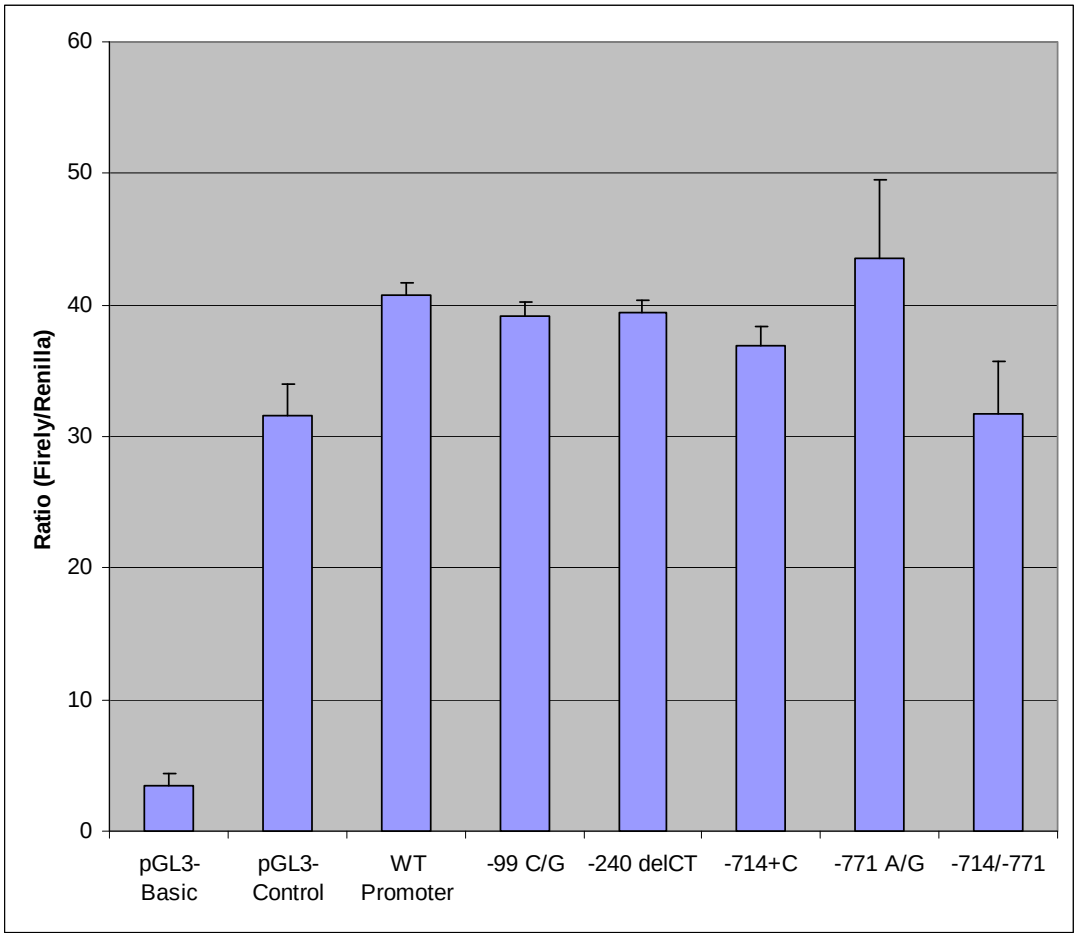

60
50
40
30
20
10
0
Ratio (Firely/Renilla)
pGL3-Basic
pGL3-Control
WT Promoter
-99 C/G
-240 delCT
-714+C
-771 A/G
-714/-771

Figure 2.2.5

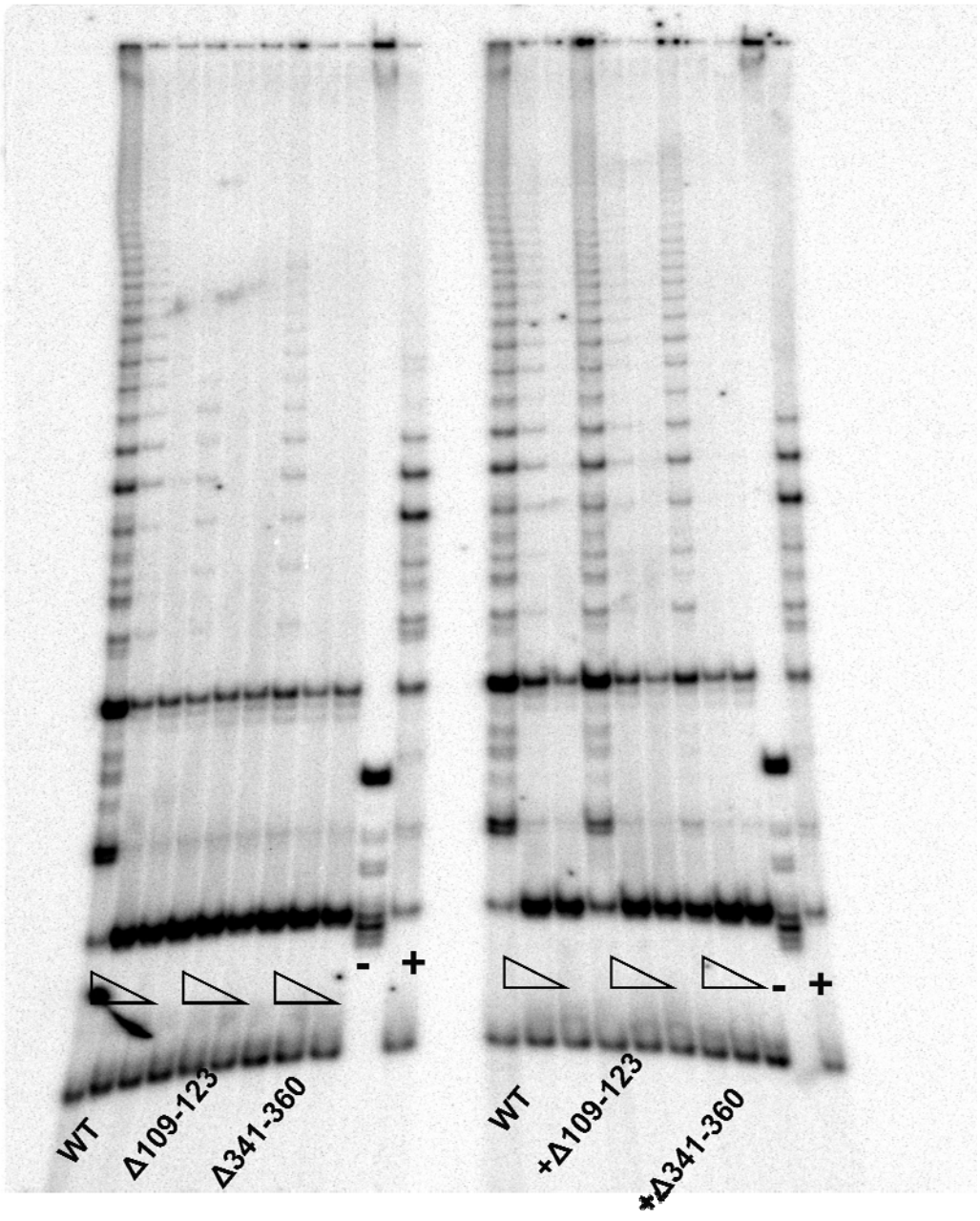
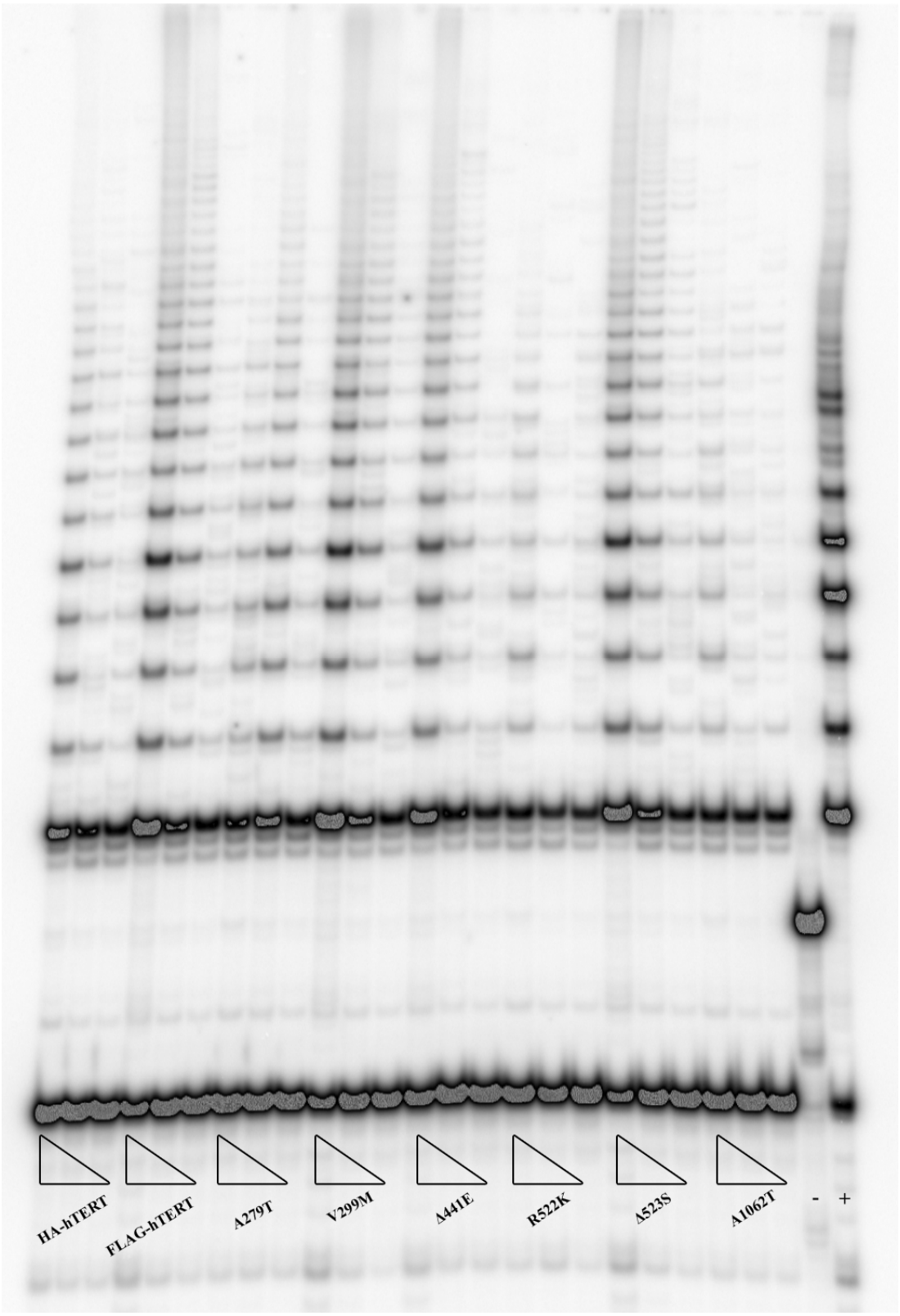


Figure 2.2.6



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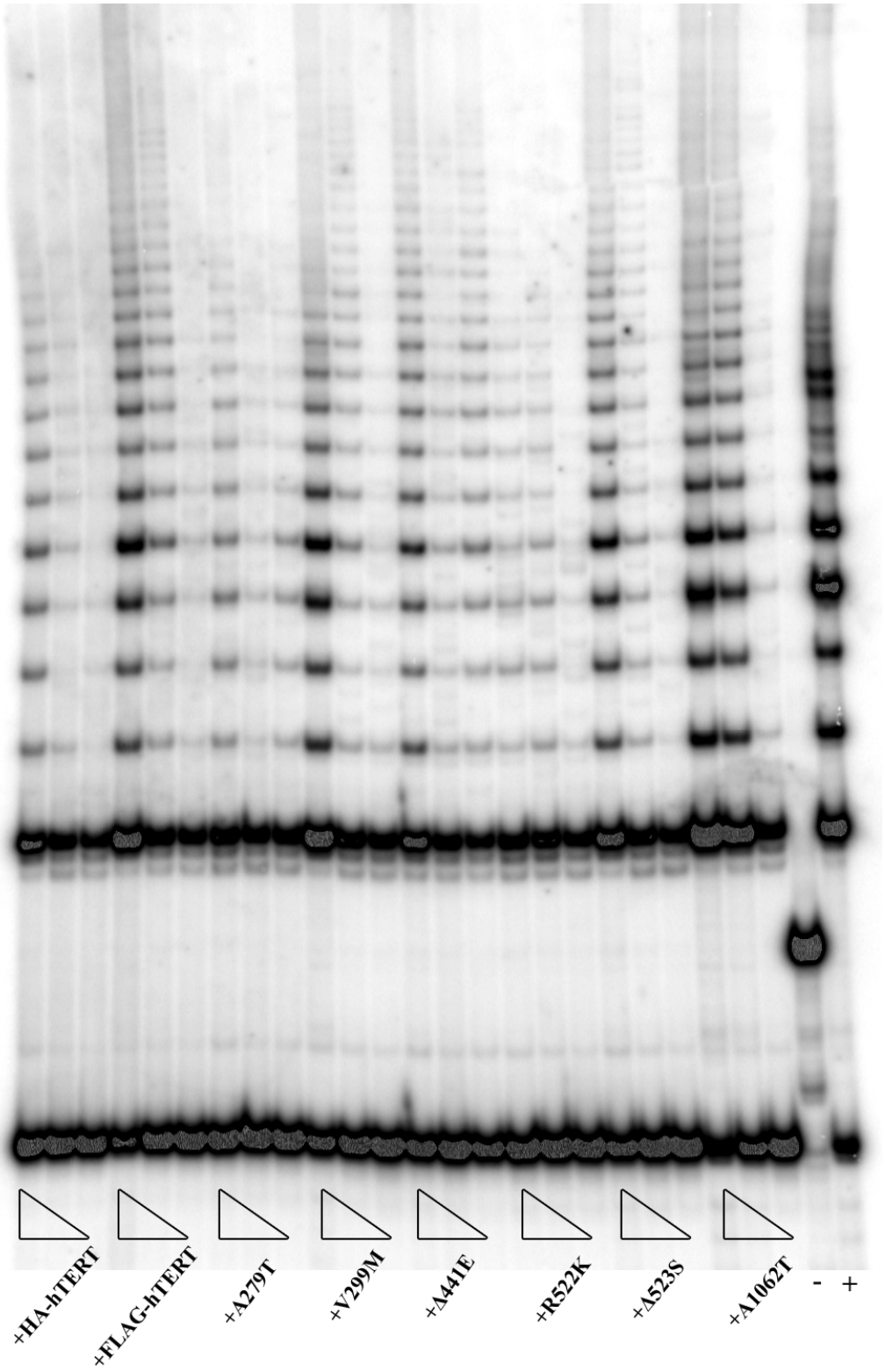

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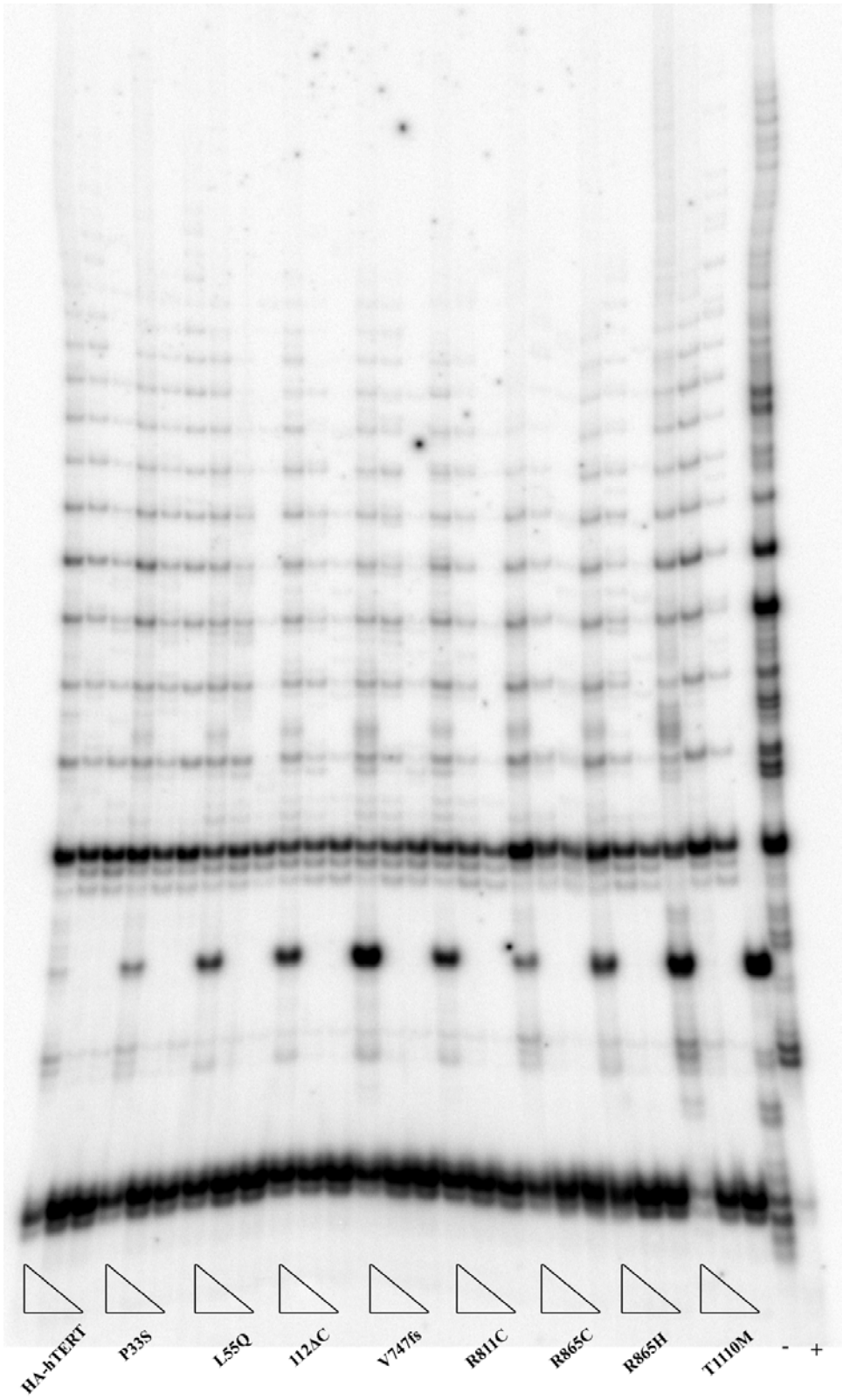

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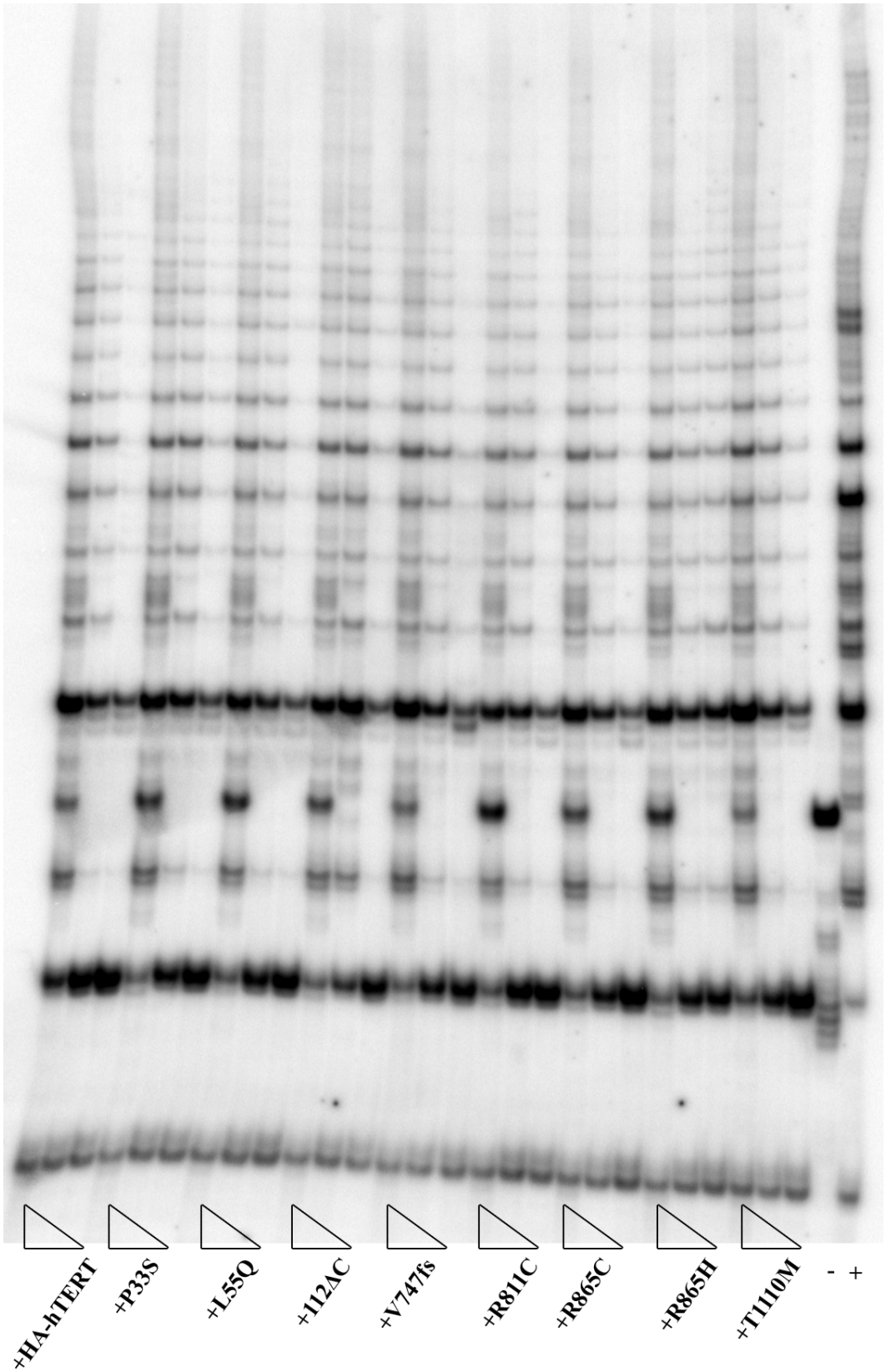

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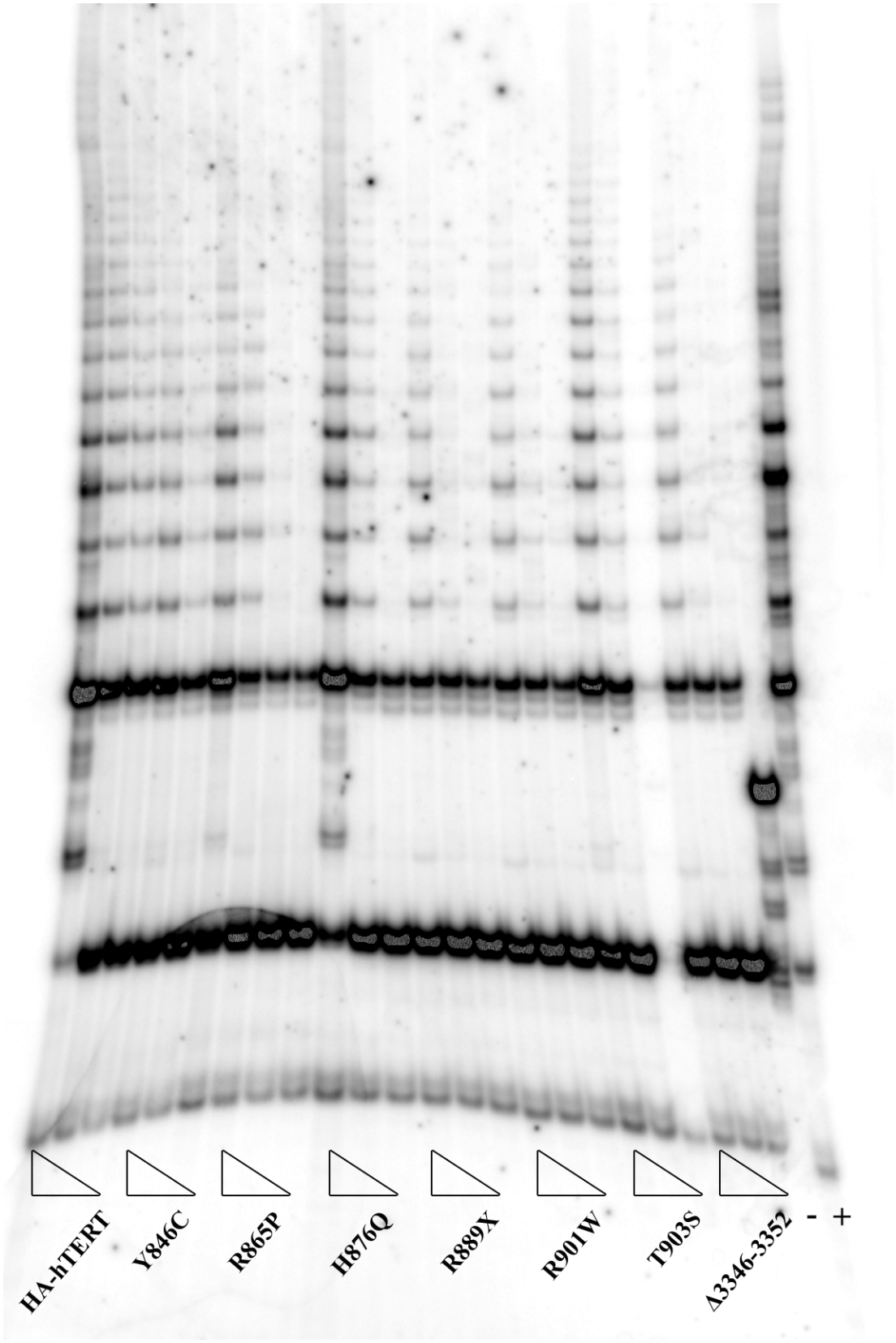

Figure 2.2.11

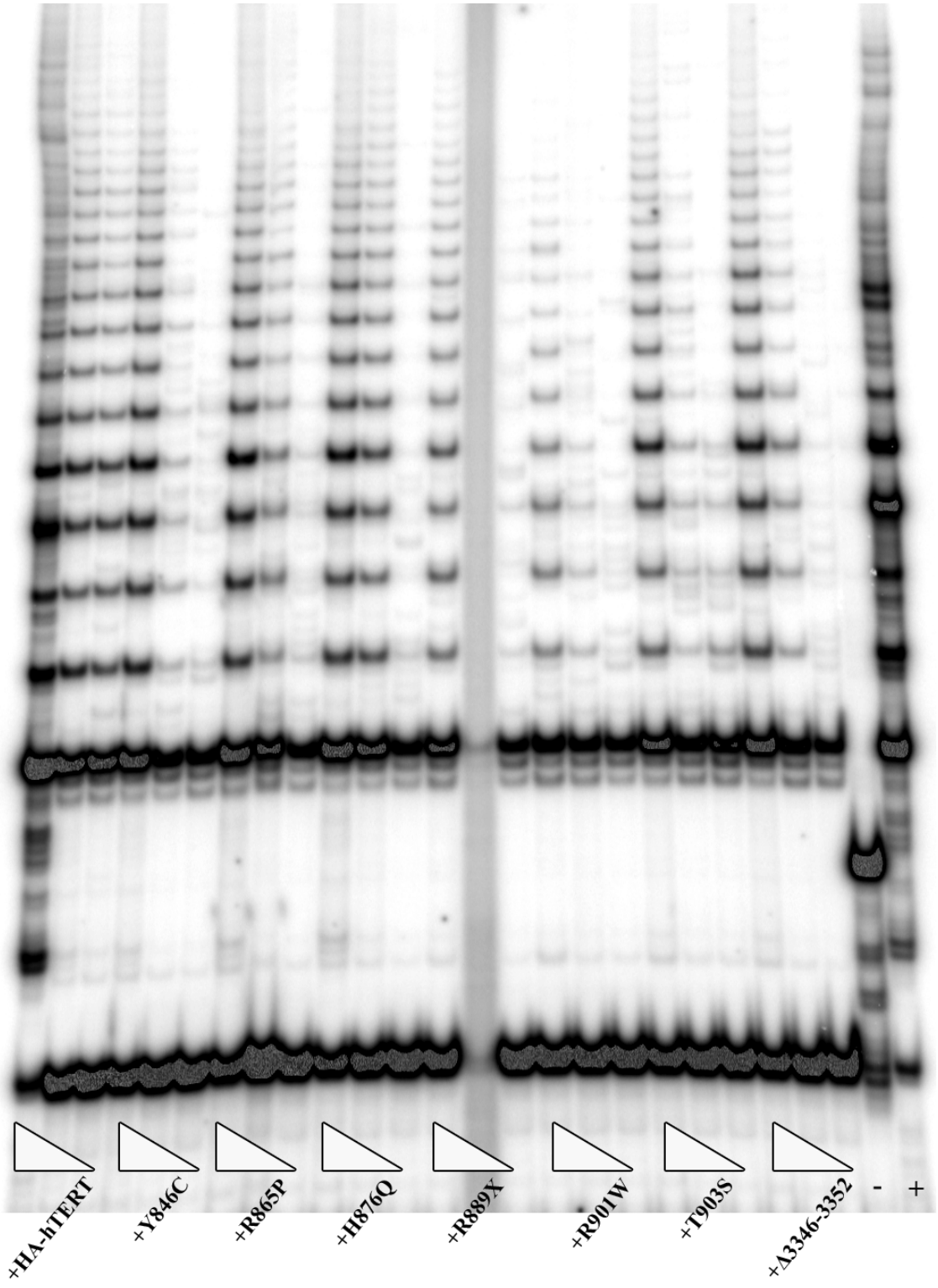

Figure Legends

Figure 2.2.1: Schematic of the hTERC RNA. Schematic depiction of the predicted secondary structure of hTERC as proposed by Chen et al. [189]. The 8-base template sequence (rectangle) and other structural features are indicated, including the pseudoknot, CR4-CR5, box H/ACA, and CR7 domains, as well as the hypervariable paired region. The two patient-identified mutations (Δ109-123 and Δ341-360) are denoted by red boxes, indicating the deleted nucleotides.

Figure 2.2.2: Schematic of the hTERT Protein. Map of the hTERT protein, showing the naturally occurring sequence changes tested in this study, color-coded by the disorder in which they were first identified. Novel variants, tested for this first time by our lab, are indicated by asterisks. The remaining amino acid substitutions have been tested by other groups using methods different from ours. V144M, G260D, R486C, R631Q, and P704S have not been tested yet.

Figure 2.2.3: Naturally Occurring hTERC Promoter Variants Show No Significant Decrease in Telomerase Enzymatic Activity. Telomerase enzymatic activities as determined in VA13+hTERT cells for the naturally occurring hTERC promoter mutations. A representative TRAP gel showing the relative telomerase enzymatic activities obtained from the promoter sequence changes is shown. Serial ten-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lane 19 shows a negative control composed of wild-type cell lysate denatured at 95°C for 5 minutes prior to assay. Lane 20 shows

positive control PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit.

Figure 2.2.4: Naturally Occurring hTERC Promoter Variants Show No Significant Decrease in hTERC Transcriptional Activity. The name of each reporter construct was assigned according to the hTERC promoter variant nucleotide upstream of the ATG initiation codon. pGL3-Basic is a promoter-less construct, included as a negative control. The positive control pGL3-Control construct consists of the firefly luciferase gene driven by the strong SV40 promoter. For each transfection, the firefly luciferase activity was normalized to the *Renilla* activity expressed from the co-transfected pRL-CMV expression vector. The means from three independent experiments are shown for each construct; *bars*, SD.

Figure 2.2.5: Naturally Occurring hTERC Variants Cause a Significant Decrease in Telomerase Enzymatic Activity. Telomerase enzymatic activities as determined in VA13+hTERT cells for the naturally occurring hTERC deletions. A representative TRAP gel showing the relative telomerase enzymatic activities obtained from the hTERC sequence changes is shown. "+" before the sequence change denotes that the indicated mutant hTERC was co-transfected with a plasmid expressing a wild-type copy of the hTERC RNA to assess any possible dominant-negative effects. Serial ten-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lanes 10 and 21 show a negative control composed of wild-type cell lysate denatured at 95°C for 5 minutes prior to assay. Lanes 11 and 22 show positive control PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit.

Figure 2.2.6: Naturally Occurring hTERT Variants Have Varying Effects On Telomerase Enzymatic Activity: Set 1. Telomerase enzymatic activities as determined in VA13+hTERC cells for the naturally occurring hTERT variants. A representative TRAP gel showing the relative telomerase enzymatic activities obtained from the sequence changes is shown. Serial ten-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lane 25 shows a negative control composed of wild-type cell lysate denatured at 95°C for 5 minutes prior to assay. Lane 26 shows positive control PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit.

Figure 2.2.7: Naturally Occurring hTERT Variants Show No Dominant-Negative Effects On Telomerase Enzymatic Activity: Set 1. Telomerase enzymatic activities as determined in VA13+hTERC cells for the naturally occurring hTERT variants. A representative TRAP gel showing the relative telomerase enzymatic activities obtained from the sequence changes is shown. "+" before the sequence change denotes that the indicated mutant hTERC was co-transfected with a plasmid expressing a wild-type copy of the hTERC RNA to assess any possible dominant-negative effects. Serial ten-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lane 25 shows a negative control composed of wild-type cell lysate denatured at 95°C for 5 minutes prior to assay. Lane 26 shows positive control PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit.

Figure 2.2.8: Naturally Occurring hTERT Variants Have Varying Effects On Telomerase Enzymatic Activity: Set 2. Telomerase enzymatic activities as determined

in VA13+hTERC cells for the naturally occurring hTERT variants. A representative TRAP gel showing the relative telomerase enzymatic activities obtained from the sequence changes is shown. Serial ten-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lane 28 shows a negative control composed of wild-type cell lysate denatured at 95°C for 5 minutes prior to assay. Lane 29 shows positive control PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit.

Figure 2.2.9: Naturally Occurring hTERT Variants Show No Dominant-Negative Effects On Telomerase Enzymatic Activity: Set 2. Telomerase enzymatic activities as determined in VA13+hTERC cells for the naturally occurring hTERT variants. A representative TRAP gel showing the relative telomerase enzymatic activities obtained from the sequence changes is shown. "+" before the sequence change denotes that the indicated mutant hTERC was co-transfected with a plasmid expressing a wild-type copy of the hTERC RNA to assess any possible dominant-negative effects. Serial ten-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lane 28 shows a negative control composed of wild-type cell lysate denatured at 95°C for 5 minutes prior to assay. Lane 29 shows positive control PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit.

Figure 2.2.10: Naturally Occurring hTERT Variants Have Varying Effects On Telomerase Enzymatic Activity: Set 3. Telomerase enzymatic activities as determined in VA13+hTERC cells for the naturally occurring hTERT variants. A representative TRAP gel showing the relative telomerase enzymatic activities obtained from the

sequence changes is shown. Serial ten-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lane 28 shows a negative control composed of wild-type cell lysate denatured at 95°C for 5 minutes prior to assay. Lane 29 shows positive control PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit.

Figure 2.2.11: Naturally Occurring hTERT Variants Show No Dominant-Negative Effects On Telomerase Enzymatic Activity: Set 3. Telomerase enzymatic activities as determined in VA13+hTERC cells for the naturally occurring hTERT variants. A representative TRAP gel showing the relative telomerase enzymatic activities obtained from the sequence changes is shown. "+" before the sequence change denotes that the indicated mutant hTERC was co-transfected with a plasmid expressing a wild-type copy of the hTERC RNA to assess any possible dominant-negative effects. Serial ten-fold dilutions of the transfected cell lysates (indicated by triangles) were assayed for each sample to ensure linearity of the assay. Lane 34 shows a negative control composed of wild-type cell lysate denatured at 95°C for 5 minutes prior to assay. Lane 35 shows positive control PCR products amplified from the non-hTERC control TSR8 DNA template supplied in the TRAP kit.

CHAPTER 3:

Transcriptional Activation of *TINF2*, a Gene Encoding the Telomere-Associated Protein TIN2, by Sp1 and NF-κB Factors

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Running Title: *TINF2* transcriptional regulation

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Abstract

The expression of the telomere-associated protein TIN2 has been shown to be essential for early embryonic development in mice and for development of a variety of human malignancies. Recently, germ-line mutations in *TINF2*, which encodes for the TIN2 protein, have been identified in a number of patients with bone-marrow failure syndromes. Yet, the molecular mechanisms that regulate *TINF2* expression are largely unknown. To elucidate the mechanisms involved in human *TINF2* regulation, we cloned a 2.7 kb genomic DNA fragment containing the putative promoter region and, through deletion analysis, identified a 406 bp region that functions as a minimal promoter. This promoter proximal region is predicted to contain several putative Sp1 and NF-κB binding sites based on bioinformatic analysis. Direct binding of the Sp1 and NF-κB transcription factors to the TIN2 promoter sequence was demonstrated by electrophoretic mobility shift assay (EMSA) and/or chromatin immunoprecipitation (ChIP) assays. Transfection of a plasmid carrying the Sp1 transcription factor into Sp-deficient SL2 cells strongly activated TIN2 promoter-driven luciferase reporter expression. Similarly, the NF-κB molecules p50 and p65 were found to strongly activate luciferase expression in NF-κB knockout MEFs. Mutating the predicted transcription factor binding sites effectively reduced TIN2 promoter activity. Various known chemical inhibitors of Sp1 and NF-κB could also strongly inhibit TIN2 transcriptional activity. Collectively, our results demonstrate the important roles that Sp1 and NF-κB play in regulating the expression of the human telomere-binding protein TIN2, which can shed important light on its possible role in causing various forms of human diseases and cancers.

Introduction

Telomeres are complex nucleoprotein structures at chromosome ends that function to prevent chromosome fusions and genomic instability (reviewed in [198]). Mammalian telomeres consist of repetitive (T_2AG_3)n DNA sequence and associated proteins that are collectively known as the shelterin complex. The shelterin complex consists of at least six proteins TRF1, TRF2, Rap1, TIN2, POT1, and TPP1 that are required for telomere protection and length control (reviewed in [10]). The first of these proteins, the telomere-repeat binding factor 1 (TRF1), was isolated based on its ability to bind double-stranded TTAGGG repeats [199,200], followed soon after by the identification of its paralog TRF2 [201,202]. TRF1-interacting nuclear protein 2 (TIN2) and Rap1 were found through yeast two-hybrid screens for proteins that could interact with TRF1 and TRF2, respectively [40,203]. Finally, a search for TIN2-interacting proteins yielded TPP1 [204], and POT1 was pulled out based on sequence homology to similar telomere-protecting proteins in unicellular eukaryotes [43].

TIN2 is an important component of the shelterin complex as it binds directly to the double-stranded telomeric DNA binding proteins TRF1 and TRF2 and indirectly interacts with the single-strand telomeric DNA binding protein POT1 via the intermediary protein TPP1 [203,204]. Overexpression of TIN2 can shorten telomere length in telomerase-positive human cells, similar to the effect of overexpressing the TRF1 protein, implicating both proteins as a negative regulators of telomere length [203]. In contrast, TIN2 depletion via shRNA disrupts TRF1 and TRF2 binding and causes cell death, even in the absence of p53 function [205,206]. While TIN2 remains at telomeres in growth-arrested cells, it appears to form large complexes outside the telomeres,

implying that TIN2 may play other important roles in mammary epithelial differentiation [207], a hypothesis supported by the identification of a novel isoform of TIN2 which can localize to the nuclear matrix [208]. Furthermore, knock-out of TIN2 in a mouse model results in early embryonic lethality prior to embryonic day 7.5 in a telomerase-independent manner [171]. Such important roles of TIN2 have prompted several laboratories to screen patients with degenerative bone-marrow failure syndromes that are known to be associated with telomere dysfunction for natural mutations in this gene. These efforts have led to the identification of several natural sequence variations in the *TINF2* gene [124,125,126,127]. However, the specific mechanisms through which these mutations may act to affect disease pathology remain unknown.

In addition to the experimental alterations in TIN2 protein levels, which clearly demonstrate that TIN2 level changes can disrupt telomere end structure and result in cell distress and/or death, several studies have shown that the changes in the endogenous expression level of several telomere-binding proteins (including TIN2) may be associated with various forms of human cancer [209,210,211,212,213]. As over 90% of cancers have also been shown to upregulate the catalytic component of the telomere-elongating enzyme telomerase, more careful studies of the transcriptional regulation of the telomere-binding proteins that have been directly implicated in telomere maintenance are warranted. Furthermore, it has recently been shown that the telomere-binding protein hRap1 and the transcription factor NF-κB positively regulate each other through a feed-forward loop [214]. To this end, we have for the first time characterized both the *cis*-elements and *trans*-acting factors that regulate the transcription of the human *TINF2* gene, which encodes for the TIN2 protein. This comprehensive examination of the

transcriptional regulation of the *TINF2* gene will shed important light on the role(s) this gene plays in the pathogenesis of human hematological diseases and cancer.

Materials and Methods

Cloning of the TINF2 functional promoter region

Using the available genomic sequence of the *TINF2* gene in the GenBank database (NT 026437), we designed an appropriate primer set (sequences available upon request) to amplify a 2.7 kb DNA fragment corresponding to a region immediately upstream of the known initiation codon of the gene by polymerase chain reaction (PCR). The PCR reaction was performed using the following reaction conditions: 200 ng of genomic DNA isolated from HeLa cells using the DNeasy Tissue Kit (Qiagen), 1U of Phusion Hot-Start DNA Polymerase (FINNZYMES, 2 U/µl), 1×Phusion GC buffer, 0.5µM primers, 200 µM dNTPs, and 3% v/v DMSO per 50µl reaction volume. The PCR cycling program was the following: 98 °C for 30sec; 35 cycles of 98 °C for 10sec, 72°C for 90sec; 72 °C for 10min.

Generation of luciferase reporter constructs

The 2.7 kb PCR product was digested with HindIII and NcoI (New England Biolabs, NEB), gel-purified (Qiagen), and cloned into the same restriction enzyme sites of the pGL3-Basic vector (Promega) to allow transcription of the firefly luciferase reporter gene under control of this DNA fragment. To generate a series of deletion constructs (Fig. 3.1A), the plasmid P2731 that contained the 2.7 kb TINF2 promoter sequence was digested with HindIII and either AflII, PvuII, AleI, BIpI, or AvrII, end-

polished by the Mung bean nuclease and then self-ligated with T4 DNA Ligase to generate the plasmid constructs P2201, P1668, P553, P450, P351, P248, P148, and P74. All enzymes were purchased from NEB. All other truncation plasmids (Fig. 3.1B and 3.1C) were obtained by PCR mutagenesis using the QuickChange method (Stratagene) and appropriate primer sets (sequences available upon request). All plasmid DNAs were confirmed to have the intended sequences by direct sequencing, and their quantity and quality routinely checked by spectrophotometric analysis and agarose gel electrophoresis.

Luciferase assays in mammalian cells

293T cells were seeded at a density of 4×10^5 cells per well (or HEK293 cells at a density of $2x10^5$ cells per well) in 12-well plates 24 hours prior to transfection. The cells were transfected with 700 ng of the *TINF2* promoter-driven luciferase plasmid(s) and 100 ng of either the pRSV-β-galactosidase plasmid or the pRL-CMV plasmid as an internal control of transfection efficiency, using Lipofectamine 2000 transfection reagent (Invitrogen) for 293T cells and SuperFect (Qiagen) for HEK293 cells according to the manufacturer's instructions. Cells were harvested 24 hours after transfection and lysed in 200µl reporter lysis buffer (Promega). Luciferase activity was measured using the Luciferase Assay System (Promega), and β-galactosidase activity was determined by Beta-Glo Assay System (Promega). Luciferase activity (arbitrary units) was divided by the internal control in the same sample to normalize for transfection efficiency and expressed as relative luciferase activity. Transfection data represents at least three independent experiments, each performed in triplicate. The wild-type P450 plasmid

construct was also transfected into the Jurkat and K562 human leukemic cells lines using similar conditions as outlined above for 293T cells.

NIH 3T3 p65/p50 double-knockout cells (a kind gift of Dr. David Baltimore at the California Institute of Technology) were maintained in Dulbecco's Modified Minimal Essential Medium (DMEM) with 10% fetal bovine serum under 5% CO_2 at 37°C. These cells were seeded at a density of 5×10^4 cells per well in 12-well plates 24 hours prior to being transfected with 500ng of the appropriate *TINF2* promoter-driven luciferase plasmids, 500 ng of either pCMV4-p50 or pCMV4-p65, or 500 ng of pCMV4 empty vector using SuperFect transfection reagent (Qiagen). Luciferase activities were normalized to the total amount of cellular protein as determined by the Bio-Rad Protein Assay.

Luciferase assay in Drosophila melanogaster SL2 cells

Drosophila melanogaster Schneider SL2 cells, known to lack expression of the Sp1/Sp3 transcription factors, were cultured in HyClone SFX-Insect serum-free medium (HyClone). On the day of transfection, cells were collected, washed once with 1X PBS, and seeded at a density of 1×10^6 cells/ml in 6-well plates. Cells were then transfected using SuperFect transfection reagent (Qiagen) with 1μg of the appropriate *TINF2* promoter-driven luciferase reporter plasmid(s) along with varying amounts of pPac-Sp1 plasmid (a kind gift of Dr. Robert Tjian, University of California, Berkeley) or pPac-Sp3 plasmid (a generous gift of Dr. G. Suske, University of Marburg, Germany), and the total amount of DNA brought up to 2μg per well with the empty pPac plasmid. Forty-eight hours after transfection, cells were harvested in Reporter Lysis Buffer (Promega) and

assayed for luciferase activity as suggested by the manufacturer (Promega). Luciferase activities were normalized to the total level of cellular protein as measured by the Bio-Rad Protein Assay.

Electrophoretic mobility shift assay (EMSA)

Human Sp1 and AP2 proteins were *in vitro* synthesized by the TNT T7 Quick Coupled Transcription/Translation System (Promega) using pcDNA3.1-Sp1 and pcDNA3.1-AP2 (kind gifts of Dr. Ceshi Chen at Emory University) as templates, respectively. Double-stranded DNA oligonucleotides representing the transcriptional binding sites were prepared by denaturing complementary single-stranded oligonucleotides (synthesized by Invitrogen) at 90°C for 10 min and then cooling to room temperature gradually before end-labeling with [γ-^{32}P]dATP by T4 polynucleotide kinase (NEB). Oligo sequences are as follows:

412-382 WT: CTACAGCTCCGCTGGGGCGTGGCCTTCTGACG

412-382 Mut: CTACAGCTCCGCTGGAACGTGGCCTTCTGACG

95-59 WT: GTTGCCAGAAGCCCCGCCCCTAGGAGTGATCGGAAAG

95-59 Mut: GTTGCCAGAAGCCCCGTTCCTAGGAGTGATCGGAAAG.

In vitro translated protein was incubated in 10 mM Tris-HCl (pH 7.5), 4% glycerol, 1 mM $MgCl_2$, 25 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA, 50 mM NaCl, and 0.5 mg/ml poly(dI-dC) in a final volume of 20 µl for 10 min at room temperature. The incubation was continued for an additional 30 min at room temperature after the addition of 50,000 cpm ^{32}P -labeled probes. For competition experiments, 100-fold molar excess of unlabeled DNA oligonucleotides were added to the binding reaction

mixture 10 minutes prior to the addition of the labeled probes. For super-shift experiments, 2 μg of antibody against Sp1 (Santa Cruz Biotech) were incubated with the binding reaction mixture on ice for 1 hour before the labeled probe was added. The DNA–protein complexes were separated by electrophoresis on a 4% native polyacrylamide gel in 0.5X TBE at 200 V for 2 hours at 4°C, vacuum-dried, and then autoradiographed.

Chromatin immunoprecipitation

293T cells in 100 mm cell culture plates (cell number at ~2.5×10^7) were cross-linked for 10 min by adding formaldehyde directly into tissue culture medium (final concentration of 1%) at room temperature with mild shaking. The reaction was stopped by adding glycine (125 mM), and the cells were kept at room temperature for 5 minutes. The cross-linked cells were then washed twice with cold PBS, scraped, pelleted, and resuspended in 600 μl of Nuclei Lysis Buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS) with protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin) (Sigma). The lysates were then sonicated for four cycles of 10 sec each, resting on ice for 2 min between cycles, on a Branson Sonifier 450 (settings: duty cycle = 50%; output control = 3), resulting in chromatin fragmentation to an average length of 500-1000 bps. After sonication, the samples were then centrifuged at 16,000 *g* for 10 minutes. Sheared chromatin was diluted 20-fold in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl) with protease inhibitors as described above. Then, 4 μg of Rabbit anti-Sp1 antibody (Santa Cruz Biotech, PEP 2), Rabbit anti-p65 antibody (Santa Cruz Biotech, C-20),

control antibody (Rabbit anti-c-Myc, Santa Cruz Biotech, A14), or no antibody was added to each aliquot of chromatin extract and the reaction mixture incubated overnight at 4°C on a rotary shaker. Complexes were captured by incubation with protein A/G agarose beads (Santa Cruz Biotech) blocked with 250 µg/ml of sheared salmon sperm DNA (Ambion) and 1 mg/ml BSA (NEB) in ChIP dilution buffer at 4°C for 4 hours. Captured complexes were washed successively with ChIP dilution buffer, high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl wash buffer (0.5 M LiCl, 1% NP40, 1% deoxycholate, 100 mM Tris-HCl, pH 8.1), and TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Complexes were washed twice in each buffer for 10 min apiece with shaking and then centrifuged to collect protein A/G agarose beads. After the final wash, 250 µl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) was added and incubated at room temperature for 15 minutes with rotation. Then, 5 M NaCl was added to reverse the formaldehyde cross-linking by heating at 65°C for 4 hours. After precipitation with ethanol, the pellets were resuspended and treated with proteinase K (NEB). DNA was recovered by standard phenol–chloroform extraction and ethanol precipitation. Pellets were resuspended in TE buffer and subjected to polymerase chain reaction (PCR) amplification using the following primers to amplify the TIN2 fragment: Forward (5'-CTTCTGACGCACCGTCACGG-3') and Reverse (5'-CACCAGGGGCGTAGCCATGG-3'). The PCR products were separated by agarose gel electrophoresis.

Generation of luciferase reporter plasmids carrying point mutations

Transcriptional binding site mutant plasmids (Fig. 3.4A and 3.4B) were generated in the minimal promoter pGL3-P406 backbone by PCR mutagenesis using the QuickChange method (Stratagene) and appropriate primer sets (sequences available upon request). Sequences of the putative binding site mutants are as follows:

P406 (397-396) WT: GCTGGGGCGTGG;

P406 (397-396) Mut: GCTGGAACGTGG;

P406 (79-78) WT: CCCCGCCCCTAG;

P406 (79-78) Mut: CCCCGTTCCTAG;

P406 (99-96) WT: CGACAGGGAGTTGC;

P406 (99-96) Mut: CGACAAAATGTTGC;

P406 (64-60) WT: TGATCGGAAAGCCTC;

P406 (64-60) Mut: TGATCAACCCGCCTC.

Numbers in parentheses denote mutated nucleotides, which are underlined. All mutated DNAs were confirmed by direct sequencing, and their quantity and quality routinely checked by spectrophotometric analysis and agarose gel electrophoresis.

Pharmacological inhibitors

293T or HEK293 cells were seeded at a density of $2x10^5$ cells per well in 12 well plates 24 hours prior to transfection. Bay11-7082 (Sigma) at 100 nM or 10 μM concentration, PDTC (Sigma) at 500 nM or 5 μM, or Mithramycin A (Sigma) at 1 nM or 100 nM was added directly to the media 1 hour prior to being transfected with 1 μg of either the minimal promoter construct (pGL3-P406), pGL3-Basic, pNFAT-Luc, or pGL3-

Control along with 70 ng of pRL-CMV using Lipofectamine 2000 (Invitrogen) for the 293T cells and SuperFect (Qiagen) for the HEK293 cells according to the manufacturers' instructions. Media was changed 4 hours post-transfection to a fresh aliquot of the media that contain the same amounts of the chemicals as shown above. 24 hours later, cell lysates were prepared for luciferase activity measurements using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase reporter readings were normalized to the co-transfected *Renilla* luciferase values and expressed as relative luciferase activity. All reactions were done in triplicate.

Statistical analysis

All statistical analyses were performed using a two-tailed Student's t test. *p<0.0.5, **p<0.01, ***p<0.001

Results

Cloning and mapping the TINF2 promoter

To study promoter activation, the region located immediately upstream of the initiation codon of the human *TINF2* gene was cloned from genomic DNA of HeLa cells by polymerase chain reaction into the pGL3-Basic luciferase reporter vector. The sequence was confirmed by DNA sequencing, which was found to completely match the corresponding TIN2 promoter proximal sequence on chromosome 14 genomic contig (NT 026437) reported by The Human Genome Project. A comparison of luciferase activity in extracts prepared from 293T cells transfected with the P2731 plasmid that contains the luciferase reporter gene under the control of the longest sequence (~2.7 kb)

of the *TINF2* promoter proximal region with those transfected with the pGL3-Control vector that contains the viral SV40 promoter-driven luciferase gene showed that the *TINF2* promoter functioned as a reasonably strong promoter (Fig. 3.1A).

To better define the *cis*-acting sequences responsible for the transcriptional activation of *TINF2*, a series of successive 5' deletions of *TINF2* promoter sequence were generated based on the available unique restriction enzyme sites on the cloned P2731 promoter sequence (Fig. 3.1A). These were again assayed for their ability to produce luciferase reporter activity in a series of transient transfection reactions of the individual plasmids into 293T cells. All constructs that contain large regions of the *TINF2* promoter sequence (i.e. P2201, P1668, P553, and P450) produced levels of luciferase activity that are comparable to those generated by the P2731 construct (Fig. 3.1A). In contrast, a significant drop in luciferase activity (~3 fold, $p<0.001$) was observed with constructs containing *TINF2* promoter sequence of less than 351 base pairs (i.e. P351, P248, and P148). Further deletion of the *TINF2* promoter region (P74) resulted in a second significant drop in reporter activity to the basal levels of luciferase activity generated by the promoter-less pGL3-Basic construct (Fig. 3.1A). Collectively, these results indicate that sequences responsible for *TINF2* promoter regulation are contained within a core region encompassing 450 bps upstream of the translational initiation site of the *TINF2* gene.

Further successive deletion analysis of the P450 proximal promoter construct generated two additional constructs (P389 and P370) that produced similar levels of luciferase activity to P351 (Fig. 3.1B), strongly suggesting that elements responsible for activating the *TINF2* gene are primarily situated between positions -406 and -389, thus

designating a minimal promoter of 406 base pairs. We also created a series of 10 deletion constructs spanning the sequence from position -148 to -74 (Fig. 3.1C). When these deletion constructs were transfected into 293T cells, robust luciferase expression was observed with P148, as well as with all constructs that contain longer than 88 bps of the *TINF2* promoter sequence (Fig. 3.1C, P148, P133, P118, P103, and P88). In contrast, all constructs with less than 74 bps of promoter proximal sequence (P74, P64, P54, P44, P34 and p24) showed levels of the luciferase activity that were comparable to that of the negative control vector pGL3-Basic (Fig. 3.1C, Vector). These observations suggest that there is another important *cis*-acting element located between positions -88 and -74. Taken together, these promoter mapping experiments revealed two major regions within the *TINF2* promoter proximal region located between positions -(406-389) and positions -(88-74) that contain essential transcriptional activating elements of the *TINF2* gene in 293T cells.

Prediction of putative regulatory elements within the TINF2 promoter and validation of the cloned TINF2 promoter sequences in different cell lines

Sequence analysis revealed that the *TINF2* promoter lacks the conventional TATA and CAAT boxes as predicted for many of the GC-rich promoters, such as that of the *TINF2* gene. When various bioinformatics methods (TESS, Genomatix, and Gene Regulation search programs) were used, they all predicted a number of potential transcription factor binding sites on the core P450 *TINF2* promoter sequence, including potential binding sites for Sp1, AP-2, and NF-κB (data not shown). The abundance of these sites suggests the possibility that *TINF2* expression may be subject to multiple

levels of control and be regulated by different factors in different cellular contexts. As such, we validated the functionality of the core *TINF2* promoter by transfecting the P450 construct into various cancer cell lines, including human leukemia K562 cells and Jurkat cells (Fig. 3.1D). As shown in Fig. 3.1E, a few of the putative transcription factor binding sites fall near the two regions where we have observed significant drops in luciferase reporter activity (Fig. 3.1B, 3.1C), allowing us to focus on these specific sequence elements.

Binding of Sp1 to TINF2 promoter in vitro

To determine whether Sp1 can bind to its putative binding sites in the *TINF2* promoter, an electrophoretic mobility shift assay (EMSA) was performed using each putative site as a DNA probe and the recombinant Sp1 protein that could be abundantly and correctly translated in the rabbit reticulocyte lysate (data not shown). A specifically strong Sp1-shifted DNA band was observed with a DNA oligo containing the putative Sp1-binding site spanning the GC-rich motif at positions -(88-74) (Fig. 3.2A, lane 9). This Sp1-shifted band could be super-shifted by the addition of an antibody against the Sp1 protein (Fig. 3.2A, lane 12). Using a second DNA oligo representing another potential Sp1-binding site at positions -(406-389) and a similar amount of the *in vitro* translated Sp1 protein, a weak but consistent Sp1-shifted band was observed (Fig. 3.2A, lane 3) that could also be super-shifted by the addition of the anti-Sp1 antibody (Fig. 3.2A, lane 6), demonstrating that Sp1 can bind with relatively high affinity to both of these sequences *in vitro*.

The specificity of the interactions was demonstrated by the disappearance of the shifted bands when 100-fold molar excess of unlabeled wild-type probe was added to the reactions as competitors (Fig. 3.2A, lanes 4 and 10). In contrast, no competition was observed when using unlabeled probes that contain 2-bp substitutions of conserved core nucleotides mutations in these putative Sp1-binding sequences (Fig. 3.2A, lanes 5 and 11). Further Sp1-binding specificity was demonstrated by the failure to generate a shifted complex with any of these DNA oligos with the *in vitro* translated AP-2 protein or with the radiolabeled DNA probes containing 2-bp substitution mutations in these two motifs using similar gel shift conditions (data not shown). A strong band located between the unbound oligos and the Sp1-shifted oligos (i.e. the RRL binding band) was observed in most of the reactions, even in a reaction when the pcDNA3.1 control vector (Fig. 3.2A, C-Control) was used in the rabbit reticulocyte lysates (RRL), suggesting that some endogenous transcription factors in the RRL can also bind to the radiolabeled DNA probes. Collectively, these data show that Sp1 can indeed specifically bind *in vitro* to two DNA elements located within positions -(406-389) and -(88-74) of the *TINF2* promoter proximal region.

Binding of endogenous Sp1 proteins to the native TINF2 promoter in cells

Chromatin immunoprecipitation (ChIP) was next used to determine whether endogenous Sp1 protein localizes to the native *TINF2* promoter. Sheared DNA from 293T cells was immunoprecipitated using antibodies specific to the large subunit of Sp1, c-Myc, or control IgG. *TINF2* proximal promoter DNA was detected by PCR using primers that amplify a 407 bp product. Figure 3.2B shows that anti-Sp1 antibody could

effectively and specifically precipitate proteins bound to the *TINF2* promoter proximal region encompassing the minimal 406 base pair promoter, which contains the two putative Sp1-binding sites (Fig. 3.2B, lane 7), whereas anti-c-Myc antibody and non-specific IgG antibody failed to precipitate any protein-DNA complexes (Fig. 3.2B, lanes 4 and 5). These findings clearly demonstrate that Sp1 indeed can bind to the *TINF2* promoter proximal region in 293T cells.

Sp1 is a transcriptional activator of the TINF2 promoter

Sp1 and the related Sp3 are major factors of the Sp family of transcription factors which can serve redundant roles in cells. They are expressed in most mammalian cells except in the *Drosophila melanogaster* SL2 cells, which allows for a convenient means to determine whether Sp1 or Sp3 specifically activates the *TINF2* promoter in cell culture. To do this, we co-transfected SL2 cells with the minimal *TINF2* promoter-driven luciferase reporter construct P406 and either the *Drosophila* expression vector pPac-Sp1 or pPac-Sp3. As shown in Figure 3.2C, upon addition of the Sp1 transcription factor, we observed a very strong and dose-dependent induction of luciferase reporter expression. In contrast, while Sp3 appeared to also able to active *TINF2* promoter-driven luciferase activity, the effect was much weaker as compared to that obtained with the Sp1 transcription factor. These data strongly argue that Sp1, rather than Sp3, is the major transcriptional activator of *TINF2*.

NF-κB binds to and transactivates the TINF2 promoter

Various bioinformatic methods predicted two potential NF-κB binding sites residing near position -(82-78) (Fig. 3.1E). A chromatin immunoprecipitation (ChIP) assay was carried out using an antibody to the major p65 subunit of the NF-κB complex and lysate prepared from the 293T cells as described in the Materials and Methods section. We observed binding of endogenous NF-κB to the native *TINF2* promoter proximal region *in vivo* (Fig. 3.3A, lane 5). In order to validate these findings, we co-transfected the minimal *TINF2* promoter-driven luciferase reporter plasmid P406 with plasmids encoding the two major NF-κB components p50 and p65, either individually or simultaneously, into NF- κB knockout NIH 3T3 ($p50^-/p65^-$) cells. Relative to a control lysate of cells transfected with an empty expression vector, luciferase activities were found to be minimally increased when cells were transfected with either the p50 or p65 components but were significantly increased when plasmids containing both factors were co-transfected into cells (Fig. 3.3B). These data suggest that, in addition to Sp1, NF-κB also serves an important role in regulating *TIN2* promoter activity.

Mutating the putative binding sites abolishes Sp1 and NF-κB transcriptional activation of the TINF2 promoter

In order to determine whether the predicted Sp1 and NF-κB binding sites are important for transcriptional activation of the *TINF2* promoter, core consensus nucleotides (see Materials and Methods) were mutated in the P406 plasmid which contains the minimal 406 bp promoter sequence. When transfected into HEK293 cells, plasmids carrying single substitutions in either Sp1 or NF-κB binding sites showed

significantly decreased levels of *TINF2* promoter-driven luciferase activity relative to the P406 wild-type construct (Fig. 3.4A and 3.4B). This reduction in promoter activation is consistent with both our ChIP and EMSA data (Fig. 3.2A, 3.2B, and 3.3A) and supports the hypothesis that not only can Sp1 and NF-κB bind to and transactivate the *TINF2* promoter, but they do so by binding to the specific sites predicted using bioinformatics programs. However, as plasmids carrying double substitutions in both Sp1 or NF-κB binding sites do not show synergistic reductions in luciferase activity (Fig. 3.4A), the different putative transcription factor binding sites may serve redundant roles or be utilized under different cellular contexts.

Pharmacological inhibitors can interfere with Sp1- and NF-κB-activated TINF2 transcription

Various pharmacological inhibitors exist which have been shown to interfere with transcription factor binding and/or activation of their target promoters. Mithramycin A, a GC-specific DNA-binding drug [215] prevents binding of Sp1 to its consensus GC-rich binding sites [216]. When added to 293T or HEK293 cells transfected with the minimal promoter reporter construct P406 or control plasmid constructs (pGL3-Basic, pNFAT-Luc, or pGL3-Control), Mithramycin A was able to specifically and significantly reduce the reporter activity of both the *TINF2* promoter and the SV40 promoter-containing pGL3-Control plasmid, both of which contain Sp1 binding sites. No effect was observed in chemically treated cells transfected with the negative control vectors pGL3-Basic or pNFAT-Luc (Fig. 3.5A). Similar results were obtained in Mithramycin A-treated HEK293 cells that were transfected with a similar set of plasmids (data not shown).

Bay11-7082 (Bay 11), which has been shown to irreversibly inhibit NF-κB activation by blocking TNF-α-induced phosphorylation of IκB [217], also specifically and significantly reduced NF-κB-mediated activation of the *TINF2* promoter in a strong dose-dependent manner (Fig. 3.5B). A similar effect was seen with pyrrolidine dithiocarbamate (PDTC; Fig. 3.5C), an antioxidant which also inhibits NF-κB by inhibiting IκB degradation [218]. None of the control vectors (pGL3-Basic, pGL3-Control, or pNFAT-Luc) were impacted by these two NF-κB inhibitors. Similar results were obtained in HEK293 cells transfected with a similar set of plasmids and treated with Bay 11 or PDTC (data not shown). Collectively, these studies provide further evidence that Sp1 and NF-κB indeed play important roles in regulating the transcriptional activation of the *TINF2* gene.

Discussion

Both Sp1 and NF-κB have been shown to be misregulated in the disease state, including various types of human cancer. For example, Sp1 mRNA and DNA-binding activities are increased in epithelial tumors, suggesting that increased activity of this transcription factor contributes to tumor progression in the skin [219], and Sp1 has been shown to be constitutively overexpressed in pancreatic and gastric cancers [220,221]. Also, Sp1 site-dependent transcription is involved in many signal transduction pathways linked to cancer progression (reviewed in [222]). Constitutive IKK and NF-κB signaling have been implicated in the development of several cancers as well, particularly breast cancer [223,224,225,226]. As maintaining telomeres in order to escape cellular senescence is an important aspect of cancer development and progression, it is possible that alterations in the expression levels and/or activation of these transcription factors

could be an early step in a signaling pathway designed to hijack the cellular machinery in order to create the optimal environment for cancer cell growth.

In this study, we have explored the mechanisms which regulate *TINF2* transcription and identified a core promoter region of approximately 406 base pairs, which appears to be at least partially regulated by the Sp1 and NF-κB transcription factors. Our research is in keeping with the finding that Sp1 interacts with components of the transcription machinery to help initiate the transcription of TATA-less promoters, such as the *TINF2* promoter [227,228,229]. Furthermore, Sp1 has been implicated in the transcriptional regulation of both core components of human telomerase, hTERT and hTR [148,230,231]. Of particular interest is the identification of a functional mutation in a putative Sp1 binding site in the hTR promoter in a patient with paroxysmal nocturnal hemoglobinuria (PNH), a rare blood disorder [148]. As it has been hypothesized that telomere dysregulation is involved in the pathogenesis of several human diseases, including bone-marrow failure disorders (reviewed in [1]) and cancer (reviewed in [232]), future research should be directed toward careful screening of the promoter proximal regions of genes important for telomere maintenance in order to identify any functional sequence changes. In addition, as Sp1 binding sites are GC-rich, there is a strong probability that the transcriptional regulation of these proteins may be subject to epigenetic regulation as well. Therefore, characterization of the methylation status of the endogenous TIN2 promoter may also be warranted.

Teo and colleagues have recently uncovered an unexpected relationship between NF-κB and the shelterin component, hRap1 [214]. They have shown that not only can NF-κB activate transcription of the *hRap1* promoter, but hRap1 itself can positively

regulate NF-κB activation through its interaction with IKK. The NF-κB pathway has also been found to be involved in the upregulation of hTERT in HTLV-I-transformed cells [233], as the viral Tax protein can activate NF-κB which in turn stimulates Sp1-dependent transcription [234,235], a process already known to be important for hTERT regulation. Taking all these data together, it is reasonable to speculate a global regulation mechanism for proteins important for telomere maintenance; however, a detailed exploration of the promoter regions of genes that encode all known components of the telomere binding and maintenance complexes is still necessary.

Figure 3.1

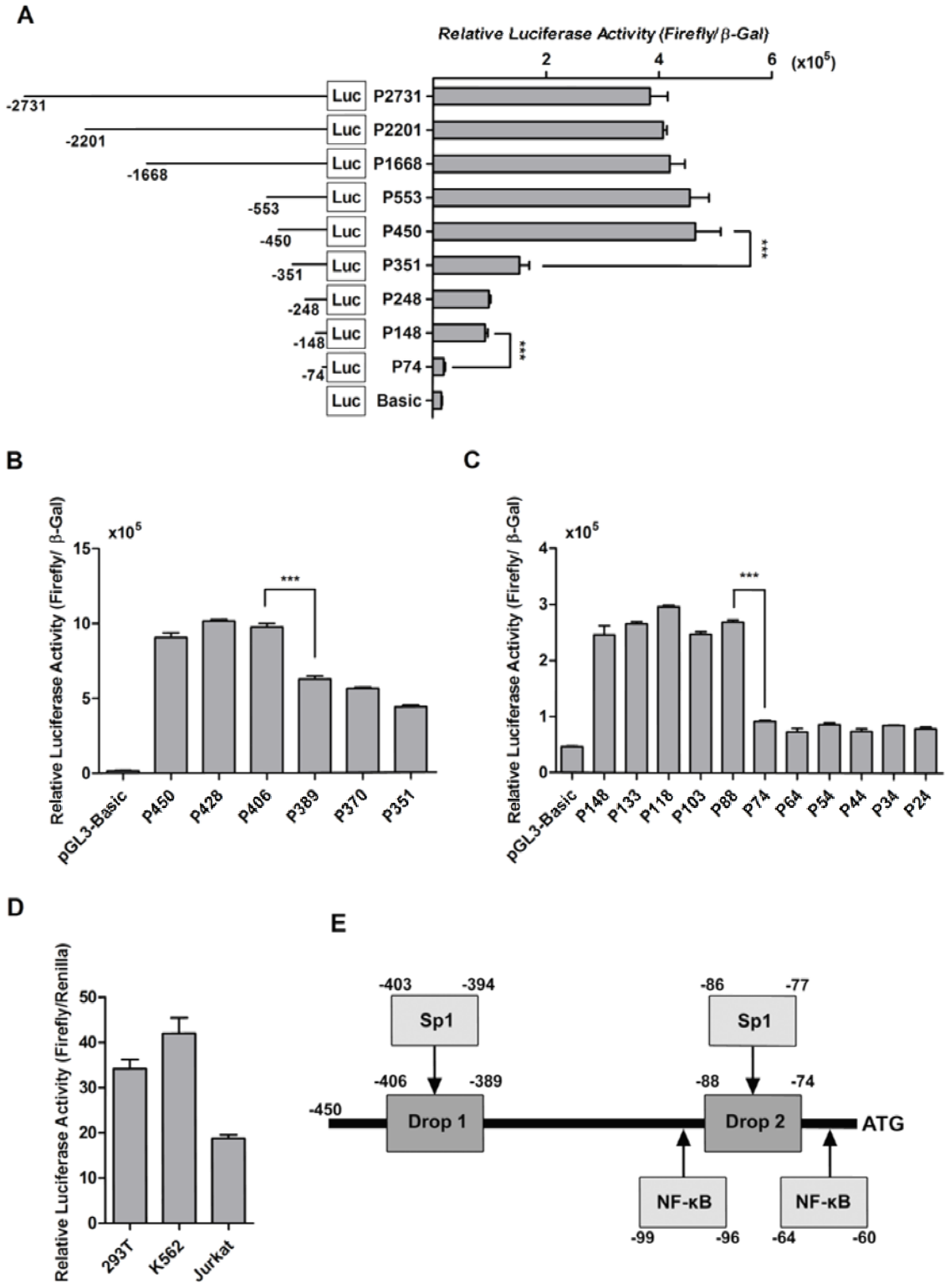
A
Relative Luciferase Activity (Firefly/ β-Gal)
2
4
6
(x10^5)
-2731
-2201
-1668
-553
-450
-351
-248
-148
-74
Luc
P2731
P2201
P1668
P553
P450
P351
P248
P148
P74
Basic
***
***
B
Relative Luciferase Activity (Firefly/ β-Gal)
x10^5
15
10
5
0
***
pGL3-Basic
P450
P428
P406
P389
P370
P351
C
Relative Luciferase Activity (Firefly/ β-Gal)
x10^5
4
3
2
1
0
***
pGL3-Basic
P148
P133
P118
P103
P88
P74
P64
P54
P44
P34
P24
D
Relative Luciferase Activity (Firefly/Renilla)
50
40
30
20
10
0
293T
K562
Jurkat
E
-403
-394
Sp1
-406
-389
Drop 1
-450
-86
-77
Sp1
-88
-74
Drop 2
ATG
NF-κB
-99
-96
NF-κB
-64
-60

Figure 3.2

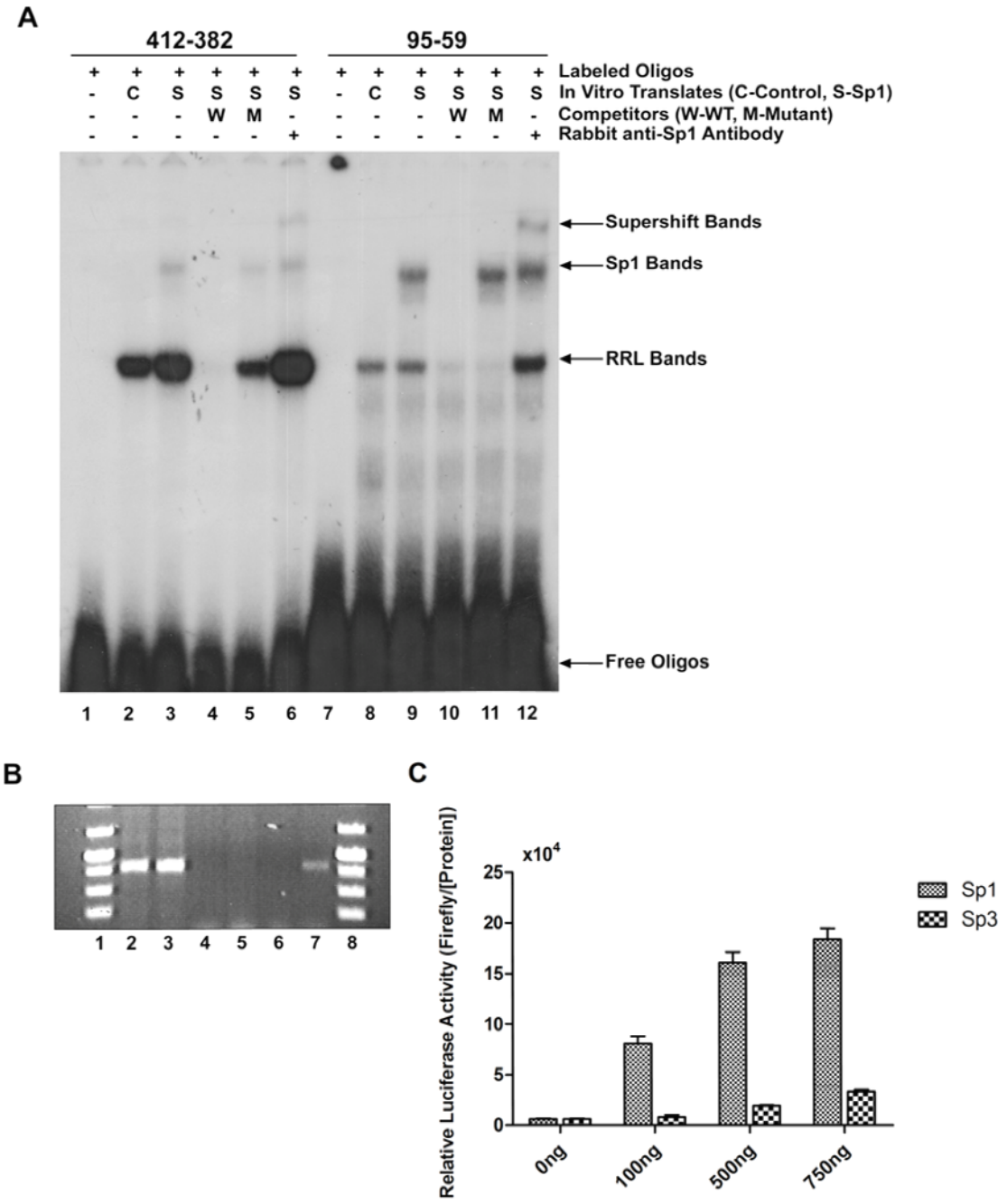
A
412-382
95-59
Labeled Oligos
In Vitro Translates (C-Control, S-Sp1)
Competitors (W-WT, M-Mutant)
Rabbit anti-Sp1 Antibody
Supershift Bands
Sp1 Bands
RRL Bands
Free Oligos
1 2 3 4 5 6 7 8 9 10 11 12
B
1 2 3 4 5 6 7 8
C
Relative Luciferase Activity (Firefly/[Protein])
x10^4
25
20
15
10
5
0
0ng
100ng
500ng
750ng
Sp1
Sp3

Figure 3.3

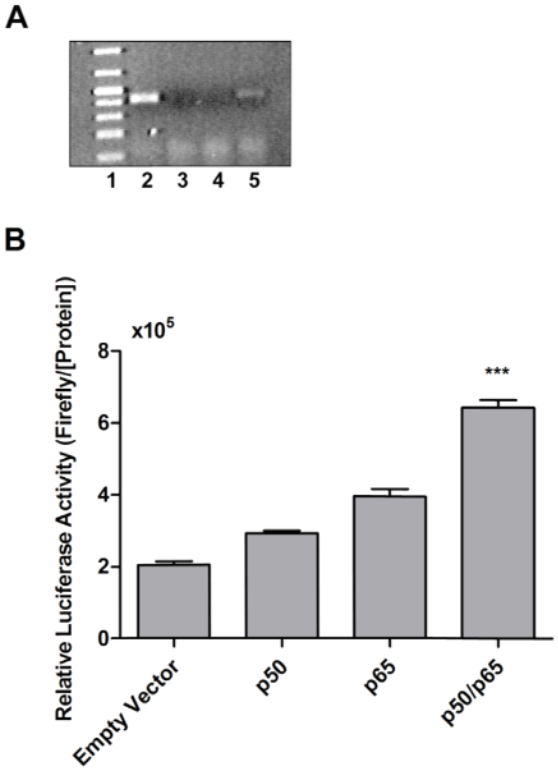
A
1 2 3 4 5
B
Relative Luciferase Activity (Firefly/[Protein])
x10^5
8
6
4
2
0
***
Empty Vector
p50
p65
p50/p65

Figure 3.4

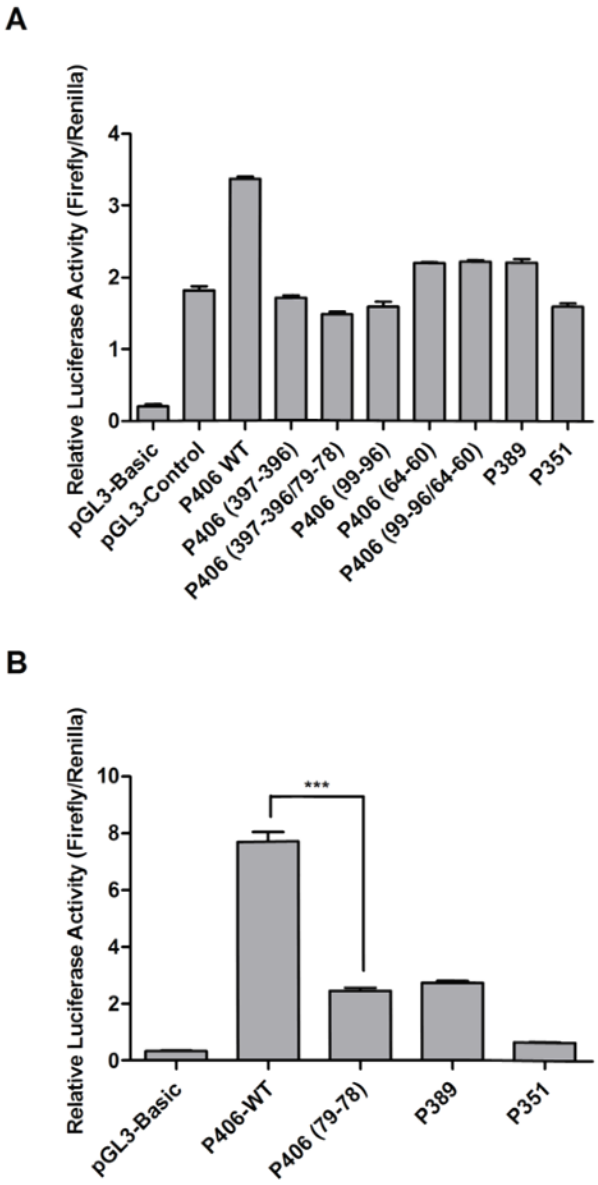
A
Relative Luciferase Activity (Firefly/Renilla)
0
1
2
3
4
pGL3-Basic
pGL3-Control
P406 WT
P406 (397-396)
P406 (397-396/79-78)
P406 (99-96)
P406 (64-60)
P406 (99-96/64-60)
P389
P351
B
Relative Luciferase Activity (Firefly/Renilla)
0
2
4
6
8
10
***
pGL3-Basic
P406-WT
P406 (79-78)
P389
P351

Figure 3.5

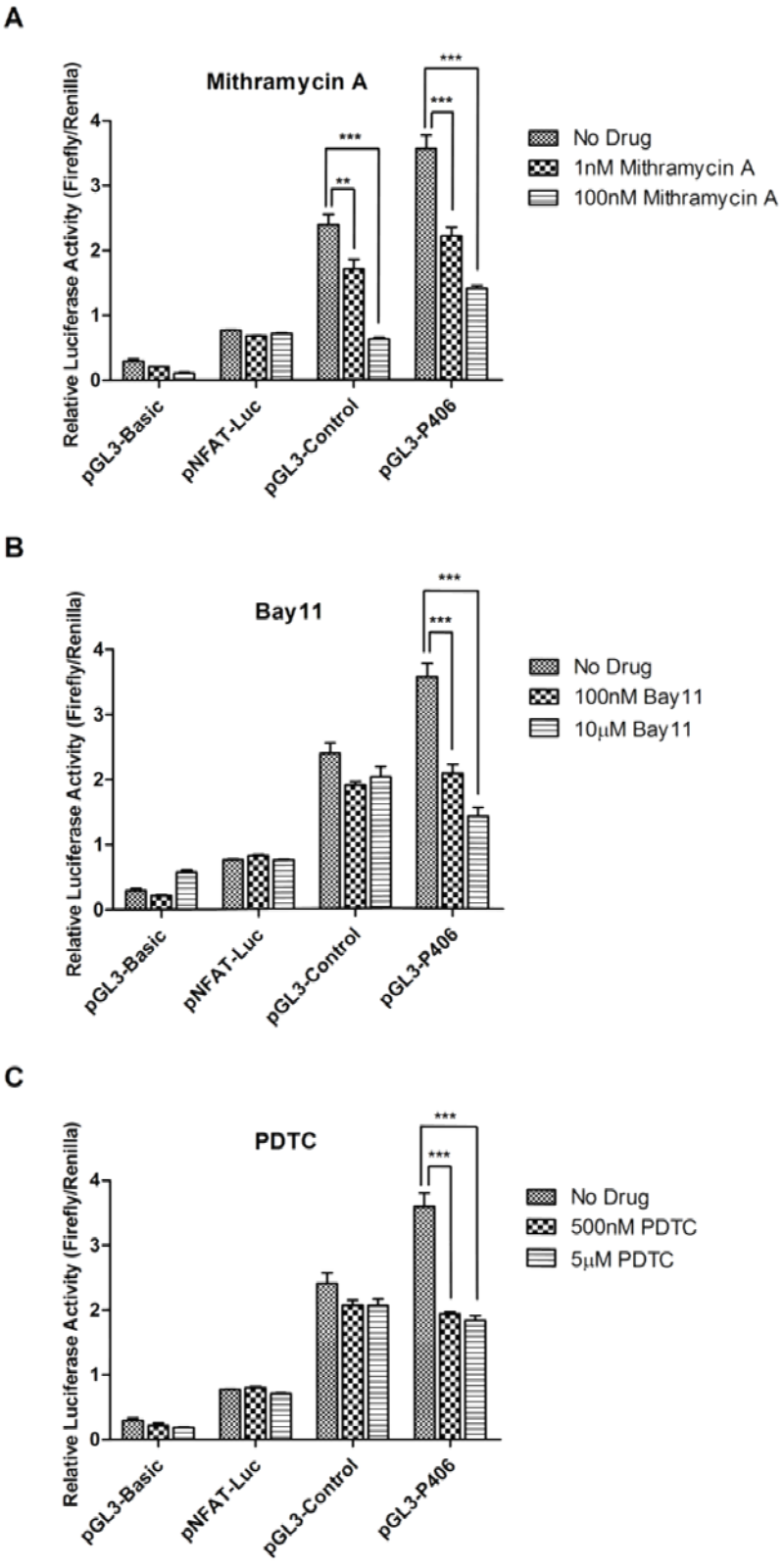
A
Mithramycin A
Relative Luciferase Activity (Firefly/Renilla)
No Drug
1nM Mithramycin A
100nM Mithramycin A
pGL3-Basic
pNFAT-Luc
pGL3-Control
pGL3-P406
B
Bay11
No Drug
100nM Bay11
10μM Bay11
C
PDTC
No Drug
500nM PDTC
5μM PDTC

Figure Legends

Figure 3.1. Luciferase assay analysis of *TINF2* promoter truncations defines two important elements that control promoter activity. (A) The name of each TIN2 reporter construct was assigned according to the 5'-end nucleotide numbers of the promoter sequences inserted upstream of the ATG initiation codon. Basic refers to the pGL3-Basic vector. For each transfection, the firefly luciferase activity was normalized to the β-galactosidase activity expressed from a co-transfected β-galactosidase expression vector. The means from three independent experiments are shown for each construct; *bars*, SD. (***$p<0.001$). (B) Finer promoter mapping of the region between -450 and -351, expressed as in panel A. (C) Finer promoter mapping of the region between -148 and -24, expressed as in panel A. (D) Verification of the functionality of the *TINF2* promoter construct in various cell lines. For each transfection, the firefly luciferase activity was normalized to the *Renilla* activity expressed from the co-transfected pRL-CMV expression vector and expressed as in panel A. (E) Schematic of the core *TIN2* promoter construct showing putative Sp1 and NF-κB binding sites predicted using bioinformatic tools. "Drop 1" and "Drop 2" refer to the drops in luciferase reporter activity shown in panel A.

Figure 3.2. Sp1 binds to its putative binding sequences in the *TINF2* promoter *in vitro* and *in vivo*. (A) EMSA showing the ability of Sp1 to bind to its two predicted sites *in vitro*. Sp1 protein was *in vitro* translated using the rabbit reticulocyte lysate (RRL) system and incubated with end-labeled DNA oligos encompassing the putative binding

sites. These complexes (lanes 3 and 9) could be competed away with unlabeled wild-type oligos (lanes 4 and 10), but not with a mutant form (lanes 5 and 11). Furthermore, the complexes could be super-shifted using an anti-Sp1 antibody (lanes 6 and 12). (B) The ability of Sp1 to bind to the endogenous *TINF2* promoter *in vivo* was shown using ChIP. The 407 base pair fragment could be amplified from a reaction including the anti-Sp1 antibody (lane 7), but not from reactions containing anti-c-Myc, normal IgG, or no antibody (lanes 4-6). Lanes 1 & 8: GeneRuler 1kb Plus DNA Ladder; Lane 2: Input (No Ab); Lane 3: Input (Normal Rabbit IgG); Lane 4: Rabbit anti-c-Myc Ab; Lane 5: Normal Rabbit IgG; Lane 6: No Ab; Lane 7: Rabbit anti-Sp1 Ab. (C) Sp1 is the major Sp transcription factor that can activate the *TINF2* promoter. *Drosophila melanogaster* SL2 cells were co-transfected with the minimal promoter reporter construct pGL3-P406 and various amounts of expression vectors encoding either Sp1 or Sp3. For each transfection, the firefly luciferase activity was normalized to the total protein concentration. The means from three independent experiments are shown; *bars*, SD.

Figure 3.3. NF-κB can bind to and activate the *TINF2* promoter. (A) The ability of NF-κB to bind to the endogenous *TINF2* promoter *in vivo* was shown using ChIP. The 407 base pair fragment could be amplified from a reaction including the anti-p65 antibody (lane 5), but not from reactions containing no antibody or normal IgG (lanes 3 and 4). Lane 1: GeneRuler 1kb Plus DNA Ladder; Lane 2: Input (No Ab); Lane 3: No Ab; Lane 4: Normal Goat IgG; Lane 5: Goat anti-p65 Ab. (B) The ability of NF-κB to activate the minimal *TINF2* promoter was verified by co-transfection of vectors encoding p50 and/or p65 protein and pGL3-P406 into NIH 3T3 ($p50^-/p65^-$) cells. For each

transfection, the firefly luciferase activity was normalized to the total protein concentration. The means from three independent experiments are shown; *bars*, SD. (***p<0.001).

Figure 3.4. Mutating the putative binding sites abolishes Sp1 and NF-κB transcriptional activation of the *TINF2* promoter. (A) Mutating core consensus nucleotides in predicted Sp1 or NF-κB binding sites (see Materials and Methods) results in reduced *TINF2* promoter-driven luciferase activity in a minimal promoter context. For each transfection, the firefly luciferase activity was normalized to the *Renilla* activity expressed from the co-transfected pRL-CMV expression vector. The means from three independent experiments are shown; *bars*, SD. (p<0.001). (B) Mutating core consensus nucleotides in the predicted Sp1 binding site at position -(88-74) results in reduced *TINF2* promoter-driven luciferase activity as compared to wild-type control. Results are shown as in panel A. (***p<0.001).

Figure 3.5. Pharmacological inhibitors can interfere with Sp1- and NF-κB-mediated *TINF2* promoter activation. (A) Mithramycin A, an Sp1 inhibitor, reduces *TINF2* promoter-driven luciferase activity. 293T cells are transfected with either the promoter-less pGL3-Basic plasmid, the NFAT-responsive pNFAT-Luc plasmid, the SV40 promoter-containing pGL3-Control plasmid, or the minimal TIN2 promoter P406 plasmid and incubated with the indicated concentrations of drug for 24 hours. For each transfection, the firefly luciferase activity was normalized to *Renilla* luciferase activity expressed from a co-transfected *Renilla* luciferase expression vector. The means from

three independent experiments are shown; *bars*, SD. (**p<0.01, ***p<0.001). (B) Bay11-7082, an NF-κB inhibitor, reduces *TINF2* promoter-driven luciferase reporter activity. Cells are processed and values expressed as in panel A. (***p<0.001). (C) PDTC, an NF-κB inhibitor, reduces *TINF2* promoter-driven luciferase reporter activity. Cells are processed and values expressed as in panel A. (***p<0.001).

CHAPTER 4: DISCUSSION

In this body of work, we have explored several areas in order to gain insight into the mechanisms of telomere maintenance. While analyzing patient-identified mutations can help shed important light on critical residues of telomerase required for telomere maintenance, currently available technologies make it challenging to nail down the precise defect conferred by each sequence change. The most popular method by which to test telomerase enzymatic activity, the TRAP assay, involves PCR amplifying the elongation products created by incubating assembled enzyme with a synthetic telomeric probe. As such, this assay will mask any slight defects in a mutant telomerase's ability to elongate telomeres. While slight defects in telomerase enzymatic activity might not render telomeres dramatically shortened after a single round of replication, successive rounds of replication, as occur in hematopoietic stem cells and other highly proliferative tissues, could create telomeres short enough to induce replicative senescence. Though replicative senescence is absolutely necessary to limit the proliferation of "old" and presumably highly-mutated cells, premature death of the stem cell compartments of these tissues can create various premature aging phenotypes. Furthermore, these telomerase defects are compounded by a phenomenon called disease anticipation, whereby shortened telomeres are inherited from generation to generation [101]. Thus, while an entire family may carry the same sequence change in a telomere maintenance gene that causes the same decrease in enzymatic activity, disease phenotypes may not manifest until later generations, when the inherited telomeres are short enough to allow them to reach a critical length through insufficient elongation. In addition, it is currently believed that

telomerase is not recruited to every telomere during each round of replication, but rather only to the shortest ones, maintaining all telomeres at a certain "set point" [61,236]. There are currently no assays available to test whether mutant forms of telomerase can still be recruited to the correct telomeric end, and therefore we currently have no knowledge of what domains or possibly what molecules are important for this activity. A single critically short telomere can induce replicative senescence so it is imperative for a cell's survival that telomerase be recruited to the correct telomere. It is reasonable to consider that some type of immunofluorescence-FISH assay might be used to investigate such defects. However, these technologies would only show that telomerase colocalizes with the shortest telomere, not necessarily that the enzyme is able to efficiently elongate it. Finally, telomerase possesses two different types of processivity: the first is the enzyme's ability to add all six nucleotides of a TTAGGG repeat onto the chromosome end and the second is its ability to add multiple repeats before falling off (reviewed in [237]). Aside from sequencing individual telomeres after the enzyme has elongated them to check for abnormal telomeric sequences, it is very difficult not only to identify if an enzyme has a processivity defect, but also to distinguish between the two types.

While creating mouse knock-ins of patient-identified sequence changes may seem like a logical prospective next step, as it would allow for examination of the effects of each mutation in an *in vivo* system, the differences between human and mouse telomere maintenance make such comparisons quite difficult. Mouse telomeres are significantly longer than those of humans, 40-60 kb versus 5-15 kb. Thus, it seems improbable that telomeric shortening serves the same "cellular clock" role in mice that it does in humans, as mice have to be bred for several generations in the absence of telomerase activity to

achieve telomeres short enough to recapitulate any human disease phenotypes. As such, telomere length and even gene expression of various telomere maintenance genes may be under less stringent control or even utilize completely different regulatory mechanisms all together in the mouse. In support of this, mice express two paralogs of the human single-stranded DNA binding protein POT1, POT1a and POT1b, which appear to serve independent roles in maintaining mouse telomeres and protecting them from an aberrant DNA damage response [174,175,176,177]. Furthermore, while homozygous loss of either the hTERC RNA or hTERT protein has never been reported for humans, presumably because such a loss would be incompatible with life, knock-outs of either essential telomerase component in the mouse are completely viable and must be bred for several generations before any disease symptoms manifest [138,139,140,141,162,163,164,165]. Therefore, while mouse experiments and models of human telomere deficiency syndromes are valuable and have provided important insights into possible mechanisms of human telomere regulation, the interpretation of these data should be conducted conservatively as these differences are not slight. The development and advancement of cell culture experiments involving human stem cells may be able to help overcome the shortcomings of mouse studies. There have been a few studies demonstrating that nuclear reprogramming, achieved either by somatic cell nuclear transfer (SCNT) or by expression of defined transcription factors to create induced pluripotent stem cells (iPS), increases telomerase activity, leading to telomere length restoration. Moreover, this transformation towards an ES cell-like state involves epigenetic changes towards a more open and transcriptionally active state, increasing the expression of telomeric repeat-containing RNAs (TERRA) (summarized in [238]). It has

also been shown that fibroblast cells from patients with mutations in *Dkc1* can be reprogrammed to a pluripotent state restoring telomere maintenance and self renewal capacity [239]. However, the mechanisms for the telomerase activation and for the chromatin remodeling remain unknown, and furthermore, it remains to be seen whether these iPS cells are more susceptible to cancer development.

It is also believed that the hTERT protein may serve roles in the cell aside from its role in the telomerase elongation complex. Conditional transgenic induction of TERT in mouse skin epithelium causes rapid differentiation and proliferation of hair follicle stem cells, independent of its ability to elongate telomeres [170,240]. While this study is conducted in a mouse model and the hTERT protein may not serve the same functions in human stem cell compartments, it raises the possibility that some of the defects we observe in human patients with various BMFS are due to mutant hTERT's inability to fulfill its non-telomeric functions. All of the patients were identified as having abnormally shortened telomeres. However, even though the simplest and most plausible explanation is that the sequence variants cause some direct defect in the ability of telomerase to efficiently elongate telomeres, misregulated differentiation and/or proliferation of the stem cell compartments as a result of telomerase mutations has never been ruled out as a possible telomere shortening mechanism. Therefore, mutant versions of hTERT which appear to possess wild-type telomerase enzymatic activities may be deficient not only in the untested activities listed above, but also in their proposed extracurricular roles (summarized in [241]). Since the mouse mTERT and human hTERT proteins are interchangeable, these non-telomeric functions could be tested, although laboriously so, by repeating the skin epithelium study using mutant versions of

the TERT protein and observing if they could still cause the rapid differentiation and proliferation induced by the wild-type protein. Such studies may reveal what domains or residues are important for these activities. Furthermore, it is also possible that mutations in any of the telomerase holoenzyme components may affect the RNP's ability to assemble in an *in vivo* setting, as the components must be trafficked from their sites of transcription and translation to the nucleolus in order to form a fully functional enzyme. In the case of X-linked DC, where patients carry mutations in dyskerin, as this protein has been shown to play a role in ribosomal RNA processing, it is plausible that it is a defect in this activity which causes the disease phenotype with telomere shortening occurring as a secondary feature.

With each cellular replication, each newly synthesized telomeric end must be bound by its own telomere-binding shelterin complex for protection from an aberrant DNA damage response and for proper regulation of its length [10]. Therefore, the expression levels of the six components of the shelterin complex, TRF1, TRF2, Rap1, TIN2, TPP1, and POT1, must be tightly regulated and their stoichiometric relationship maintained [242]. As it has been shown that the expression levels of these proteins can be altered in the cancers, it is important to study the transcriptional regulation of these proteins in order to better understand how deviations from the norm can influence the disease state [209,210,211,212,213,243]. When taken together with recent studies showing a role for Rap1 in a feed-forward loop NF-κB signaling [214], our data suggest that NF-κB, and perhaps Sp1, may serve as a global regulatory mechanism for genes important for telomere maintenance. In support of this hypothesis, it has been shown that c-Myc and Sp1 cooperate to activate transcription of the hTERT promoter [231] and,

furthermore, that activation of the NF-κB pathway and subsequent increased binding of Sp1 to the hTERT promoter can increase hTERT transcription in response to HTLV-1 infection [233].

Further studies on the transcriptional regulation of the remaining shelterin components are necessary in order to solidify this model. To date, no promoter mapping studies of any kind have been published for TRF1, TRF2, TPP1, or POT1, although the GC-rich nature of these promoters raises the possibility for potential Sp1 binding sites. It is reasonable to speculate that at least the proteins of the shelterin complex would be regulated by a common mechanism, as all six components are required to protect the telomere end. In order to maintain the proper ratio of these important telomere-binding factors, their promoters would all need to be activated either by a common mechanism or, as Rap1 has been shown to activate the NF-κB pathway [214], perhaps the shelterin components themselves can regulate each other's expression.

In conclusion, our study provides the first step in assessing the validity of such a common regulatory model. As the basics of this important telomere-protection complex and its individual components are fairly well established, attention must now be turned to how these proteins are regulated at transcription, through post-translational modifications, and possibly through intracellular trafficking or telomere recruitment. Recent studies have examined the issue of how the shelterin proteins TRF1 and TRF2 are regulated through post-translational modifications. It has been shown that Polo-like kinase 1 (Plk1), which is expressed and active in mitosis, phosphorylates TRF1 at Ser-435 and that this phosphorylation increases the ability of TRF1 to bind telomeric DNA in a cell cycle-dependent manner [244]. Furthermore, TRF1 can be ADP-ribosylated by

tankyrase 1 and 2, under the modulation by TIN2, releasing it from telomeres, and allowing telomerase access to the chromosome end [206,245,246]. Finally, a preliminary study reports that Aurora-C, the least understood of the Aurora serine/threonine kinases, phosphorylates TRF2 at Thr-358 *in vitro,* although the significance of this modification has yet to be proven *in vivo* [247]. It is highly likely that the modification and regulatory mechanisms of the shelterin proteins will vary with developmental state, cell type, and disease state, so the experimental systems which researchers choose to study must be selectively considered, and the data generated from these studies must be carefully interpreted. Regardless, understanding how these pathways are regulated is the next step in our quest to understand the important issue of telomere maintenance under normal and pathological states in humans.

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