THE REGULATION OF HTERT BY ALTERNATIVE SPLICING

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DEDICATION

I would like to thank the members of my Graduate Committee and mentors, Jerry Shay and Woodring Wright, all of the members of the Shay/Wright lab, my collaborators, the UTSW MSTP, my friends, my family, my significant other and my two cats, Kerrigan and Tofu.

THE REGULATION OF HTERT BY ALTERLATIVE SPLICING

by

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DISSERTATION

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THE REGULATION OF HTERT BY ALTERNATIVE SPLICING

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Telomeres are non-coding DNA hexameric repeats (TTAGGG in mammals) located at the ends of linear chromosomes that, along with their associated proteins, protect against the loss of genomic material during cell division and prevent the recognition of chromosome ends as double-strand breaks. Human telomeres shorten with continued cell proliferation but are maintained by human telomerase reverse transcriptase (hTERT), an enzyme that synthesizes telomeric repeats using an RNA template.

The regulation of telomerase has been studied at many levels—from epigenetic and transcriptional regulation to the alternative splicing of hTERT pre-mRNA into catalytically

inactive splice variants. Our hypothesis is that if the regulation of telomerase reverse transcriptase splicing is necessary for telomere length homeostasis, altering telomerase splicing to decrease the production of full-length hTERT and will result in decreased telomerase activity and subsequently telomere shortening. We focused our efforts on identifying splicing factors are involved in hTERT splicing and characterized the role of two splicing factors, NOVA1 and PTBP1, in regulation of hTERT splicing in non-small cell lung cancer cells.

We show that these splicing factors are important for full-length hTERT, telomerase activity and telomere length maintenance in vitro. Xenograft studies suggest that NOVA1 is also important for tumor growth in vivo. We found that these splicing factors are able to directly interact with hTERT in a region our group previously identified to be important for hTERT splicing.

Altogether, our work suggests that splicing factors are important for hTERT regulation and telomerase activity in cancer. Since telomerase activity is undetectable in most somatic tissues but is increased in the vast majority of human cancers, dependence on telomerase represents a key vulnerability in cancer tissues which could be therapeutically targetable.

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Gao, Z., Ure, K., Ding, P., Nashaat, M., Yuan, L., Ma, J., Hammer, R. E., & Hsieh, J. (2011). The master negative regulator REST/NRSF controls adult neurogenesis by restraining the neurogenic program in quiescent stem cells. J Neurosci.,31 (26), 9772-86.

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ATM – ataxia-telangiectasia mutated

Bcl-x – B-cell lymphoma x

CDC40 - cell division cycle 40

CLIP – cross-linking immunoprecipitation

CLIP-ddPCR – crosslinking immunoprecipitation followed by RT-ddPCR

CLK – CDC like kinase

CMML – chronic myelomonocytic leukemia

CRISPR – clustered regularly interspaced short palindromic repeats

CTD – C-terminal domain

ddPCR – droplet digital polymerase chain reaction

DDR – DNA damage response

ddTRAP – droplet digital telomerase repeat amplification protocol

DR8 – direct repeat 8

DSB – double strand break

E2A –Equine rhinitis A virus 2A

EGF – epidermal growth factor

ESE – exonic splicing enhancer

ESYT2 – extended synpatotagmin 2

ETS – E26 transformation specific

EZH2 – enhancer of zeste homologue 2

GABPA – GA binding protein transcription factor alpha

GLRA2 – glycine receptor alpha 2

HAT – histone acetyltransferase

HBEC – human bronchial epithelial cell

HDAC – histone deacetylase

HEAT – Huntingtin, elongation factor 3, protein phosphatase 2A, targets of rapamycin 1)

HER2 – human epidermal growth factor receptor 2

hESC – human embryonic stem cell

HITS-CLIP – high-throughput sequencing of RNA isolated by crosslinking

immunoprecipitation

hnRNP – heterogeneous nuclear ribonucleoprotein

hTERT – human telomerase reverse transcriptase

IPF – idiopathic pulmonary fibrosis

iPS – induced pluripotent stem cell

IκBα – inhibitor of kappa B alpha

Mad1 – mitotic arrest deficient 1

Mcl-1 –myeloid cell leukemia 1

MDS – myelodysplastic syndrome

MRN – Mre11-Rad50-Nbs1

MZF2 – myeloid zinc finger 2

NFAT – nuclear factor of activated T-cells

NF-κB – nuclear factor kappa-light chain-enhancer of activated B cells

NHEJ – non-homologous end-joining

NMD – nonsense-mediated decay

NOVA1 – neuro-oncological central antigen 1

NSCLC – non-small cell lung cancer

NUMB – protein numb homolog

POMA – paraneoplastic opsoclonus-myoclonus ataxia

PTBP1 – polypyrimidine tract binding protein 1

QKI – Quaking

RBM – RNA-binding motif

REN – restriction endonuclease

RFLP – restriction fragment length polymorphism

RRM – RNA recognition motif

SEAP – secreted embryonic alkaline phosphatase

SF3B1 – splicing factor 3b subunit 1

shRNA – short hairpin RNA

siRNA – short interfering RNA

snRNP – small nuclear ribonucleoprotein

SNRPB – small nuclear ribonucleoprotein-associated protein B

Sp1 – specificity protein 1

SRPK – Serine/threonine-protein kinase

SRSF – serine/arginine-rich splicing factor

T2A – Thosea asigna virus 2A

TERC – telomerase RNA component

TPE – telomere position effect

TRRAP – transformation/transcription domain-associated protein

U2AF1 – U2 small nuclear RNA auxiliary factor 1

UV-IP – UV-crosslinking and immunoprecipitation

VEGF – vascular endothelial growth factor

VNTR – variable nucleotide tandem repeat

WT1 – Wilms tumor 1

ZRSR2 – zinc finger CCCH-Type, RNA binding motif and serine/arginine rich 2

CHAPTER ONE
Introduction

TELOMERES AND TELOMERASE

Introduction to telomeres and telomerase

First observed by Leonard Hayflick, normal primary human cells are only able to divide a limited number of times in culture (Hayflick & Moorhead, 1961). The clock or timing mechanism of this limited replication span was unknown for many years but was demonstrated to be located in the nucleus (Wright & Hayflick, 1975). Telomeres were known to be present at the ends of chromosomes and functionally defined as required for replication and stability and eventually found to be made up of TTAGGG repeats (Moyzis et al., 1988). Years later, telomeres were found to shorten with increasing age in vitro and in vivo (Harley, Futcher, & Greider, 1990; Hastie et al., 1990). Eventually, the solution to this telomere shortening problem, also called the end replication problem, was found in the form of telomerase, a reverse transcriptase that elongates telomeres (Greider & Blackburn, 1985). Finally, evidence that telomere shortening causes cellular senescence was demonstrated by the introduction of telomerase into cells to extend the replicative life span of cells (Bodnar et al., 1998).

Since those initial observations, through the efforts of many labs around the world, understanding of telomeres and telomerase in normal biology and disease states has grown tremendously. Telomeres are now known to be specialized DNA-protein complexes found at

the ends of linear chromosomes and are made up of telomeric repeat sequences (TTAGGG in humans) bound by a group of proteins called the shelterin complex (Palm & de Lange, 2008) (Fig 1 A). Together, these structures serve a number of functions in cells-the most basic of which are protecting the ends of chromosomes from being recognized as double strand breaks (end protection) and serving as a buffer to protect protein coding genes from being lost during DNA replication (end replication) (Blackburn, 2001).

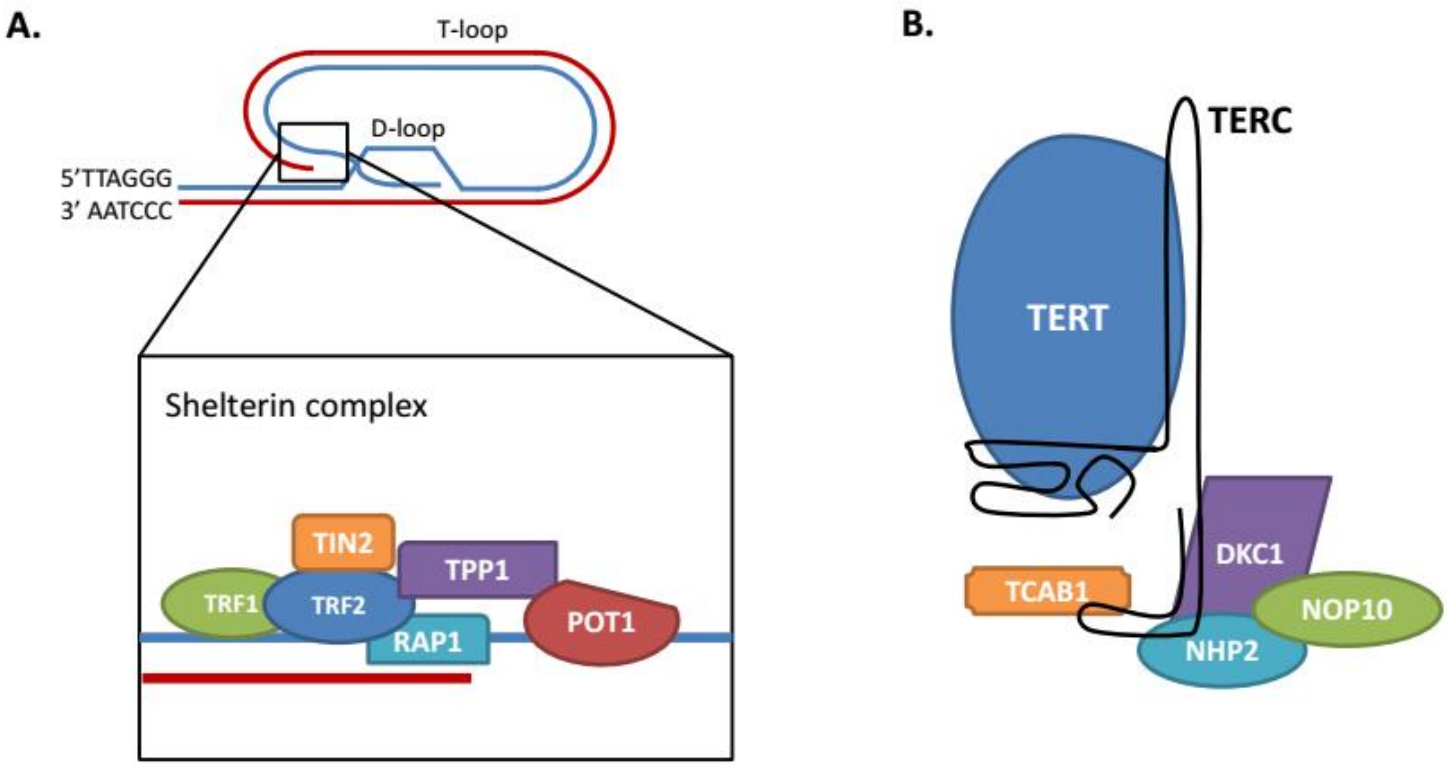

Fig 1. Structure of telomeres and telomerase (Adapted from Roake and Artandi, 2017)
A. The shelterin complex is made up of TRF1, TRF2, TPP1, TIN2, RAP1 and POT1. Each component has its own specialized function in telomere biology but overall this complex protects chromosome ends and is a major regulator of telomerase.
B. Telomerase is a ribonucleoprotein enzyme complex which uses TERC as an RNA template and as a scaffold for the various protein components TERT, DKC1, NHP2, NOP10 and TCAB1. TERT is the catalytic subunit while the other components are involved in trafficking, TERC biogenesis and stability.

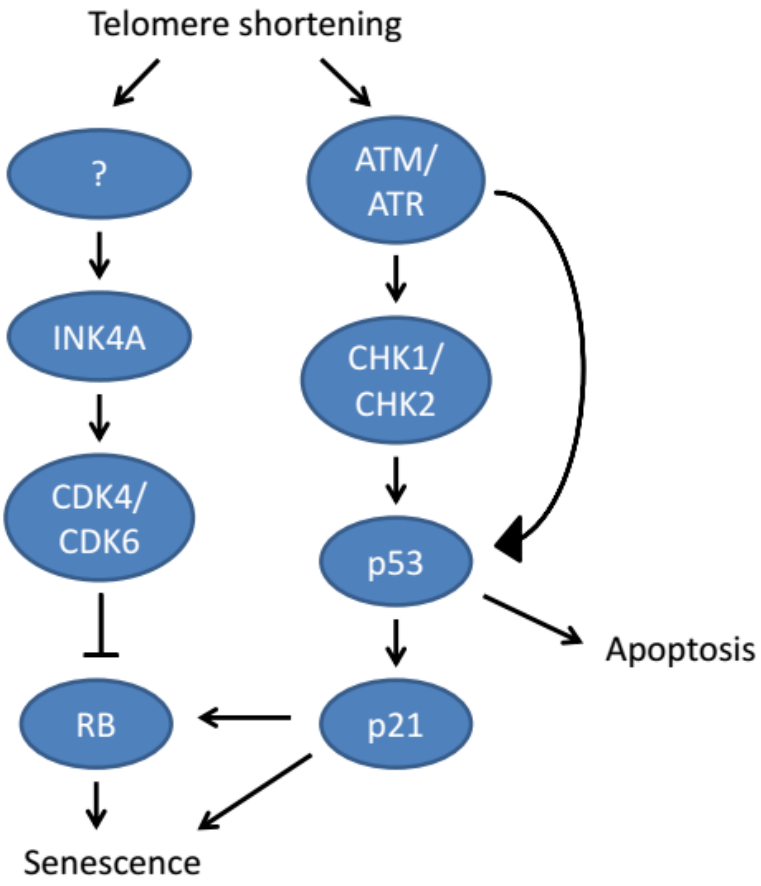

Fig 2. Telomere-associated DNA damage signals (adapted from Deng, Chan & Chang 2008)
Telomere shortening is detected and can trigger the activation of ATM/ATR and downstream signaling to result in p53-induced apoptosis or senescence. Another less-studied pathway is the INK4A-RB pathway which results in cellular senescence.

The end protection problem is based on observations that breaks in chromosomal DNA are readily recognized by the cell and can trigger a DNA damage response (DDR) (Fig 2). Double strand breaks (DSBs) can lead to genomic rearrangements and must be repaired quickly. The ends of linear chromosomes would be sensed as broken DNA and mistakenly repaired if not for telomeres and their associated proteins (Bae & Baumann, 2007; Bombarde et al., 2010). In fact, the Mre11-Rad50-Nbs1 (MRN) complex, one of the main DSB sensors in eukaryotes, interacts with telomeres and plays a key role in facilitating telomerase activity at telomeres (Chai, Sfeir, Hoshiyama, Shay, & Wright, 2006; Verdun, Crabbe, Haggblom, &

Karlseder, 2005). Furthermore, MRN is crucial for ATM activation and non-homologous end-joining (NHEJ) of dysfunctional telomeres (Deng, Guo, Ferguson, & Chang, 2009).

The end replication problem stems from the fact that telomeres shorten with cell division due to the inability of eukaryotic DNA replication to fully copy the ends of linear chromosomes. Since telomeres are repeat sequences which can be elongated by telomerase, this mechanism allows cells to circumvent the otherwise problematic loss of important genomic material.

Telomerase reverse transcriptase

Telomerase is a ribonucleoprotein enzyme complex that uses telomerase reverse transcriptase (TERT) as the catalytic subunit and telomerase RNA component (TERC) as an RNA template along with a number of protein components to add new DNA onto telomeres (Greider & Blackburn, 1985) (Fig 1 B). Unlike TERC and other factors which are generally ubiquitously expressed, the catalytic subunit of telomerase, TERT, is more tightly regulated and has much more limited expression (Avilion et al., 1996; Meyerson et al., 1997; Yi, Shay, & Wright, 2001). In normal human tissues, telomerase activity is abundant in early fetal/embryonic development but quickly restricted to low levels in proliferative cells and almost undetectable levels in most somatic cells (N. W. Kim et al., 1994; Wright, Brasiskyte, Piatyszek, & Shay, 1996). This tight repression of telomerase in normal tissues results in telomere shortening with age in most somatic cells (Fig 3). Thus, telomere shortening can activate DDR and result in replicative senescence, acting as an initial deterrent for tumor formation.

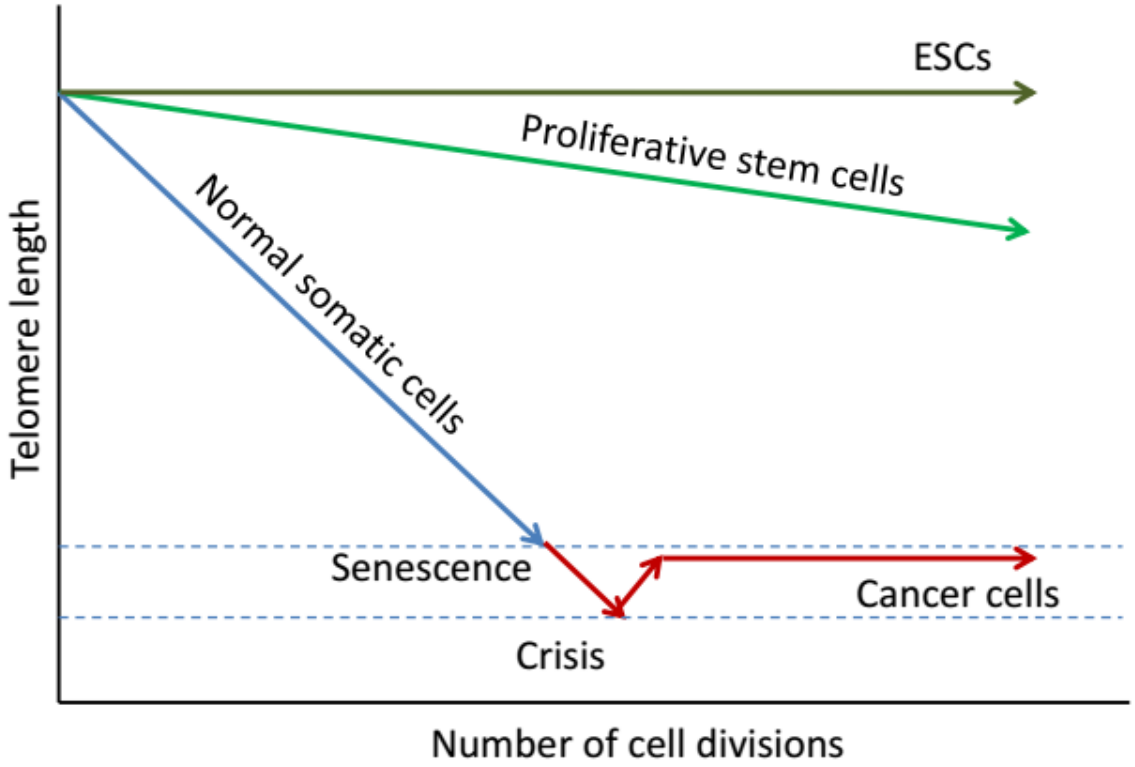

Fig 3. Telomere length in normal and cancer cells (adapted from Buseman, Wright & Shay, 2012)
Embryonic stem cells (ESCs) have infinite replicative potential and sufficient telomerase activity to completely prevent loss of telomeres. Proliferative stem cells have limited telomerase activity and gradually lose telomeres with time. Normal somatic cells are telomerase negative and shorten telomeres at the fastest rate. At a critical shortness, DDR is activated and normally results in replicative senescence. However, if cells are able to bypass this checkpoint, for example by inactivating p53, they can continue to proliferate until they reach crisis. At crisis, telomeres are too short to protect chromosomes, resulting in chromosomal fusion and apoptosis. Cancer cells that are able to reactivate telomerase will survive and generally maintain their telomeres at very short lengths.

However, approximately 90% of cancers find ways to reactivate telomerase, allowing them to maintain their telomeres and giving them limitless replicative potential (N. W. Kim et al., 1994; Shay & Bacchetti, 1997) (Fig 3). Thus, telomerase reactivation has been characterized as a major hallmark of cancer (Hanahan & Weinberg, 2000, 2011). Telomerase is highly regulated even in telomerase positive cells such as proliferative stem cells and cancer cells.

Long-term inhibition of telomerase has been shown to result in cell death in cancer cells (Dikmen et al., 2005; Frink et al., 2016; Gellert, Dikmen, Wright, Gryaznov, & Shay, 2006). Thus, regulation of TERT has been the subject of great interest to the telomerase field. Specifically, the mechanisms of telomerase reactivation and potential as a therapeutic target have been of particular interest to the cancer biology field.

TERT regulation

TERT expression is tightly regulated by transcriptional, epigenetic and post-transcriptional regulation. Transcriptional regulation was believed to be the major regulatory mechanism of telomerase for many years. Indeed, TERT promoter-luciferase experiments showed that the promoter is inactive in normal cells but activated in immortal cells (Cong, Wen, & Bacchetti, 1999). Additionally, TERT promoter mutations, as well as mutations in other genes involved in telomere and telomerase activity, have been identified by a number of groups to have causal connections in familial melanoma (Horn et al., 2013; Huang et al., 2013; Robles-Espinoza et al., 2014; Shi et al., 2014). These mutations were found to generate novel binding sites for the ETS family of transcription factors and mutated promoters showed an increase in transcription in reporter assays (Horn et al., 2013; Huang et al., 2013). Another group found that mutant TERT promoters recruited GABPA, which mediated long-range chromatin interactions and heterochromatin changes to increase TERT transcription (Akincilar et al., 2016). Additionally, TERT promoter mutations have been observed in a wide variety of cancer types including non-melanoma skin cancer, glioma, medulloblastoma, hepatobiliary cancer, thyroid cancer and urinary tract cancer (Heidenreich & Kumar, 2017).

Transcriptional regulation of TERT

The initial findings in cancer were extremely promising. Since then, TERT promoter mutations have also been studied in other contexts. One group used CRISPR/cas9 to engineer TERT promoter mutations into the endogenous TERT locus in human embryonic stem cells (hESCs) and found them to have modest increases in TERT expression without changes in telomerase activity (Chiba et al., 2015). However, when they differentiated hESCs into somatic cells, cells with TERT promoter mutations had increased TERT expression and telomerase activity and had longer telomeres after differentiation.

Another group has observed some of the same cancer-associated TERT promoter mutations in somatic blood cells of a subset of patients with idiopathic pulmonary fibrosis, a condition caused by loss of function mutations in telomere/telomerase-related genes (Maryoung et al., 2017). Patients with the promoter mutation had higher telomerase activity and TERT gene expression than family members with the same TERT coding mutation. Additionally, they generated lymphoblastoid cell lines from families and found that TERT promoter mutation was associated with increased proliferation and was selected for over time. This finding along with the observation that no patients developed cancer suggests that TERT promoter mutation and telomerase activation confer a growth advantage but are not necessarily oncogenic. The study also suggests that the TERT promoter mutations in this disease context may counteract the heterozygous germline loss of function mutation in telomerase-related genes.

Numerous factors that are involved in TERT transcription have been identified. Two of the most well characterized activating transcription factors are c-Myc and Sp1. The TERT promoter region critical for immortalization and cancer cell growth was identified and found to contain E-boxes and GC-boxes, which are the binding sites for Myc and Sp1 respectively (Takakura et al., 1999). Myc and Sp1 were confirmed to bind to the TERT promoter and activate transcription (Kyo et al., 2000). Ectopic expression of c-Myc is able to induce TERT expression and telomerase activity in some normally telomerase-negative cells (Khattar & Tergaonkar, 2017; J. Wang, Xie, Allan, Beach, & Hannon, 1998). Sp1 is a zinc finger transcription factor that binds to GC-boxes found in the TERT promoter and is important along with c-Myc for transcriptional activation of the TERT promoter (Kyo et al., 2000). Since those early studies, a number of other transcription factors have been identified to activate TERT transcription including NFAT and ETS proteins (Chebel et al., 2009; Maida et al., 2002).

Repressors of TERT transcription have also been proposed to be critical for regulating TERT expression since cell fusions of telomerase positive cells with telomerase negative cells resulted in telomerase repression (Wright et al., 1996). Many direct transcriptional repressors have been identified, including Mad1, WT1, MZF-2 and Menin, while many more factors have been observed to repress TERT transcription but may be more indirect repressors (Daniel, Peek, & Tollefsbol, 2012; Fujimoto et al., 2000; S. Y. Lin & Elledge, 2003; S. Oh, Song, Yim, & Kim, 1999).

Another aspect of transcriptional regulation is the epigenetic control of the TERT gene locus and chromatin structure. Histone modifications can alter the ability of transcription factors to access target genes but can also be recruited by transcription factors. For example, Myc is known to recruit TRRAP, a common component of histone acetyltransferase (HAT) complexes, and this recruitment is required for activating TERT transcription (McMahon, Wood, & Cole, 2000; Nikiforov et al., 2002). Other studies have demonstrated that histone deacetylases (HDACs) play a repressive role in the transcription of TERT as HDAC inhibitors can activate TERT transcription (Cong & Bacchetti, 2000).

Alternative splicing of TERT

While transcriptional regulation of TERT is clearly a key step in controlling TERT expression, there is evidence that transcriptional control is not sufficient to completely repress TERT expression. Post-transcriptional regulation, for example alternative splicing, is also important for telomerase activity and is less well understood.

TERT has been known to be alternatively spliced into a number of isoforms in normal and cancer cells (Hrdlickova, Nehyba, & Bose, 2012; Kilian et al., 1997; Ulaner et al., 2000; Wong, Wright, & Shay, 2014). Interestingly, when looking at developmental telomerase activity and TERT expression, the loss of telomerase activity during development in fetal kidney coincided with a shift in TERT splicing in fetal kidney (Ulaner, Hu, Vu, Giudice, & Hoffman, 1998). Indeed, this same group later found that expression of TERT splice variants

was tissue dependent and directly correlated with telomerase activity and telomere length (Ulaner, Hu, Vu, Giudice, & Hoffman, 2001). Our lab has also confirmed that while TERT transcription is very low in normal somatic cells, it is detectable and spliced into alternative isoforms that do not code for active telomerase. Additionally, we observed that when telomeres are short, the TERT promoter becomes more permissive to transcription but alternative splicing is able to function as a downstream bock by splicing TERT transcripts into inactive isoforms (W. Kim et al., 2016).

To date, 22 TERT isoforms have been identified (Hrdlickova et al., 2012). The only isoform with reverse transcriptase activity is the full-length isoform that contains all 16 exons. Many alternatively spliced isoforms have been shown to have dominant negative effects when overexpressed in cells but their function in normal cells or cancer cells at endogenous expression levels is currently unknown (Colgin et al., 2000; Listerman, Sun, Gazzaniga, Lukas, & Blackburn, 2013; Zhu et al., 2014).

How hTERT alternative splicing is regulated during development and cancer progression to produce catalytically active full-length (FL) hTERT transcripts is unknown. Recently, three splicing proteins, SRSF11, hnRNPL and hnRNPH2, when overexpressed in cancer cells were identified to potentially regulate hTERT minus beta splicing choice using an hTERT minigene (Listerman et al., 2013). There are more than 500 RNA binding proteins encoded in the genome and splicing is the result of cellular context, RNA secondary structure, RNA editing, and competition for splice sites (Barash et al., 2010; Castello et al., 2012;

Gerstberger, Hafner, Ascano, & Tuschl, 2014). Thus, there is still much to be learned about the protein networks that regulate the alternative splicing of FL hTERT during development and transformation.

The low abundance of TERT expression, even in telomerase positive cells, has made the study of TERT isoforms challenging. Current methods rely on gel-based splicing assays and primers specific to alternatively spliced isoforms to detect TERT expression and splicing as there are currently no reliable TERT antibodies. Still, studies to date strongly support the importance of alternative splicing as another regulatory aspect of TERT expression that directly controls telomerase activity in cells.

The importance of telomeres and telomerase has fascinated researchers for decades from a basic biology perspective and from therapeutic perspectives. Since telomeres were first thought to serve as a cellular "clock" which can be reset by telomerase, many have sought to harness its potential to reverse ageing. Additionally, since telomerase has been observed to be overexpressed in a large percentage of cancers and is not expressed in most normal somatic tissue, it has also been of great interest as a potential cancer therapeutic. Current telomerase-based therapies include a competitive inhibitor that binds to the telomerase RNA template in the form of an oligonucleotide called GRN163L or Imetelstat, a nucleoside analogue called 6-thio-2'-deoxyguanosine (6-thio-dG) that is incorporated into telomeres by telomerase and is able to specifically uncap telomeres in telomerase positive cells, anti-telomerase immunotherapy, and telomerase-based viral therapies that use the TERT promoter

to drive apoptotic genes or adenovirus replication (Buseman, Wright, & Shay, 2012; Mender, Gryaznov, Dikmen, Wright, & Shay, 2015; Mender, Gryaznov, & Shay, 2015) (Supplemental Table 1). Better understanding of TERT regulatory mechanisms should open other potential pathways with which to pursue this almost universal target in human cancers. The mechanism of TERT/telomerase regulation that is the focus of this dissertation is the alternative splicing regulation of full-length hTERT mRNA.

CHAPTER TWO
Review of the Literature

SPLICING IN CANCER

Introduction to splicing

Pre-mRNA splicing is a critical step of mRNA processing which involves the removal of introns and joining of exons to form mature mRNA. This process is catalyzed by a ribonucleoprotein complex called the 'spliceosome,' which is composed of core factors and over 300 accessory factors. The major spliceosome consists of five small nuclear RNAs (U1, U2, U4, U5, and U6) that interact with protein factors to catalyze assembly and splicing the vast majority of human introns. During the initial steps of splicing, U1 recognizes the 5' splice site and U2 is recruited to the branch point. U1 and U2 interact to form Complex A. The U4, U5 and U6 tri-snRNP complex interacts with the initial spliceosome complex to form Complex B. U4 is displaced, allowing U6 to replace U1 at the 5' splice site. This results in a catalytically active complex, resulting in two transesterification reactions which result in the removal of the intron as a lariat and the ligation of exons (Will & Luhrmann, 2011). A very small percentage of introns are spliced by the minor or U12-dependent spliceosome which has been less studied but also has recently been implicated in a number of diseases (Madan et al., 2015; Onodera et al., 2014; Reber et al., 2016; Turunen, Niemela, Verma, & Frilander, 2013).

The regulation of these processes is extremely complex. Indeed, over 90% of multi-exon genes are alternatively spliced, producing multiple mRNA isoforms from a single gene (Pan, Shai, Lee, Frey, & Blencowe, 2008; E. T. Wang et al., 2008). This greatly increases the protein diversity of a cell by allowing a single gene to produce multiple mRNA isoforms and proteins (Nilsen & Graveley, 2010). The different mRNA isoforms can alter mRNA stability, localization or translation while the proteins generated from these mRNA isoforms may have similar, different or even opposite functions in cells. Alternative splicing is modulated by RNA binding proteins, which can by interacting with enhancer or silencer elements located in introns and exons to promote or repress their inclusion. The process of alternative splicing is extremely dynamic and is well established to contribute to organ development, cell differentiation and tissue identity maintenance throughout the human body (Baralle & Giudice, 2017; Mauger & Scheiffele, 2017; E. T. Wang et al., 2008).

Splicing plays a key role in determining the function of transcribed genes. This process has been known to be altered in disease states including cancer. For example, a number of splicing factors have been identified to be mutated in cancer and can have gain or loss of function effects on cell biology (Figure 4). Genetic mutations can also indirectly result in aberrant expression of splicing factors, leading to spliced isoforms of genes which promote cell proliferation, metastasis, or angiogenesis (Figure 5). Other aspects of splicing regulation such as transcriptional and epigenetic changes can also be abnormal in cancer, promoting cancer progression and survival via splicing.

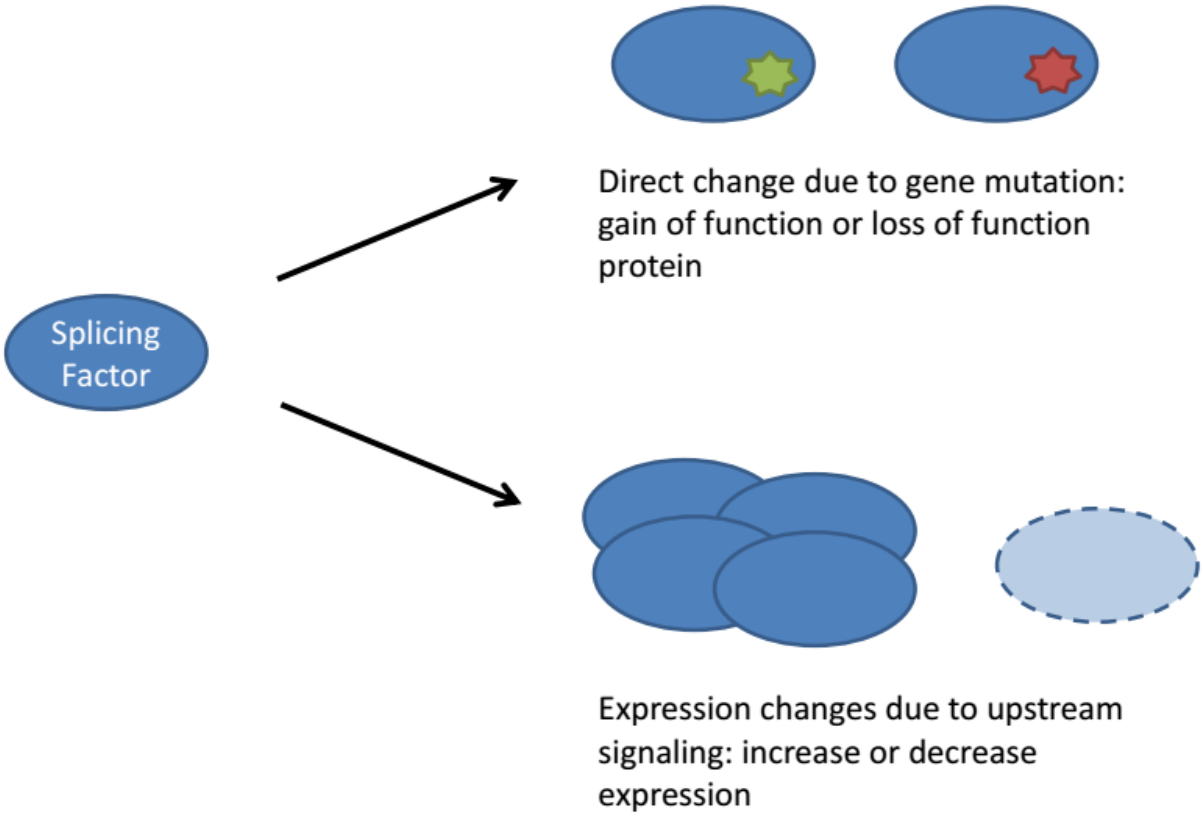

Fig 4. Splicing factor changes in cancer
Splicing factors can be directly mutated in cancer, resulting a gain or loss of function protein. They can also have dramatic changes in expression levels due to changes in upstream signaling during cancer progression.

Mutated splicing factors as oncogenes

Large scale sequencing efforts have identified a number of recurrent mutations in splicing factors, especially in hematological malignancies, suggesting splicing factors could play a critical role in cancer progression. Of the mutations found in splicing factors, the best characterized and most commonly detected are SF3B1, SRSF2 and U2AF1. The pattern of mutation in these genes suggests gain or change of function, leading to the theory that these genes are functioning as proto-oncogenes in cancer.

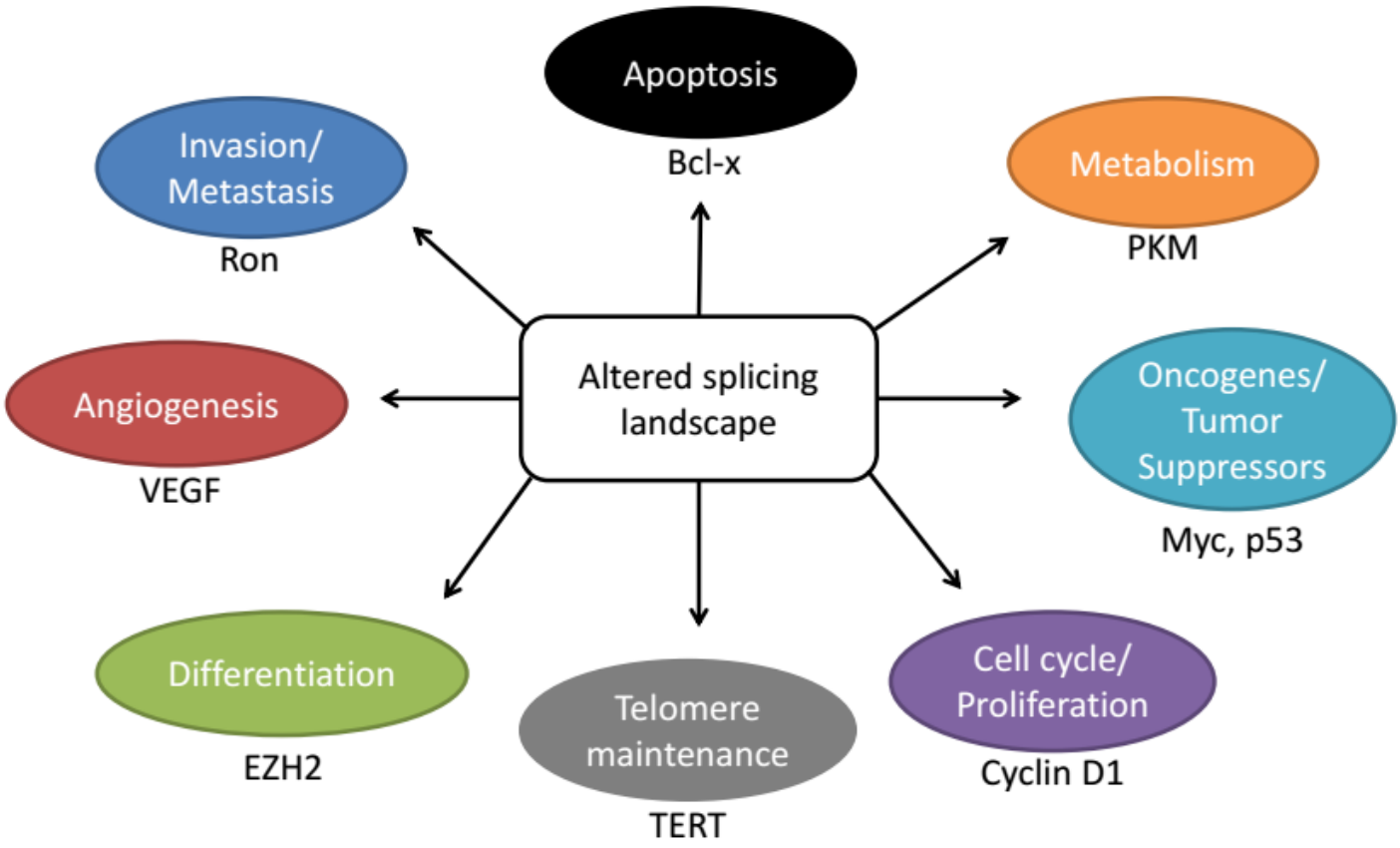

Fig 5. Effects of altered splicing on cancer biology
Altered splicing by mutational changes or changes in expression have been shown to have vast effects on cancer biology. They have been specifically found to affect apoptosis, metabolism, oncogenes/tumor suppressors, cell cycle control/proliferation, telomere maintenance, differentiation, angiogenesis and invasion/metastasis. These are examples of direct targets but generally multiple targets have been identified to be affected by splicing in most of these critical functions.

SF3B1: Splicing factor 3b subunit 1

The single most commonly detected mutated splicing factor in cancer, SF3B1 was identified many years ago to have oncogenic properties in hematological and solid cancers. SF3B1 is a component of the U2 snRNP complex that is binds near the branch point and is key in 3' splice site recognition for many genes (Gozani, Potashkin, & Reed, 1998; Wahl, Will, & Luhrmann, 2009). Mutations tend to cluster in the HEAT (Huntingtin, Elongation factor 3, protein phosphatase 2A, Targets of rapamycin 1) repeats but the function of these domains is

not well understood. Multiple groups have suggested that SF3B1 mutants may be able to alter 3' splice site choice or recognize cryptic 3' splice sites (Darman et al., 2015; DeBoever et al., 2015). Later, other groups have discovered that SF3B1 mutants are able to promote usage of alternative branch point and to recognize normally inaccessible cryptic 3' splice sites (Alsafadi et al., 2016; Kesarwani et al., 2017) (Figure 6). Interestingly, while many other proteins are also involved in U2 snRNP function and 3' splice site selection, SF3B1 mutation seems to be specifically advantageous for oncogenesis as it is so frequently detected.

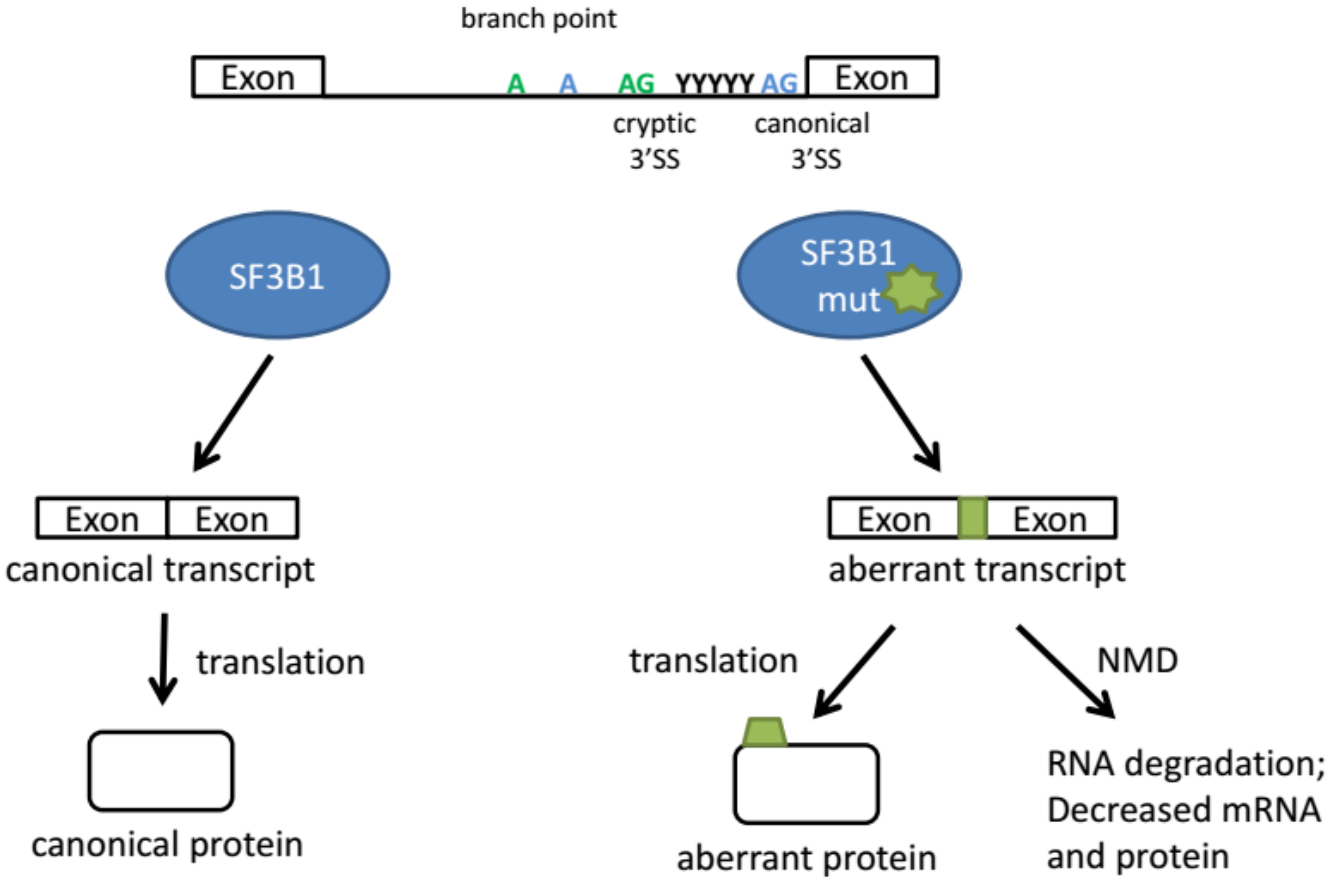

Fig 6. Effects of SF3B1 mutation in cancer (Adapted from Darman et al. 2015)
Cancer-associated mutations in SF3B1 allow SF3B1 to recognize cryptic 3' splice sites. This results in aberrant transcripts which can then be translated into aberrant proteins or degraded by NMD. This will then have downstream effects on those targets by altering functionality or decreasing target expression.

SRSF2: Serine/arginine-rich splicing factor 2

SRSF2 is another commonly detected mutated splicing factor in cancer, most commonly found in chronic myelomonocytic leukemia (CMML). SRSF2 binds to exonic splicing enhancers (ESE) and promotes exon recognition for constitutive and alternative splicing. All detected SRSF2 mutations to date involve the P95 residue, located near its RNA recognition motif (RRM) (Komeno et al., 2015; Thol et al., 2012; Yoshida et al., 2011). RNA-seq results suggest that mutations shifts its preferred binding sequence to CCNG compared to wild-type SRSF2 which binds CCNG and GGNG with similar affinity (J. Zhang et al., 2015). Mutant SRSF2 is sufficient to drive myelodysplasia and results in aberrant splicing of hundreds of downstream genes, including enhancer of zeste homologue 2 (EZH2) (E. Kim et al., 2015). Specifically, mutant SRSF2 causes the inclusion of an in-frame stop codon and consequent nonsense-mediated decay (NMD) of EZH2 which disrupts hematopoietic differentiation.

U2AF1: U2 small nuclear RNA auxiliary factor 1

U2AF1 is mutated in both hematologic and solid cancers-most commonly in myelodysplastic syndromes (MDS) and lung cancer (Imielinski et al., 2012; Yoshida et al., 2011). It normally binds the 3’ splice site in a sequence-specific manner but is only required for a subset of AG-splice sites. Mutations in hematologic cancers have been shown to shift 3’ splice site recognition and affects splicing of genes involved in DNA damage response and apoptosis (Ilagan et al., 2015; Okeyo-Owuor et al., 2015).

Dysregulated splicing factors as oncogenes

In addition to somatic mutations, splicing factor expression changes have also been identified in many cancer types. Some of the most studied splicing factors that are overexpressed or have growth-promoting effects in cancer include hnRNP A1, SRSF1, SRSF3 and PTBP1.

hnRNP A1: heterogeneous nuclear ribonucleoprotein A1

The hnRNP family of proteins plays a key role in exon recognition and numerous members have been found to be overexpressed in different types of cancer. hnRNP A1 is one of the most abundant and has been observed to be overexpressed in many human cancers including colorectal, hepatocellular, and lung cancer (Liu, Zhou, Lou, & Zhong, 2016; Ma et al., 2009; Zhou et al., 2013). hnRNP A1 has many functions in cells from transcription and splicing to mRNA stability, export and translation (Jean-Philippe, Paz, & Caputi, 2013). Interestingly, hnRNP A1 has also been shown by multiple groups to have important functions in telomere biology including physical association with telomere ends and an important role in telomere capping after DNA replication (Flynn et al., 2011; Jean-Philippe et al., 2013; Le, Maranon, Altina, Battaglia, & Bailey, 2013; Q. S. Zhang, Manche, Xu, & Krainer, 2006). Its functions in cancer biology have been studied and attributed, in part, to its ability to activate NF-κB by directly interacting with IκBα, to its inhibitory effects on apoptosis by promoting anti-apoptotic splicing of Bcl-x and Mcl-1, and to its regulatory effects on cell metabolism through the alternative splicing of pyruvate kinase (M. Chen, Zhang, & Manley, 2010; Kedzierska & Piekielko-Witkowska, 2017; Sahu, Sangith, Ramteke, Gadre, & Venkatraman,

2014). Knock down of hnRNP A1 in various cancer cell lines have been associated with increased apoptosis , decreased cell growth, and colony formation (Liu et al., 2016; Patry et al., 2003). Recently, hnRNP A1 has also been implicated as a downstream target of EGF signaling. Specifically, EGF activation results in ubiquitination of hnRNP A1, resulting in altered splicing of cytoskeletal factors and increased cell migration (F. Wang et al., 2017).

SRSF1: Serine/arginine-rich splicing factor 1

The SRSF family of proteins functions predominantly as RNA-binding proteins required for constitutive splicing and major regulators of alternative splicing. Generally they have been characterized as factors that promote exon inclusion but they have other functions throughout RNA metabolism from transcription and nuclear export to stability and translation (Kedzierska & Piekielko-Witkowska, 2017). Many members of this family of proteins were observed to be overexpressed in cancer and found to act as oncoproteins. SRSF1 was the first to be characterized as having oncogenic properties and has been well studied and reviewed (Das & Krainer, 2014; Kedzierska & Piekielko-Witkowska, 2017). Overexpression of SRSF1 was sufficient to transform immortal rodent fibroblasts which were able to form sarcomas in xenograft models and can cooperate with MYC to transform mammary epithelial cells (Anczukow et al., 2012; Karni et al., 2007). In fact, SRSF1 is a transcriptional target of MYC and is responsible for part of the oncogenic activity of MYC (Das, Anczukow, Akerman, & Krainer, 2012). Various groups have identified a number of processes which are affected by SRSF1 which contribute to its oncogenic properties including apoptosis, cell cycle

regulation, metastasis and angiogenesis (Kedzierska & Piekielko-Witkowska, 2017). Inhibition of SR proteins like SRSF1 has been largely done by targeting kinases that phosphorylate SR proteins like SRPK and CLK. For example, SRSF1 is able to effectively shift VEGF splicing from anti-angiogenic isoforms to a highly pro-angiogenic isoform (Guyot et al., 2017). SRPK inhibitors have similarly been tested and found to be effective at shifting VEGF splicing and can trigger apoptosis in leukemia models (Batson et al., 2017). These inhibitors have had some preclinical success but there is still a lot to learn about whether or not more specific inhibitors targeting SRSF1 will work as a cancer therapeutic.

SRSF3: Serine/arginine-rich splicing factor 3

SRSF3 is another member of the SRSF family which has strong association with cancer-specifically oral squamous cell carcinoma and ovarian cancer (Guo, Jia, & Jia, 2015; He et al., 2011). Additionally, it has been shown to be important for other cancer types including breast and colon cancer (Kurokawa et al., 2014). Important targets of SRSF3 include HER2, whose splice variants switch from oncogenic to tumor suppressive with loss of SRSF3, and p53, which can also be spliced to induce cellular senescence in the absence of SRSF3 (Gautrey et al., 2015; Tang et al., 2013).

PTBP1: Polypyrimidine tract binding protein 1

PTBP1, also known as hnRNP I, is an RNA binding protein which binds pyrimidine-rich sequences and is involved in a number of mRNA processing including splicing,

polyadenylation and IRES-mediated translation (Castelo-Branco et al., 2004; Grover, Ray, & Das, 2008; Mitchell et al., 2005; Xue et al., 2009; Zheng, Huynen, & Baker, 1998). PTBP1 has been studied extensively as a major repressor of neuronal differentiation and has also been shown to be a regulator of microRNA function-able to repress and enhance microRNA activity on different targets (Xue et al., 2013). PTBP1 has been observed to be overexpressed in a number of cancer types including breast and colorectal cancers (He et al., 2014; Takahashi et al., 2015). It has a wide variety of functions and targets which allow it to promote cancer development, from promoting cell survival by shifting BCL-X splicing toward an anti-apoptotic isoform, to altering cell motility by alternative splicing of cortactin, to promoting aerobic glycolysis over oxidative phosphorylation by splicing pyruvate kinase into the M2 isoform (Bielli, Bordi, Di Biasio, & Sette, 2014; M. Chen, David, & Manley, 2010; M. Chen, Zhang, et al., 2010; Z. N. Wang et al., 2017). Knockdown of PTBP1 in cancer cell lines was able to decrease cell proliferation, migration and invasion but overexpression was not sufficient to transform normal cells (Jiang et al., 2017; C. Wang et al., 2008).

Splicing factors as tumor suppressors

In addition to promoting cancer growth and progression, a number of splicing factors have been identified with loss of function or decreased expression in cancer, suggesting that they may be tumor suppressive. The most well-studied include ZRSR2, QKI, and a number of RBM proteins.

ZRSR2: Zinc Finger CCCH-Type, RNA Binding Motif and Serine/Arginine Rich 2

ZRSR2 mutations have been observed at low frequency in a number of hematologic cancers. Normally ZRSR2 functions as a critical component of the minor spliceosome, which is important for splicing U12-type introns. Unlike other mutated splicing factors which have clustered mutations that alter function, ZRSR2 mutations are distributed throughout the gene and include frameshift and nonsense mutations. Mutant ZSRS2 and direct knock down results in increased U12-type intron retention and had significant effects on MAPK signaling, ErbB signaling and hematopoietic differentiation (Madan et al., 2015). Together with the fact that it is located on the X chromosome and is associated with male cancer patients, ZRSR2 seems to be functioning as a tumor suppressive splicing factor which can be lost due to mutation in cancer.

QKI: Quaking

QKI has been found to be frequently down regulated in a number of cancers including testicular, lung, bladder, cervical, breast and ovarian cancers (Novikov et al., 2011). It is normally involved in a number of mRNA-related processes from splicing to mRNA transport and stability. QKI was found to be able to inhibit lung cancer growth in vitro and in vivo. This may be in part through QKI's role in the alternative splicing of macroH2A1.1, a histone variant that is suppressed in many cancer types, NUMB, a regulator of Notch signaling, and ESYT2, a cytoskeleton regulator with differential splicing in cancer (de Miguel et al., 2016;

Novikov et al., 2011; Zong et al., 2014). Low QKI expression has been reported to be a prognostic factor associated with worse survival in gastric, lung and prostate cancer (Bian et al., 2012; de Miguel et al., 2016; Y. Zhao et al., 2014).

RBM: RNA-binding motif proteins

RBM proteins are characterized by the presence of one or more RNA binding motifs (RBMs). A number of these have been found to regulate the splicing of apoptotic genes (Sutherland, Rintala-Maki, White, & Morin, 2005). For example, RBM4 has been found to be downregulated in a number of cancers and is able to promote pro-apoptotic isoforms of BCL-X (Y. Wang et al., 2014). Other RBM proteins like RBM5 are able to alternatively splice CASP2 and FAS (Bonnal et al., 2008; Fushimi et al., 2008). Overexpression of RBM5 in a lung cancer cell lines was able to suppress cell growth and induce apoptosis (J. J. Oh et al., 2006). Another RBM protein, RBM10, has been found to be frequently mutated in lung cancer (Imielinski et al., 2012). Follow up studies found that cancer-associated mutations result in loss of function RBM10 and is able to disrupt NUMB splicing, inducing a pro-growth isoform (Hernandez et al., 2016; J. Zhao et al., 2017).

Other regulatory factors

In addition to ‘cis’ (RNA binding sites) and ‘trans’ (RNA binding proteins) elements, alternative splicing is also regulated by transcriptional regulatory processes due to the tight coordination between splicing and transcription (Gornemann, Kotovic, Hujer, & Neugebauer,

2005). Thus, factors that affect transcription, for example RNA Pol II phosphorylation, nucleosome positioning and epigenetic markers, may also have direct effects on splicing (Batsche, Yaniv, & Muchardt, 2006; Munoz, de la Mata, & Kornblihtt, 2010; Tilgner et al., 2009). For example, RNA Pol II has highly conserved repeats in its C-terminal domain (CTD) which can be differentially phosphorylated and affect splicing (Munoz et al., 2010). Additionally, splicing factors have also been shown to have other effects on cellular processes outside of their canonical functions in splicing. Many splicing factors have been shown to have critical roles in genomic stability, DNA damage response, mRNA export and translation, and nonsense mediated decay. There is still much to understand about the complex relationships between these cellular processes and how they can influence each other.

Additionally, the function of a given splicing factor is not always consistent between cancers or even within a certain cancer type. Numerous splicing factors have been observed to act as tumor suppressors in some contexts and oncogenic in others. It is important to consider the importance of the splicing landscape and cellular context of cancers when studying the complex process of splicing. Another complicating aspect of splicing factors is their widespread effects on cell biology due to the diverse functions of their targets. This could be harmful in a therapeutic setting, potentially introducing negative side effects, but it could also be beneficial and allow one drug to target a number of pathways important for cancer growth. Targeting splicing factors could potentially allow us to manipulate otherwise difficult to

target genes. Better understanding of the interaction within splicing factors and between splicing factors and other cellular processes will allow us to manipulate them for therapeutic purposes.

CHAPTER THREE
Methods and Materials

Plasmids

Human telomerase (hTERT) exon and intron sequences were inserted into pcDNA5/FRT expression vector (Invitrogen, Carlbad, CA) and were modified to exclude exons 5 and 10 and introns 5 and 9 to shorten the construct (Wong et al., 2013). The hTERT minigene was modified with renilla and firefly luciferase fragments. Lentiviral plasmid for SEAP (secreted embryonic alkaline phosphatase, Clontech), GFP pGIPZ shRNA plasmids for control (non-targeting), NOVA 1 (Openbiosystems, NOVA 1 – 5'- TTGGACTTAGACAGCTTGA), and PTBP1 (Openbiosystems, PTBP1 – 5'-TCTGGAAGAACTTGAATCC) were obtained. Lentivirus was made by co-transfecting 5 μg of proviral shRNA plasmids and 2 μg of packaging plasmids pMD2.G and psPAX2 using Polyjet transfection reagent (SignaGen Laboratories) into 293FT cells. CCSB-Broad lentiviral human NOVA 1 full length cDNA with a C-terminal V5 tag and blasticidin selection in mammalian cells (accession: BC075038, clone ID: ccsbBroad304_01104) was purchased and sequence verified by our group (GE, Dharmacon). Viral particles were produced as above. ShRNA resistant NOVA 1 cDNA was generated by site directed mutagenesis (Agilent Quickchange). Retroviral particles were generated in 293 FT packaging cells with pUMVC and pVSVG.

Cell culture and cell lines

All non-small cell lung cancer cell lines (H1299, H920, Calu6, H1819, H1993, H2882, H2887, and HCC1359) were cultured at 37ºC in 5% CO2 in 4:1 DMEM:Medium 199

containing 10% calf serum (HyClone, Logan, UT). HBECs were maintained as previously described in low oxygen conditions (Ramirez et al., 2004).

Human lymphocytes

A human peripheral blood sample were obtained from a healthy donor (32 years old, female) after informed consent and in accordance with the Institutional Review Board (IRB) at UT Southwestern Medical Center. The subject included in this study was a nonsmoker with no history of alcohol abuse or drug consumption. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation with Ficoll-Paque Plus (GE Healthcare) and T cells were further isolated by negative selection. T cells were cultured in RPMI+GlutaMAX-I with 10% fetal bovine serum, 1% penicillin, streptomycin and amphotericin B and 10 ng/mL interleukin-2. Cells were stimulated 24 hours after isolation by adding Dynabeads Human T-Activator CD3/CD28 (Life Technologies) in a 1:1 ratio. The percent of live cells was determined every day by trypan blue exclusion using a TC20 Automated Cell Counter (BioRad). When the cell density reached $\geq 1.5\times10^6$/ml, cells were diluted with fresh complete RPMI medium to a density of below 1.0×10^6/ml. Following stimulation cells were collected for RNA and TRAP analysis at 1, 3, 5, 7, and 10 days.

Cancer growth assays

Stable knockdown cells and controls (non-targeting shRNA) were suspended in 0.375% Noble agar (Difcon, Detroit) in supplemented basal medium at two densities (1,000 and

2,000 cells) and overlaid on 0.75% Noble agar in 24-well plates. Each density was seeded in triplicate and each assay was performed twice. Colony formation efficiency was calculated the by the average number of colonies counted per cell divided by the number of cells seeded. Colonies larger than 0.1 mm were measured and counted after 10 days of growth and the average of the counts was used. Data are plotted as fold change over non-targeting shRNA cells. Data were analyzed with two tailed Student's t-tests (L. Zhang, Komurov, Wright, & Shay, 2013).

Invasion was determined using Boyden chamber assays. Briefly, cells were serum starved overnight (~16hrs) prior to assays for invasion. 24-well Matrigel-coated transwell filters (BD Biosciences, San Jose, CA) were thawed and rehydrated according to manufacturer's instructions. Cells were collected and re-suspended in serum free media and added to the top chamber in duplicate. The bottom chamber was filled with 2% serum containing media (4:1 DMEM:Medium 199) as a chemoattractant. Cells were incubated overnight. Non-invaded cells were scraped off with a cotton swab and wells were washed with PBS. Invaded cells were fixed for 5 min with 10% neutral buffered formalin and stained for 10 min with Hoechst (Invitrogen). Images were taken at 10X magnification (Ly et al., 2012).

For colony formation assays, cells were plated at clonal density (30-70 cells per 2.5cm2 dish) in 10% serum containing media. Cells were analyzed seven days after plating by staining with Hoechst (Invitrogen) (Ly et al., 2013).

Xenograft

All animal experiments were approved by the University of Texas Southwestern (UTSW) Institutional Animal Care and Use Committee (IACUC) and conducted as per institutional guidelines. Athymic NRC nu/nu nude mice (~4-6 weeks old, Charles River) were purchased. Tissue culture cells from H920 control shRNA, H920 NOVA 1 shRNA, Calu6 control (wild type with empty lentiviral vector), and Calu6 plus NOVA 1 cDNA were cultured and injected subcutaneously into the hind flanks. For H920 cells, 5 million cells in 100 microliters of 1X PBS were injected. For Calu6 cell 1 million cells in 100 microliters of 1X PBS were injected. Tumor growth was monitored by caliper measurement once or twice weekly. Tumor volume was calculated (Volume = $(\text{width})^2$ x length/2).

Transient siRNA experiments

For transient knockdown experiments cells were plated in 6-well plates (150,000 cells per well) and were transfected with non-silencing controls (Santa Cruz Biotechnology, sc-37007) or a pool of three siRNAs targeting NOVA 1 (Santa Cruz Biotechnology, sc-42142: sense RNA sequences – 1. 5'-GACAGACAAUUGUUCAGUUtt, 2. 5'-GAACGGUUGAAGCACUGAAtt, 3. 5'-GACCACCGUUAAUCCAGAUtt) or PTBP1 (Santa Cruz Biotechnology, sc-38280: sense RNA sequences – 1. 5'-CCAAGAACUUCCAGAACAUtt, 2. 5'-CUUGUGGUAUUACCUUGUAtt, 3. 5'-GCAAUUCCAGGCUCAGUAUtt). Cells were plated 24hrs prior to transfections. On the

day of transfection, media was switched to 2% serum and transfection complexes were prepared with 50nM of siRNAs using MEM (Gibco, Invitrogen) and RNAi max (Invitrogen) following manufactures procedures. Following 72hrs of exposure to siRNAs, cells were washed, trypsinized, counted and pelleted for RNA extraction and telomerase activity assays.

Western blot analysis

Total protein lysates were extracted from tissue culture cells using Laemmli buffer, boiled and the protein concentration determined (BCA protein assay, Pierce). Thirty micrograms of protein was resolved on SDS-PAGE gels, transferred to PVDF membranes and detected with a rabbit monoclonal antibody for NOVA1 (Abcam, EPR13847, ab183024, 1:1000 dilution in 5% BSA) or PTBP1 (Abcam, EPR9048B, ab133734, 1:10,000 dilution in 5% NFDM). Protein loading was determined with antibodies against with beta actin (Sigma) or histone H3 (Sigma).

Reverse transcriptase-droplet digital PCR

Tissue panel RNAs were purchased (Clontech, 20 tissue panel II). Three sets of cDNAs were made with a 1:1 mixture of random hexamer and oligo-dT priming with three different reverse transcriptases: 1. iScript advanced (42ºC, Bio-Rad), 2. Superscript III (55ºC, Invitrogen,) and 3. AMV (50ºC, Invitrogen). All RNA samples were spiked with a known amount of MS2 bacteriophage RNA to enable normalization of absolute molecule counts from droplet digital PCR. For tissue panel hTERT and NOVA 1 mRNA analysis we used

three RTs because we observed differences in detection of hTERT using different RTs so to be able to eliminate spurious measures of low abundance targets we averaged data for all three RTs. All cDNAs were diluted 1:4 before use and stored at -80ºC. For hTERT splicing analyses we used iScript Advanced (Bio-Rad) to generate cDNAs, diluted 1:4 and used within 48 hrs of production in ddPCR measures. Primers and probe sequences for TERT are listed in Supplemental Table 2.

Droplet digital TRAP assay (telomerase activity)

Quantitation of telomerase enzyme activity was performed as described in (Ludlow et al., 2014).

Telomere length analysis

The average length of telomeres (terminal restriction fragment lengths) was measured as described in (Y. Zhao et al., 2009) with the following modifications. DNA was transferred to Hybond-N+ membranes (GE Healthcare, Piscataway, NJ) using vacuum transfer. The membrane was briefly air-dried and DNA was fixed by UV-crosslinking. Membranes were then probed for telomeres using a DIG-labeled telomere probe, detected with an HRP-linked anti-DIG antibody (Roche) and exposed with CDP-star (Roche) (Lai, Wright, & Shay, 2016).

Minigene screen set up and reporter assays

HeLa cells harboring the hTERT splicing reporter minigene were plated in 96 well plates

24hrs prior to transfection. Plates were transfected using RNAi max (Invitrogen) with 1 nM of pools of four siRNA (sequences in available by request) and cells were analyzed 72 hrs after transfection. Conditioned media (20 μL from the siRNA transfected cells) was analyzed for SEAP (Great EscAPe SEAP Chemiluminescence kit) and cells were lysed in passive lysis buffer and analyzed for renillia and firefly luciferase following manufactures instructions (Dual-luciferase reporter assay system, Promega). Assays were performed on two separate days and data were averaged.

Ultra-violet immunoprecipitation reverse transcriptase droplet digital PCR (UV-IP RT-ddPCR)

UV-IP was performed as described (Sei & Conrad, 2014) with slight modifications. Briefly, 90% confluent cells were crosslinked with UV-C (254 nm, 250 mj/cm2), scrapped, washed and pelleted. Pellets were then lysed in RIPA buffer containing an RNase inhibitor (Ambion), mixed with antibodies conjugated to magnetic beads (rabbit IgG or rabbit monoclonal NOVA 1, Abcam, EPR13847, ab183024), and washed. RNA was extracted and RT-ddPCR performed with a series of primers designed near and around the in silico identified potential NOVA 1 binding sites (Figure 10 A and B).

Cross-linking immunoprecipitation (CLIP RT-ddPCR)

To perform CLIP we UV (UV-C, 254 nm, 250 mj/cm2) crosslinked cells in 15 cm dishes with 3 mL 1 x PBS. Seven milliliters of ice cold 1 x PBS was added to the crosslinked cells

and the cells were the scrapped and collected by centrifugation (4ºC, 0.2 x G for 5 minutes). Supernatant was removed and 1 mL of ice cold 1 x PBS was added and the cell spun and pelleted a second time in a 1.5 mL microcentrifuge tube. The cells were lysed in 0.5% SDS lysis buffer (Tris HCl, pH 7, 1 mM EDTA, 1 mM DTT, RNase inhibitor (Ambion), and 1 mM phenylmethylsulfonyl fluoride; 140 µL per sample) and heated to 65ºC for 5 min and immediately placed on ice for 5 minutes. The lysate was volume corrected to 700 µL in RIPA correction buffer (1.25% NP40, 0.625% sodium deoxycholate, 62.5 mM Tris HCl, pH 8, 2.25 mM EDTA, 187.5 mM NaCl, RNase inhibitor (Ambion)), and 1 mM phenylmethylsulfonyl fluoride; 560 µL per sample). The lysate (700 µL) was then passed over a Qiashredder® column twice (centrifuged for 30 sec at 13,000 x G at 4ºC). The entire lysate was then centrifuged for 15 min at 16,000 x G, 4ºC for 15 min and the supernatant transferred to a new microcentrifuge tube. The lysates were then treated with micrococcal nuclease (0.15 Units in 50 mM CaCl2 buffer for 10 mins at 37ºC), immediately placed on ice and 20 mM EGTA added to quench the micrococcal nuclease activity. Antibodies and beads were prepared at room temperature in sodium phosphate buffer (0.1 M, pH 8.1; Anti-Nova1 antibody [EPR13847] (ab183024), Abcam; Rabbit IgG) and mixed with magnetic protein A/G beads (Dynabeads, 50 µL per sample) and added to the cleared lysate. The samples (NOVA 1 or IgG) were immunoprecipitated at 4ºC for four hours. The samples were then washed five times in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, Tris-HCl, pH 8, 2 mM EDTA). After the final wash the bead-protein-RNA complexes were re-suspended in 200 µL of RIPA buffer and 2 U of RNase-free DNase (Ambion) was added

in 300 μL of DNase buffer and incubated for 10 min at 37ºC with gentle agitation. Beads were collected with the magnet and supernatant removed. Proteinase K buffer was added (0.5 mg *mL-1 proteinase K, 0.5% SDS, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 8 ng of MS2 RNA, 5 μL of MRC RNA precipitation carrier; 300 μL per sample) and incubated at 37ºC for 15 min with shaking. RNA was then precipitated with sodium acetate and phenol chloroform. Following RNA precipitation reverse transcription was performed with Superscirpt III® (Invitrogen) with random hexamers. Droplet digital PCR was performed with Evagreen®.

RNA pulldown with biotinylated RNA baits

A plasmid was generated (TOPO TA) via PCR from a BAC containing hTERT (RP11-990A6, CHORI) using primers that generated a 1 kb fragment of hTERT intron 8 including DR8. Following integration into the TOPO TA vector, in vitro transcription was performed using the T7 promoter (Ampliscribe T7 kit, Ambion, Life technologies) following the manufacturer instructions including a 45 min DNase step prior to RNA precipitation. RNA was isolated and biotinylated at the 3' end (Pierce RNA 3' end biotinylation kit). Biotinylated RNA was purified with streptavidin beads. Cell lysates were prepared following the kit instructions (Peirce Magnetic RNA-protein pull-down kit). Protein-RNA complexes were immunoblotted for NOVA 1 following pulldown. To produce the smaller RNA baits, T7 promoter sequences were incorporated into the 5'end of the forward primers of each region of interest in and surrounding hTERT DR8. The same procedure was followed as above to generate the RNA baits. In both cases 293 FT cells were transfected with a full

length (exon 4 containing) V-5-tagged NOVA 1 cDNA construct using Lipofectamine 2000. After 48 hours, triplicate samples of 10 x 10^6 cell were washed, typisinized, counted, pelleted and frozen at -80ºC until analysis.

Genome editing and engineering methods (CRISPR/Cas9 methods)

To delete hTERT DR8, we designed two guide RNAs (pre-DR8 guide – 5'-ATCTGCTTGCGTTGACTCGC and post-DR8: 5'-TTATTTTCGGGAAGCGCTAT) and cloned these guides into PX458 (Addgene Plasmid #48138 - pSpCas9(BB)-2A-GFP). Cells were transfected using Lipofectamine 3000® following manufacturer's instructions for scaling up to 10 cm dish. Forty-eight hours after transfection cells were flow sorted for the top 5% GFP positive cells into individual wells of three 96-well plate. After about 14 days, wells with growing cells were scaled up to a 6 well plate and once confluent scaled up to a 10 cm dish. Cells were collected for DNA extraction and genotype analysis of CRISPR mutation validation. To validate the deletion the 480-base pair sequence containing DR8 of hTERT, we performed PCR with two different primer sets of different sizes to ensure our results were robust. We also isolated the PCR product from primer set two and performed Sanger sequencing to verify the recombination event was between two TERT alleles and not a different sequence in the genome.

To mutate endogenous hTERT we had two plasmids synthesized (SGI-DNA). The wild-type plasmid (WT – hTERT intron8 mutant PAM in pUC-SGI) contained 2000 bases of hTERT

intron 8 surrounding DR8 that had the PAM sequence of the post-DR8 guide above mutated from 5'-CCT to ACT. The mutant plasmid (MUT- 7 x 'YAAY' DR8 MUT hTERT mutant PAM in pUC-SGI) was identical to the wild type plasmid except that we mutated all seven of the 'YCAY' motifs in DR8 to 'YAAY' (changing the central CA to AA is known to block NOVA 1 recognition (Leggere et al., 2016)). We then co-transfected the guide RNA post-DR8 and either WT or MUT plasmid in the presence or absence of NHEJ inhibitor SRC7-pyrazine. We pre-treated cells with 50 nM SCR7 for 30 min prior to transfection. Cells were transfected with Lipofectamine 3000® and 48 hours later the top 5% GFP positive cells were sorted as above. Once wells with growing cells were identified we scaled up the clones as above. We isolated DNA from the clones to validate the insertion of WT or MUT plasmids. To validate WT insertion clones, we used PCR to amplify a sequence surrounding DR8 and purified and sequenced the PCR product via Sanger sequencing. To screen and validate MUT clones, we used PCR to amplify a region around DR8 and then digested the DNA with BclI. The WT sequence does not contain a BclI site but the insertion of MUT plasmid introduced a BclI site at YCAY/YAAY sites 4 and 5 of DR8 thus allowing the easy identification of MUT containing clones. We also Sanger sequence validated the MUT clones.

Study approval

A human peripheral blood sample were obtained from a healthy donor (32 years old, female) after informed consent and in accordance with the Institutional Review Board (IRB) at UT Southwestern Medical Center. All animal experiments were approved by the University of

Texas Southwestern (UTSW) Institutional Animal Care and Use Committee (IACUC) and conducted as per institutional guidelines.

Statistics

Unless otherwise noted in methods section, figure legend, or in the results section, pairwise Student's t tests (2-sided) were used to determine statistically significant differences between group means. Significant differences were accepted at a p value less than 0.05. For analysis of microarray data, we correlated the expression data of 528 RNA binding proteins across six cell lines (differential expression analysis (t tests), false-discovery rate corrected with Benjamini-Hochberg procedure).

CHAPTER FOUR
NOVA1 Results

NOVA1: Neuro-oncological ventral antigen 1

Background

Our work and the findings of others suggest that transcriptional regulation is only part of the complex repressive machinery that prevents telomerase activity in normal cells. Very few investigations into the cis- and trans-acting factors that regulate the splicing of TERT have been performed to date. Additionally, a variable nucleotide tandem repeat in intron 6 that may form RNA secondary structures with distal regions of TERT RNAs in intron 8 and regulate minus beta splicing was also identified (Wong et al., 2013; Wong, Shay, & Wright, 2014). However, little is currently known about the trans-acting factors that bind these regulatory regions. These highly conserved sequences, such as 'direct repeat 8' (called a repeat because it has 85% homology with a similar sequence in intron 6 of TERT). Thus, identification of such proteins would close a significant gap in telomerase regulation knowledge and also potentially identify protein targets to shift the splicing of TERT message to inactive forms to reduce telomerase activity and replicative capacity of tumor cells by shortening telomeres.

To address this deficiency, we took two approaches: a TERT dual-luciferase minigene splicing reporter RNAi screen and bioinformatic analyses of a panel of highly characterized

human lung cancer cell lines to identify candidate genes that regulate hTERT splicing. Based on the screen and bioinformatics analyses, we decided to focus additional experiments on NOVA1 (neuro-oncological ventral antigen 1).

In non-small cell lung cancer cells that express high levels of NOVA1, we found that stable reduction in NOVA1 levels shifted hTERT splicing toward inactive transcripts, reduced telomerase activity, which led to progressively shortened telomeres, reduced migration through extracellular matrices, and resulted in smaller tumors in vivo. Further, we demonstrate via CLIP assays, RNA pulldown, and CRISPR/Cas9 manipulation that NOVA1 directly binds hTERT. Thus, the experiments described below provide a mechanistic example of how cancer cells regulate hTERT splicing. Further, these experiments provide support to the idea that shifting hTERT alternative splicing to inactive variants with either small molecules targeting splicing proteins or with oligonucleotides that block hTERT regulatory sequences could reduce telomerase activity and shorten telomeres; potentially leading to stable cancer remissions.

Results

hTERT minigene targeted siRNA screen of 528 RNA binding proteins and splicing factors

To investigate the protein factors involved in the alternative splicing of hTERT we performed an siRNA screen in HeLa cells stably expressing an hTERT minigene splicing reporter (Figure 7). The minigene was integrated into a single locus using previously

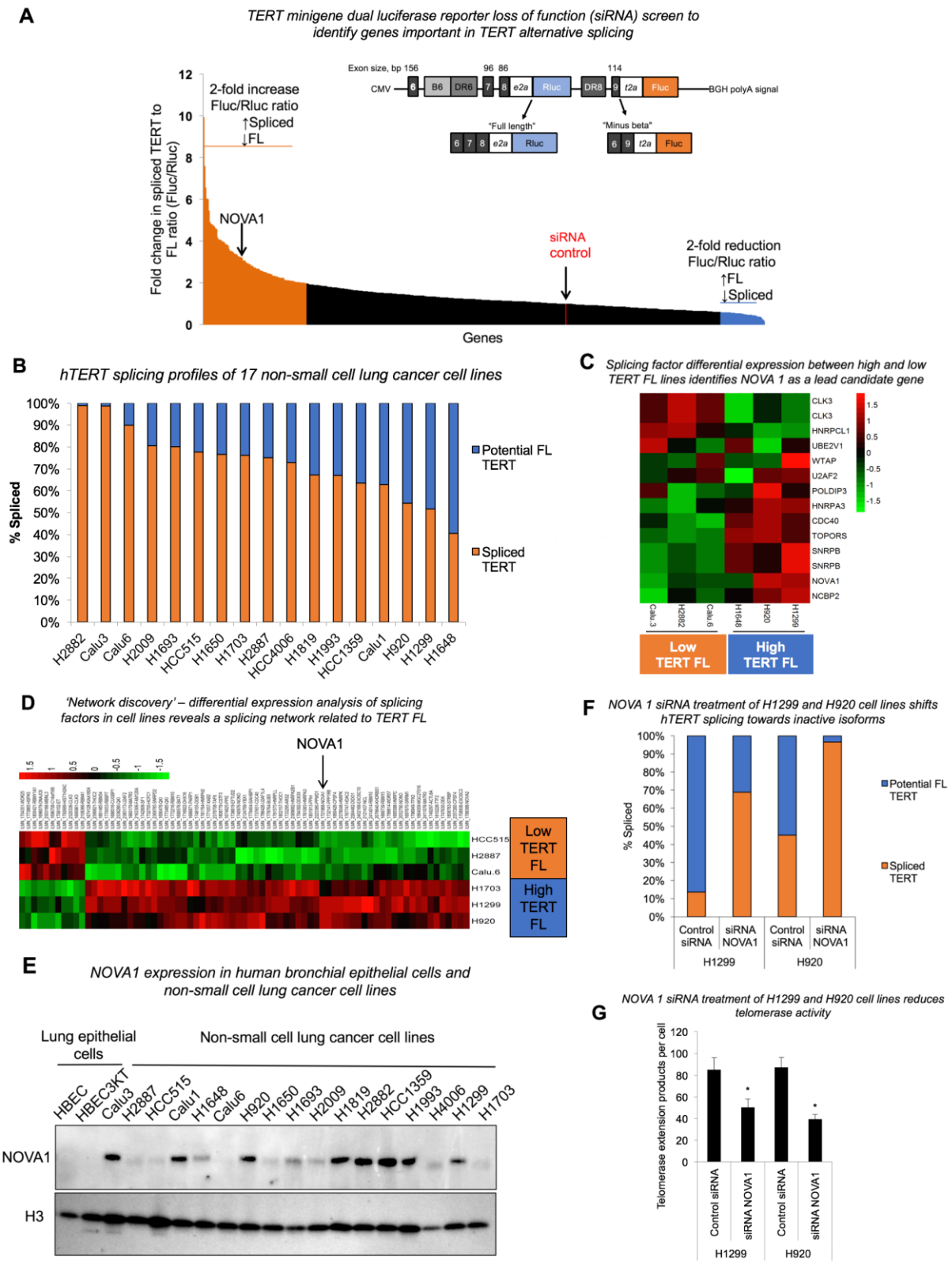

Figure 7. Telomerase/hTERT alternative splicing is regulated by a network of RNA binding proteins
A. TERT minigene cartoon showing dual luciferase reporter construct and products. Minigene screen data indicating fold-change in loss of function siRNA screen. B. hTERT steady state isoform/splicing profile in non-small cell lung cancer cell lines. C. Differential expression analysis of splicing factors related to hTERT full-length expression in the non-small cell lung cancer cell lines. D. Expanded differential expression analysis of different splicing factors related to hTERT full-length expression in the non-small cell lung cancer cell lines reveals of network of gene related to TERT splicing. E. Expression of NOVA 1 protein and histone H3 protein in normal (HBECs) and cancerous lung cell lines. F. siRNA knockdown of NOVA 1 in H1299 and H920 lung cancer lines shifts hTERT splice isoform proportions (3 biological replicates, each measured 2, Student's t test set at $p < .05$ for significance). G. siRNA knockdown of NOVA 1 in H1299 and H920 lung cancer lines reduces telomerase activity (3 biological replicates, each measured 2, Student's t test set at $p < .05$ for significance). Data are expressed as means and standard error of the mean where applicable. * = $p < 0.05$

described methods (Wong et al., 2013; Wong, Shay, et al., 2014). The minigene used in this study was modified to allow for luciferase-based measurements of hTERT 'full length' (intact exons 6-8) and hTERT minus beta (skipping of exons 7 and 8). We modified the previously used minigene to include a renilla (Rluc) and a firefly luciferase (Fluc) gene. We fused renilla luciferase and E2A (equine rhinitis A virus 2A) peptide in frame to hTERT exon 8 so that when exon 8 was included in the minigene pre-mRNA, the protein product would produce Rluc (Figure 7A) putting the Fluc out of frame. Conversely when exons 7 and 8 are skipped and splicing occurs from exon 6 to exon 9, Fluc and a T2A (Thosea asigna virus 2A) peptide are in frame. Thus, Fluc indicates minus beta splicing (inactive hTERT) and full-length (FL) hTERT is indicated by Rluc (Figure 7A). The use of the 2A peptides facilitated the generation of equal molar ratios of luciferase to hTERT (Ryan, King, & Thomas, 1991).

We used two databases (NCBI gene and Genecards) and searched the key words of 'RNA binding protein' and 'splicing factor' to generate a list of 528 genes (Table 2) and then ordered pools of 4 siRNAs to each gene (Dharmacon, GE, sequences of pools to each gene are located in Table 3). As negative controls, we used a pool of scrambled siRNAs (siRNA control), a transfection control (cells, transfection reagents, and media), and a media only control (cells plus culture media). In preliminary experiments, knock down of the core splicing factor hnRNP H1 resulted in a 4-fold induction of minus beta splicing in the minigene luciferase assay ($p < 0.05$; Supplemental Figure 1A). hnRNP H1 was included as a

minus beta-inducing positive control and ubiquitin (UBB) as a positive transfection control. Many RNA processing factors have documented effects on cell viability, so we also infected cells with a SEAP (secreted embryonic alkaline phosphatase) reporter lentivirus. Since only living cells will produce and secrete SEAP, we used SEAP as a viability control to normalize the splicing reporter gene data (Supplemental Figure 1B).

HeLa cells harboring the hTERT minigene splicing reporter were plated and transfected 24 hours later with 1nM of siRNAs, a low concentration of siRNAs which helps to reduce the potential of "off target" effects. Cells were incubated with the transfected siRNAs and luciferase measurements were made 72 hours later. The screen was repeated twice and data were averaged for each luciferase measurement. Then a ratio of minus beta to FL splice variants was calculated for each target gene (Figure 7A). Of the 528 genes tested, 97 genes resulted in a 2-fold or greater increase in minus beta splicing and 20 genes resulted in a 2-fold or greater increase in 'FL' splicing. Our objective was to find novel and perhaps more specific potential targets for telomerase inhibition; thus we focused our follow-up studies in this report on the 97 genes that when knocked down resulted in 2-fold or greater increases in the ratio of minus beta to FL. Since minigene reporter assays have special caveats concerning the representation of endogenous gene expression, we pursued a parallel bioinformatics approach to narrow down our list of candidate genes using a highly-characterized panel of human non-small cell lung cancer cell lines.

Bioinformatics analysis of 528 RNA binding proteins and splicing factors

To narrow down and validate target genes identified by the splicing reporter assay, we measured telomere biology phenotypes (telomerase activity, hTERT expression, and telomere length) in 17 well characterized lung cancer cell lines and correlated the expression of splicing factors and telomere biology between these cell lines (Frink et al., 2016). We measured telomerase activity and hTERT mRNA steady state levels with primers spanning exons 2 and 3, 7 and 8, and 15 and 16. We found that telomere length and telomerase activity were significantly correlated ($R^2 = 0.51$, $p = 0.001$) in our subset of non-small cell lung cancer cell lines (Supplemental Figure 1 C, D, and E). Further, we found that hTERT mRNA expression of exons 7/8 and exons 15/16 showed a modest but significant correlation with telomere length ($R^2 = 0.38$, $p = 0.008$ for exons 7/8 and $R^2 = 0.30$, $p = 0.02$ for exons 15/16; Supplemental Figure 1F). Also, telomerase activity correlated with exons 7/8 expression of hTERT ($R^2 = 0.27$, $p = 0.03$; Supplemental Figure 1G).

To determine the proportion of expressed hTERT splice variants, we used primers and probes specific for deletion isoforms and then calculated the percentage of each splice variant across cells lines (splice isoform assays, Figure 7B, Table 4). Based on the quantification of hTERT splice isoforms, we choose to analyze cell lines that differed significantly by the percentage of full-length (FL) hTERT expression, percentage of minus beta hTERT expression, and telomerase activity (three lines at each extreme, Figure 7C, “Gene Discovery Dataset”). We correlated the expression data of 528 RNA binding proteins across six cell lines (differential

expression analysis (t-tests), false-discovery rate corrected with Benjamini-Hochberg procedure) to the amount of potential full-length hTERT as measured by our splice isoform assays. This analysis revealed differential expression of 12 genes (heat map Figure 7C). Of these 12 genes, four also significantly increased (2-fold increase) minus beta splicing in the minigene screen (SNRPB, NOVA1, U2AF2, and CDC40). Since SNRPB, U2AF2, and CDC40 are core-splicing factors they were not pursued in this report. NOVA1 was chosen for further analysis because NOVA1 was the top-ranking tissue-specific RNA binding protein associated with cancer.

We hypothesized that NOVA1 may mark a network of splicing factors related to high levels of hTERT FL mRNA (Hsu et al., 2015). We measured protein and mRNA expression of NOVA1 across our lung cancer panel cell lines as well as two normal diploid cell lines and observed that NOVA1 protein was not expressed in normal human bronchial epithelial cells (HBECs), and was over expressed in 71% of our lung cancer cell lines (12 of 17 lines; Figure 7D and E). We found that NOVA1 mRNA expression and NOVA1 protein expression were significantly correlated in our non-small cell lung cancer cell lines ($R^2 = 0.50$, $p = 0.001$; Supplemental Figure 1I). This correlation indicates that NOVA1 mRNA maybe be useful as a proxy measure for NOVA1 protein. We also observed significant correlations of both NOVA1 mRNA and NOVA1 protein with percent FL hTERT expression in our 17 non-small cell lung cancer cell lines ($R^2 = 0.32$, $p = 0.01$; $R2 = 0.34$, $p = 0.01$, respectively; Supplemental Figure 1H).

We then used hierarchical clustering analysis (divisive) to separate and prioritize cell lines based on telomere biology (telomere length, telomerase, hTERT splicing profile) and expression of NOVA1. Using two groups of cell lines (six cell lines total: 3 high telomerase, long telomeres, high NOVA1 compared to 3 low telomerase, short telomeres, low NOVA1) we compared the expression profiles of the 528 RNA binding proteins and splicing factors. This expression analysis revealed 69 genes with different expression patterns between the two groups of lung cancer cell lines (Figure 7D; heatmap for "Network Discovery Dataset"). Further, we observed a very strong correlation of NOVA1 mRNA with hTERT percent FL in this subset of cell lines ($R^2 = 0.71$, $p = 0.03$; Supplemental Figure 1J). We compared these genes to the minigene reporter results and 7 genes also had a 2-fold or greater change in minus beta minigene splicing (Supplemental Figure 1J) including NOVA1.

To confirm the results of the minigene screen and the bioinformatics correlation analysis, we performed short-term siRNA knockdown experiments (Figure 7 F and G). Using siRNAs from a separate company (Santa Cruz) than those used in the minigene screen to avoid sequence-specific off target effects, we knocked down NOVA1 and measured hTERT splicing profiles and telomerase enzyme activity in two lung cancer cell lines (H1299 and H920). hTERT potential full-length mRNA levels were reduced by 60% in H1299 and 50% in H920 cells treated with NOVA1 siRNAs compared to cells treated with control non-targeting siRNAs (Figure 7 F and G, Supplemental Figure 1K and L). In addition, telomerase

enzyme activity as measured by droplet digital PCR TRAP (telomere repeat amplification protocol) was reduced in NOVA1 depleted cells by 2-fold ($p = 0.05$) and 2.5-fold ($p < 0.05$) in H1299 and H920 cells respectively compared to control siRNA treated cells (Ludlow et al., 2014). Efficient knockdown of NOVA1 was confirmed in both cell lines (Supplemental Figure 1L).

Importantly, hTERT steady state transcripts (exons 15/16) were not significantly decreased by knockdown of NOVA1 in either cell line (Supplemental Figure 1M), indicating that NOVA1 knockdown results in a change in splicing and not just a downregulation in transcriptional rate which is known to affect splicing (Schor, Gomez Acuna, & Kornblihtt, 2013). We did observe a reduction in exon 7/8 containing transcripts, confirming the reduction in potential full-length (Supplemental Figure 1). These data confirm and extend the minigene and bioinformatics analyses to support that NOVA1 is a key member of a potential network of genes regulating hTERT alternative splicing. To further elucidate the role of NOVA1 in hTERT regulation, we characterized its expression in other tissues and cell types.

NOVA1 expression is tissue specific and is regulated differently between cell types

NOVA1, also known as paraneoplastic Ri antigen, is an RNA binding protein originally identified in sera from patients with the paraneoplastic disease, POMA (paraneoplastic opsoclonus-myoclonus ataxia) (Buckanovich, Posner, & Darnell, 1993; Buckanovich, Yang, & Darnell, 1996). NOVA1 contains three K-homology domains and binds RNA at YCAY (Y

= C or T) clusters commonly found though out the genome (1 in 64 nucleotides) (Teplova et al., 2011; C. Zhang et al., 2010). However, NOVA1 seems to have exquisite specificity to its target genes indicating levels of regulation and cooperation amongst splicing factors that is not yet fully understood. NOVA1 has been typically studied in the context of neuronal tissues and there is limited data about the expression of NOVA1 in other cell types. NOVA1 was originally identified to be a neuron-specific RNA binding protein that plays a key regulatory role in neuronal alternative splicing (Jensen et al., 2000).

Other functions for NOVA1 have been discovered outside of the brain including a major role in alternative splicing in pancreatic beta cells and brown adipogenesis (J. C. Lin, Chi, Peng, & Lu, 2016; Villate et al., 2014). Additionally, interest in the potential effects of NOVA1 in cancer has also been growing. So far, it has been found to be overexpressed in hepatocellular carcinoma and gastric cancer (Shen et al., 2015; Y. A. Zhang et al., 2014). Knockdown of NOVA1 in glioblastoma cells resulted in decreased proliferation, migration and invasion and induced apoptosis, suggesting it may also be important in glioblastoma (Zhi et al., 2014).

The expression pattern of hTERT is such that it is expressed during human fetal development, down-regulated in adult somatic tissues at the transcriptional level while the remaining transcripts are spliced to inactive variants, and is re-expressed in human cancers. We wanted to determine if NOVA1 is regulated in a similar fashion human embryonic stem cells (H9 cell line) and human iPS cells (generous gift from Dr. Jay Schneider, UTSW). We

assayed telomerase enzyme activity, observing high levels of telomerase as expected, with extremely high levels in this particular iPS line (Figure 8A). Next, we measured steady state levels of hTERT splice variants using the ddPCR assays (Figure 8B) and an RT-PCR based assay of exons 5 to 9 (Figure 8C). We observed that the differences in the proportion of full length hTERT between H9 (38% FL) and iPS cells (70% FL), are also reflected in the gel based assay (Figure 8C), and is correlated with differences in TRAP/telomerase activity (Figure 8A).

Next, we investigated the expression of NOVA1 in a panel of normal human tissues from both adults, and human fetuses and in human stem cells (H9 embryonic and iPS). NOVA1 was found to be extremely low in most tissues, but enriched in stem cells, brain, and testis (Figure 8D). This is consistent with the concept that NOVA1 expression correlates with FL hTERT levels, as shown in telomerase positive stem cells and testis (Figure 8)(Wright, Piatyszek, et al., 1996).

One of the known regulatory mechanisms of NOVA1 is its ability to self-splice its own pre-mRNA. NOVA1 binds to exon 4 of its own pre-mRNA which results in the skipping of exon 4, thus generating two populations of mRNAs (mRNA consisting of exons 3, 4, and 5 and mRNA lacking exon 4) at least in neurons (Dredge, Stefani, Engelhard, & Darnell, 2005). We decided to characterize NOVA1 exon 4 splicing in stem cells, differentiated cells, and cancer cells. Splicing of NOVA1 in stem cells revealed that exon 4 is consistently included

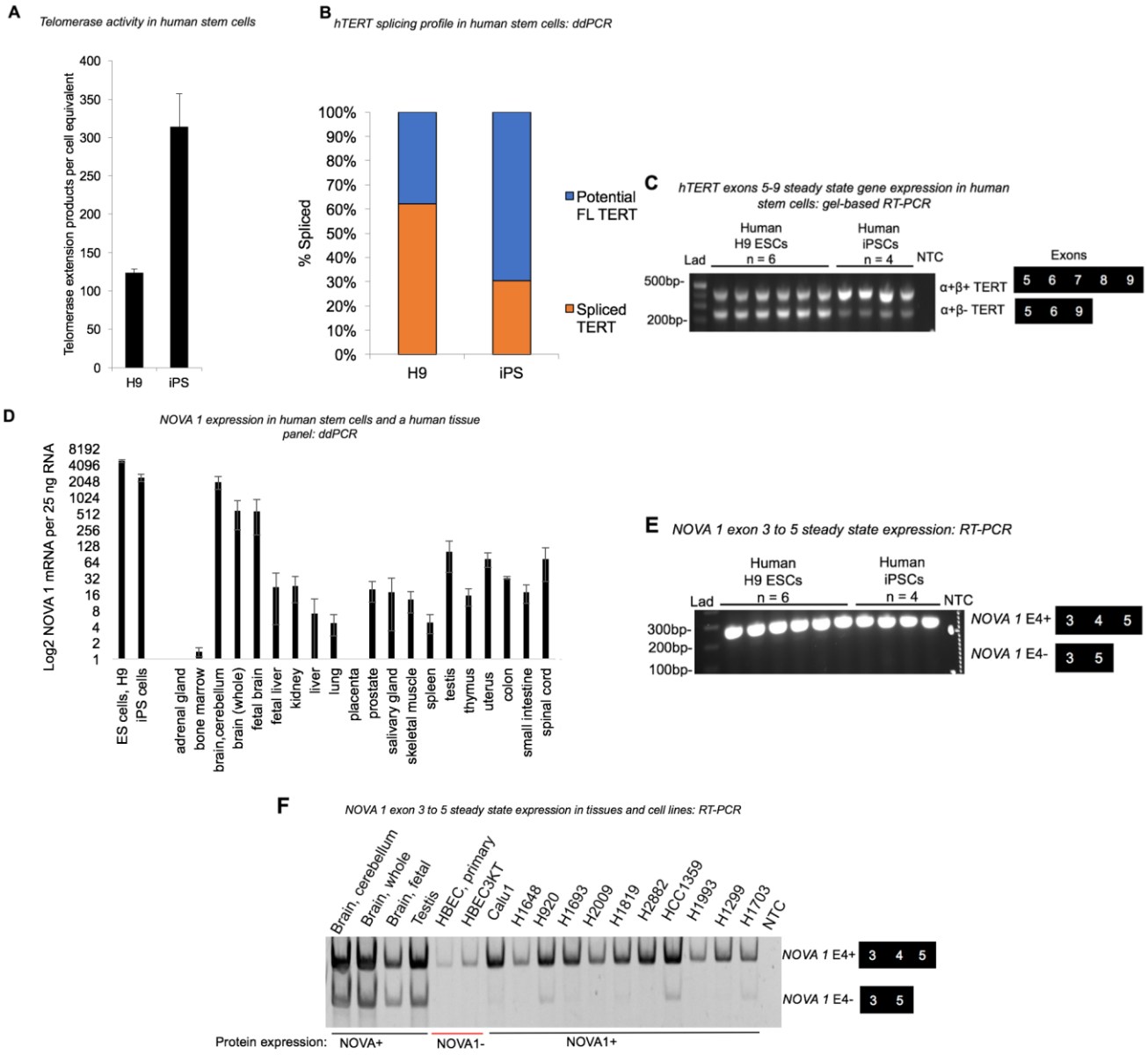

Figure 8. Characterization of NOVA 1 expression and splicing in different cell types
A. Telomerase activity in human embryonic stem cells and induced pluripotent stem cells. B. hTERT splice isoform expression in human stem cells (ES cell n = 6, iPS cell n = 4). C. hTERT expression in human stem cells as determined by RT-PCR of exons 5 – 9 (ES cell n = 6, iPS cell n = 4). D. mRNA expression of NOVA 1 in human stem cells and a panel of human tissues as determined by RT-droplet digital PCR(ES cell n = 6, iPS cell n = 4, each tissue RNA was measured 3 times from 3 different RT reactions). E. NOVA 1 expression in human stem cell lines by RT-PCR of exons 3 – 5 (ES cell n = 6, iPS cell n = 4). F. NOVA 1 expression in human tissues and cell lines by RT-PCR of exons 3 – 5 (representative image, each line was measured 3 times from 3 different RT reactions). Data are expressed as means and standard error of the mean where applicable.

(Figure 8E). Additionally, we observed the expected differential splicing of NOVA1 in the differentiated cell types (brain, testis, stimulated human lymphocytes). However, unlike in differentiated cells, human stem cells and cancer cells did not follow this splicing pattern (Figure 8F, Supplemental Figure 2 A and B). While one report found that both isoforms had similar splicing capabilities in in vitro splicing assays, the functionality of exon 4 in NOVA1 remains unknown (Dredge, Stefani, Engelhard, & Darnell, 2005). Thus, there are likely to be regulatory differences in NOVA1 splicing between normal differentiated tissues, stem cells and cancer cells.

Further, in stimulated lymphocytes NOVA1 long transcripts are produced at the same time as telomerase activity peaks (Supplemental Figure 2). This suggests that the regulatory information for NOVA1 to interact with hTERT is contained in exon 4 of NOVA1 and that protein modification (or lack thereof) of these residues may trigger the splicing of full-length hTERT. Thus, in stem cells, cancer cells, and telomerase-competent cells (i.e., stimulated lymphocytes), an additional factor may be present that modifies NOVA1 exon 4 but this factor is not expressed in telomerase-negative tissues that express NOVA1 (i.e., neurons). These data combined with the observation that NOVA1 is expressed very early in human fetal development and is then downregulated upon tissue differentiation in most tissues and is reactivated during cancer progression suggests its function in cancer is oncogenic.

Long-term depletion of NOVA1 shifts hTERT splicing to inactive variants, reduces telomerase activity, and shortens telomeres

To determine the effects of long-term reduction of NOVA1 protein levels on telomere length maintenance, we conducted shRNA mediated stable knockdown experiments. We knocked down NOVA1 in three different cell lines: two that express NOVA1 (H1299 and H920) and one that does not express NOVA1 (Calu6, as a control for off target effects of the shRNA sequence). Stable knockdown of NOVA1 reduced telomere length and telomerase activity in the two cell lines that express NOVA1 (Figure 9A, Supplemental Figure 3A). NOVA1 protein and mRNA levels were reduced by about 50% (Figure 9C, Supplemental Figure 3D). NOVA1 knockdown reduced the proportion of full-length hTERT message (Figure 9B; Supplemental Figure 3B) and decreased telomerase activity about 50% ($p = 0.05$, Figure 9D; Supplemental Figure 3C), which was sufficient to reduce telomere length in both H1299 and H920 cells. The long-term depletion of NOVA1 also reduced the steady state transcript levels of hTERT (Figure 9E, Supplemental Figure 3E).

To demonstrate that the shRNA was on target for NOVA1, we transduced H1299 cells with a retroviral 6X MYC-tagged cDNA construct coding for full-length NOVA1 with the shRNA seed sequence mutated and measured telomerase enzyme activity, hTERT splicing and hTERT transcript levels. The shRNA-resistant NOVA1 cDNA was able to rescue hTERT splicing changes, transcript levels and telomerase enzyme activity (Figure 8B, D and E). These experiments show that NOVA1 is mechanistically linked to hTERT and that the

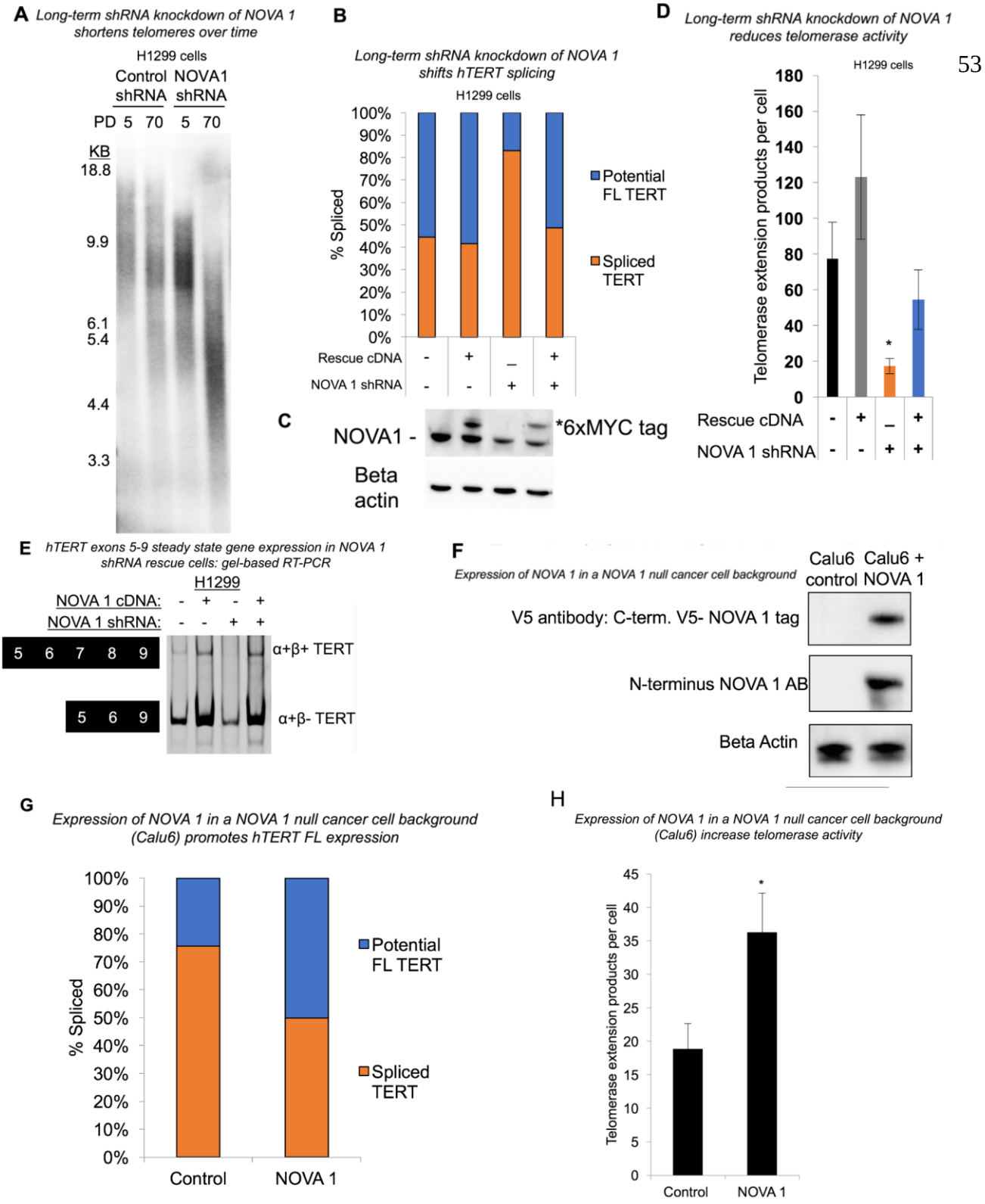

Figure 9. Long-term reduction in NOVA 1 progressively shortens telomeres while over expression of NOVA 1 in cancer promoter telomerase activity A. Terminal restriction fragment length (TRF-Southern Blot) analysis of control shRNA or NOVA 1 shRNA at two population doublings (PD). B. Rescue of shRNA knockdown of NOVA 1 with a shRNA mutant cDNA restores basal TERT splicing isoform profile in H1299 cells (stable cell lines were measured a minimum of 6 times over several passages). C. Western blot of NOVA 1 shRNA rescue in H1299 cells (representative image of stable cell lines, measured 3 times over 3 passages in culture). D. Rescue of shRNA knockdown of NOVA 1 with a shRNA mutant cDNA partially restores telomerase activity in H1299 cells (averaged data from duplicate measures of stable cell lines over 3 passages in culture, each mean is from 6 measures). E. hTERT expression in rescue H1299 cells as determined by RT-PCR of exons 5 – 9 (representative image from 3 separate measures). F. Western blot of V-5 tagged NOVA 1 expression in Calu6 cells (representative image of stable cell lines). G. hTERT splicing profile in Calu6 cells with and without NOVA 1 (biological triplicates measured in duplicate for each condition, n = 6 for each measure). H. Telomerase enzyme activity in Calu6 cells with and without NOVA 1 (biological triplicates, n = 3 for each measure). Data are expressed as means and standard error of the mean where applicable. * = $p < 0.05$

observed phenotypes associated with knockdown are on target. Further, targeting NOVA1 resulted in a potent effect on hTERT-not only by shifting the splicing away from full-length hTERT transcripts but also by reducing overall hTERT transcription. The reduced hTERT transcription is important to consider in conjunction with recent data from several labs indicating that hTERT transcripts and splice variants may have other non-canonical roles in cell growth, mitochondrial function, and anti-apoptotic pathways (Gunes & Rudolph, 2013; Kumar, Lechel, & Gunes, 2016).

The greater than 50% telomerase activity reduction observed leading to progressive telomere shortening is consistent with human diseases of hTERT haploinsufficiency like IPF that also result in clinically significant shortened telomeres. We used three sequence-independent RNAi methods in multiple cell lines and achieved a similar phenotype for NOVA1 without affecting cell proliferation, supporting on target effects in these experiments. In Calu6 cells, a cell line with no detectable NOVA1, NOVA1 stable knockdown had no effect on telomere biology (Supplemental Figure 3F, G, H, I, and J). This suggests that a separate set of splicing factors could make NOVA1 negative cancers vulnerable to splicing inhibition and our bioinformatics analysis points to some likely candidate genes. Additionally, we introduced control (non-silencing) and NOVA1 shRNAs into human bronchial epithelial cells and observed no significant growth defect (Supplemental Figure 3M). This indicates that partial reduction of NOVA1 in primary human lung epithelial cells is not toxic and that non-transformed cells that express very low levels of NOVA1 tolerate this manipulation well.

To provide further evidence that NOVA1 is important in lung cancer biology, we attempted to knock out NOVA1 with CRISPR/Cas9 genome editing methods in H1299 lung cancer cells. We were unable to obtain survival clones that had both NOVA1 alleles mutated in our assays, suggesting that NOVA1 might be critical in cell survival pathways in H1299 lung cancer cells. Additionally, when we knocked down NOVA1 with shRNAs in H2882, another non-small cell lung cancer cell line, the cells only divided twice in 90 days following selection. H2882 cells have very robust levels of NOVA1 (Figure 8F), adding strength to the idea that NOVA1 may be a critical gene to cancer cell survival (oncogene/tumor dependency). Additionally, we observed that a different shRNA targeting NOVA1 in H1299 cells significantly slowed growth and was rapidly silenced. Thus, there could be a strong selection tumors cells that have a ~50% reduction in NOVA1 protein levels since greater than 50% knockdown would be result in slowed growth and or cell death. Based on these observations we predict that partial reduction in NOVA1 protein levels would result in a potent telomerase inhibitory phenotype and progressive telomere shortening. Combined, these experiments document that hTERT splicing can be manipulated by tissue-specific RNA binding proteins such as NOVA1, suggesting a therapeutic window might exist to develop small molecules which indirectly target telomerase.

Expression of NOVA1 in cancer cells increases telomerase activity and shifts hTERT splicing toward full-length

To determine if expression of NOVA1 in a NOVA1-negative cancer cell line promotes hTERT full-length splicing and increases telomerase activity, we transduced Calu6 cells with a lentiviral vector containing NOVA1 full-length cDNA with a V5-C-terminus tag. Following transduction and selection, we confirmed overexpression of NOVA1 with both tagged (V5 epitope antibody) and N-terminus NOVA1 antibodies (Figure 9F). Next, we assayed telomerase activity and hTERT splicing. Telomerase activity increased 2-fold ($p = 0.05$, Figure 9H) as did the proportion of hTERT FL transcripts (23% vs. 50%; Figure 9G) in NOVA1 expressing cells compared to control empty vector cells. In these experiments, telomere length was not altered by overexpression of NOVA1 (Supplemental Figure 3N).

NOVA1 interacts with hTERT pre-mRNA

NOVA1 binds to pre-mRNAs in a sequence dependent fashion, binding to YCAY (Y = C or U) motifs in RNAs (Ule et al., 2006). First, we looked in silico at the hTERT locus for YCAY motifs, focusing on sequences (including introns and exons) from exons 5 to 10 (Figure 10A). It is not possible to use previous public HITS-CLIP databases from mice because of sequence element differences between mice and humans (Wong et al., 2013). Since NOVA1 is known to bind to clusters of YCAY motifs we looked for areas of highly concentrated motifs. We found several potential candidate regions in hTERT exons and introns 5 through 10 (Figure 10A). We performed UV-crosslinking and immunoprecipitation (UV-IP) of H1299 cells and observed an enrichment of the NOVA1:hTERT RNA interaction in hTERT intron 8 (direct repeat 8, DR8) of hTERT (Sei & Conrad, 2014; Wong et al.,

2013). NOVA1 appeared to bind in a region we previously observed to be involved in the regulation of hTERT alternative splicing (Figure 10 A, B, and C). Our working model is that NOVA1 binds to the DR8 region and promotes splicing of hTERT to include RT domain-coding exons 7 and 8. This idea is consistent with previous observations suggesting that NOVA1 can act as a splicing enhancer if it binds following an alternatively spliced exon (Dredge et al., 2005).

From in silico analysis we found that DR8, a 258-base pair element in intron 8 of hTERT, contains 7 YCAY NOVA1 binding motifs. Previously, we found that when cells were treated with a 2-O-methyl-antisense oligonucleotide to this region, hTERT minus beta splicing was increased and FL splicing was reduced, supporting the idea that a factor that promotes inclusion of hTERT exons 7 and 8 was blocked (Wong et al., 2013). This is consistent with our observations that hTERT minus beta splicing increases when NOVA1 levels are reduced. To confirm the UV-IP observations, we utilized our hTERT minigene series that we previously developed which excludes DR8, the intron 8 region containing the NOVA1 binding motifs, and performed crosslinking and immunoprecipitation followed by RT-ddPCR (CLIP-ddPCR) (Figure 10D). As additional controls, we included constructs that contained or excluded a highly similar (85% homology) sequence region in intron 6 called direct repeat 6 (DR6; Figure 10E). Interestingly, DR6, a 254-base pair region in intron 6, contains fewer YCAY (5) motifs than DR8.

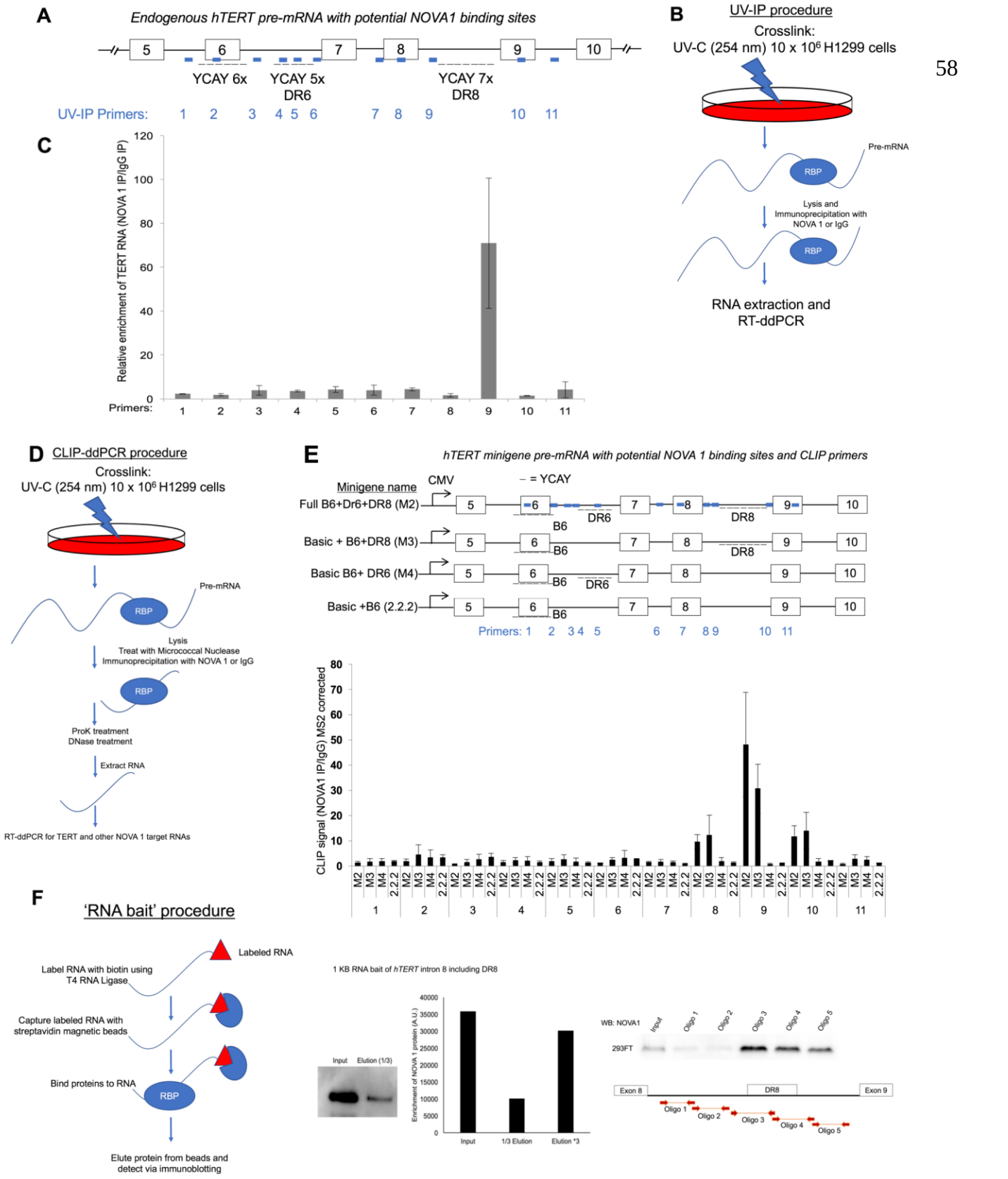

Figure 10. NOVA 1 binds to a deep intronic element in TERT precursor RNAs

A. Cartoon of TERT exons 5 through 10 showing potential NOVA 1 binding sites and the primers (blue boxes) used in the ultra-violet immunoprecipitation (UV-IP) procedure. B. UV-IP procedure schematic showing the major steps. C. Droplet digital RT-PCR showing UV-IP enrichment of NOVA 1 at TERT DR8 (n = 3 independent IPs). D. Schematic of UV cross-linking immunoprecipitation (CLIP) droplet digital PCR procedure. E. Cartoon of hTERT minigene used in the CLIP experiments, primers (blue boxes) and droplet digital PCR quantification of NOVA 1 protein and TERT RNA interaction (n = 3 independent IPs). F. Schematic of RNA bait procedure to pulldown to find proteins that interact with hTERT RNAs. We in vitro transcribed a 1 kb fragment of hTERT intron 8 that contained DR8. A second set of RNA baits were made from PCR fragments of hTERT intron 8 around and including DR8. G. Western blot and quantification of western showing pulldown of NOVA 1 protein with TERT RNA containing DR8. Representative western blot of NOVA 1 protein showing binding to DR8 at baits (oligos) 3, 4 and slight binding at bait 5. Data are expressed as means and standard error of the mean where applicable. * = $p < 0.05$

To perform CLIP, we generated stable lines expressing a V5-tagged full length (exon 4 containing, Broad ORF clone number ccsbBroad304_01104) version of NOVA1 and the hTERT minigenes, and performed CLIP-ddPCR to test for NOVA1:hTERT pre-mRNA interactions (Supplemental Figure 4). We observed that when DR8 was present we could effectively pull down hTERT minigene pre-mRNAs, but we could not when DR8 was absent. As a control, we also assayed our CLIP cDNAs for a known NOVA1 target gene, glycine receptor alpha 2 (GLRA2) (Jensen et al., 2000). First, we tested for expression of GLRA2 in HeLa and H1299 cancer cells (Supplemental Figure 4), and observed that it was expressed in both cell lines. Next, we looked in our H1299 NOVA1 rescue series to see if GLRA2 was regulated by NOVA1 in cancer cells and indeed found that GLRA2 transcripts are differentially spliced depending on NOVA1 levels (Supplemental Figure 4D) similar to previous studies (Jensen et al., 2000). Next, we assayed our CLIP cDNAs and observed that GLRA2 was effectively pulled down in all extracts regardless of hTERT status, indicating that our CLIP was efficient.

Additionally, we in vitro transcribed a 1 kb fragment of RNA containing DR8 of hTERT intron 8 and the suspected NOVA1 binding sites, labeled the RNA with biotin, attached the labeled RNA to streptavidin beads, and performed an RNA pulldown assay. When we expressed NOVA1 in 293 cells and exposed the lysate to the labeled RNA, we observed effective pulldown of NOVA1 (Figure 10F). To further confirm the CLIP, UV-IP, and RNA pulldown observations, we generated a series of small (~150 nt) RNA baits surrounding and

in DR8 of hTERT. We observed strong binding to oligos 3 and 4 (sequences located within DR8 of hTERT) and weak binding to oligos 1, 2, and 5 (sequences located just outside of DR8 of hTERT) (Figure 10F). These data combine (CLIP, UV-IP, and RNA pulldown) indicate that NOVA1 binds to hTERT RNA in a region known to regulate the splicing of exons 7 and 8. Overall, our results are consistent with the model that when NOVA1 is bound to hTERT mRNAs in intron 8, it acts as a splicing enhancer, promoting the inclusion of exons 7 and 8 to increase the production of full length hTERT mRNAs.

Deletion of endogenous hTERT DR8 or mutation of the NOVA1 binding sites in DR8 via CRISPR/Cas9 results in progressive telomere shortening, reduced telomerase activity and altered hTERT splicing

To further define the importance of hTERT DR8 in telomere biology, we deleted a 480 nucleotide fragment of hTERT intron 8 using two CRISPR guide RNAs flanking DR8 (Figure 11A). We introduced the guides with Cas9 into H1299 and sorted the top 5% GFP positive cells into 96-well plates. Positive wells (wells that had cells growing; n = 18) were scaled up to 6-well dishes and validated via PCR and Sanger sequencing. Three of the clones had the correct on-target deletion of hTERT intron 8 DNA containing DR8 (Supplemental Figure 5A).

In a parallel experiment, we introduced either WT or mutant DR8 DNA via a donor plasmid with CRISPR/Cas9 (Figure 11B). The mutant donor plasmid had all seven of the YCAY

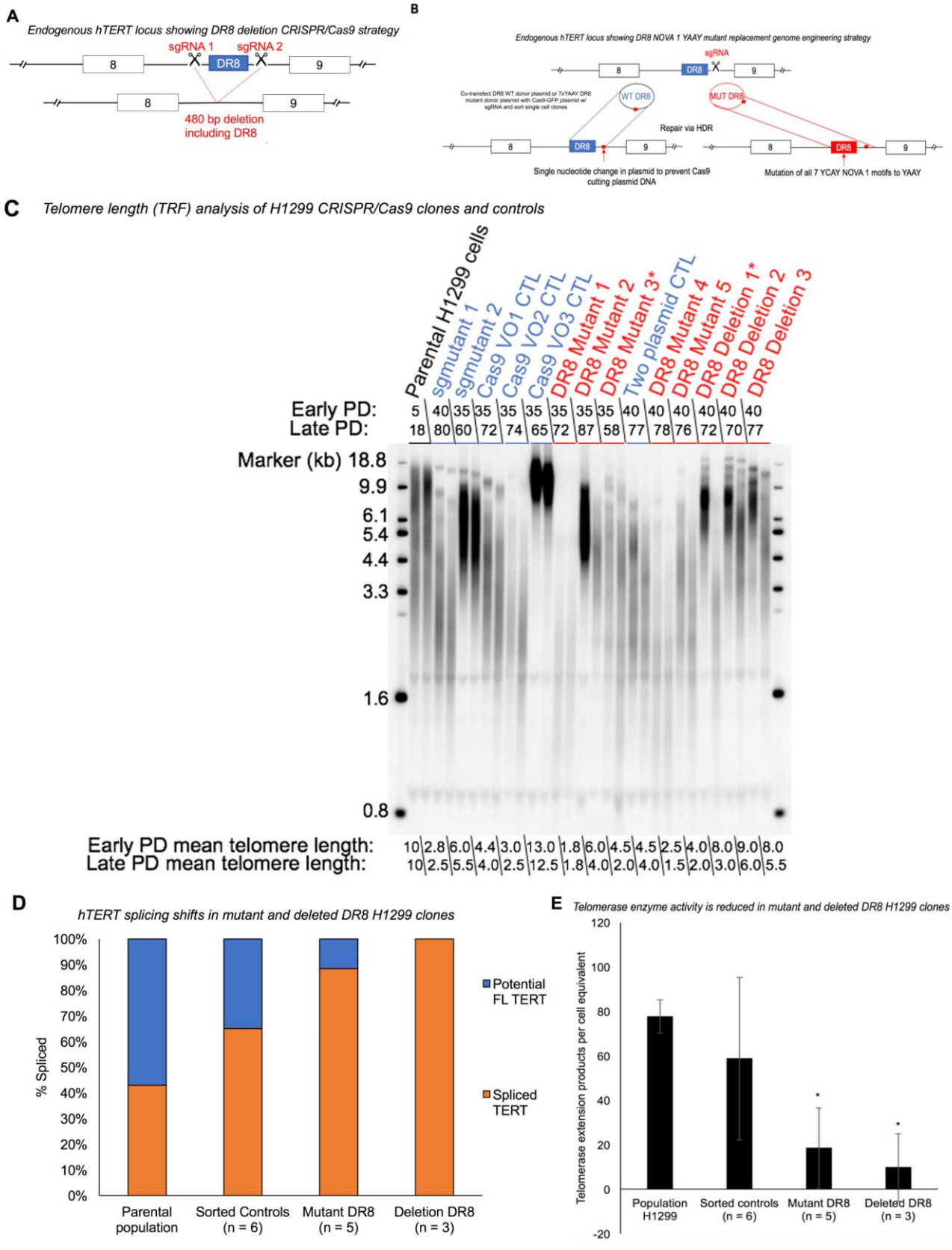

Figure 11. CRISPR/Cas9 deletion of DR8 or mutation of NOVA 1 binding sites in TERT DR8 intron 8 progressively shortens telomeres, shifts splicing to inactive isoforms and reduces telomerase activity in H1299 clones.
A. Schematic of CRISPR/Cas9 strategy to delete DR8 in TERT intron 8. B. Schematic of CRISPR/Cas9 strategy to mutate NOVA 1 binding sites in TERT DR8. C. Terminal restriction fragment length (TRF-Southern Blot) analysis of parental, sorted controls, DR8 NOVA 1 '7 x YAAY' mutants, and DR8 deleted H1299 clones. *DR8 mutant 3 and *DR8 deletion stopped growing at population doubling 57 and 85 respectively. D. hTERT splicing isoform proportion analysis of parental, averaged controls (n = 6), average DR8 mutants (n = 5), and pooled DR8 mutants (n =3). E). Telomerase activity of parental, averaged controls (n = 6), averaged DR8 mutants (n = 5), and averaged DR8 mutants (n = 3). Data are expressed as means and standard deviation where applicable. * = $p < 0.05$

motifs in DR8 mutated to YAAY, which has previously been shown to block NOVA1 recognition of target genes (Leggere et al., 2016). We pre-treated H1299 cells with the NHEJ inhibitor SCR7 for 30 min prior to transfection of the CRISPR/Cas9 and donor construct and obtained single cell clones via flow sorting GFP positive cells as above. We were able to validate two WT DR8 clones with homozygous PAM sequence mutation, indicating that homologous recombination of the donor plasmid was successful. We were also able to validate that 5 clones had homozygous integration of mutant DR8. Conveniently, the mutations in DR8 (YCAY sites 4 and 5) introduced a novel restriction enzyme site that allowed us to identify mutant clones via PCR and REN digestion (similar to RFLP analysis, Supplemental Figure 5B and C).

We also sorted a variety of controls (vector only and one control exposed to both Cas9 and mutant donor that failed to undergo cutting and repair) since it is well established that clonal heterogeneity exists in tumor cells lines (Bryan, Englezou, Dunham, & Reddel, 1998). These clones were all validated by PCR and Sanger sequencing to have WT DR8 hTERT sequences (representative Sanger sequencing in Supplemental Figure 5B). This produced 15 cell lines (14 clones and the parental H1299 population) that we then followed over time for telomere length, telomerase activity, and TERT splicing phenotypes.

We observed clonal heterogeneity for telomere length between all the clones including the controls clones, as expected. In the sorted control clones, however, we observed minimal

telomere length changes over time (14 ± 4.4 nts per doubling; average ± standard deviation), while significant telomere shortening was observed in both DR8 YAAY mutant clones (46 ± 42 nts per doubling; $p = 0.05$) and in the DR8 deleted clones (108 ± 45 nts per doubling; $p < 0.001$) (Figure 11C). All the DR8 deleted clones had longer telomeres on average compared to the mutant DR8 clones. This may indicate that the deletion of DR8 produces a strong selection pressure for clones with longer telomeres. hTERT splicing was also significantly changed, with a dramatic shift from full-length to spliced products (35% FL in sorted controls versus 12% FL in DR8 YAAY mutants, $p < 0.001$; Figure 11D, Supplemental Figure 5D).

Further, we assayed telomerase enzyme activity over time at three different population doublings and observed that, on average, the DR8 YAAY mutants had 70% less telomerase activity compared to the controls while the DR8 deletion clones had nearly undetectable telomerase activity (Figure 11E, Supplemental Figure 5D). The telomere shortening rates of 46 nts per doubling and 107 nts per cell doubling in the DR8 mutants (70% telomerase inhibition) and DR8 deleted clones (nearly 100% telomerase inhibition) correlate closely with observations that telomerase adds 50 to 150 nucleotides per cell division to maintain telomeres (Jafri, Ansari, Alqahtani, & Shay, 2016).

We also observed clonal heterogeneity for hTERT splicing: 3 clones had mostly minus beta, one clone had higher levels of hTERT FL and total hTERT mRNA which correlated to

higher telomerase activity (clone 7713; DR8 mutant 5), and one clone seemed to lack all transcripts with exons 5-9 (clone 775; DR8 mutant 3, Supplemental Figure 5D and E). The clone that completely lacked mRNA containing hTERT exons 5-9 also had no detectable telomerase activity (via ddTRAP) and eventually died in culture at population doubling 58 post sorting. Further, only one of three DR8 deleted clones had FL hTERT expression and telomerase activity, indicating that there are other mechanisms that cells can adapt to produce FL hTERT mRNA even when DR8 is not present in the pre-mRNA. After long-term passage one of the DR8 deletion clones stopped dividing at PD 85 (DEL 1).

Since there was some heterogeneity in telomerase activity and hTERT splicing, we measured the expression level of NOVA1 mRNA in these clones. We observed that on average the clones were similar for NOVA1 mRNA expression levels (Supplemental Figure 5F). However we observed heterogeneity in expression between clones with different telomere biology phenotypes. For instance, the deletion clone that died (DEL 1) and the mutant clone that died had low expression of NOVA1 compared to the average (Supplemental Figure 5). In contrast, the DR8 deletion clones with telomerase activity (DEL2/DEL 11) had very high NOVA1 levels. This suggests that NOVA1 may be helping to maintain telomerase levels via hTERT FL splicing via an alternative binding site. Overall, these data solidify the role of DR8 in the splicing choice of hTERT to produce FL or spliced products. Further, these data show that deep intronic elements have important roles in alternative splicing in addition to the well-known roles of elements closer to the exon-intron junctions.

NOVA1 knockdown reduces anchorage independent growth, invasion, and colony formation in cancer cells

To assess the potential of developing novel anti-telomerase cancer therapies targeting the hTERT splicing machinery, we assayed the tumorigenic properties of NOVA1 knockdown cells. NOVA1 has a known role in breast and lung cancers and is important during normal development for growth, survival, migration, and apoptosis (Darnell, 2010; Leggere et al., 2016; Saito et al., 2016; Villate et al., 2014). Importantly, cell growth rates were not affected in NOVA1 knockdown cells (H1299, H920, Calu6, and HBECs) in 2D tissue culture conditions (Supplemental Figure 3). To determine how NOVA1 depletion affects anchorage independent growth we performed soft agar assays comparing control shRNA H1299 cells to NOVA1 knockdown H1299 cells and rescue cells. NOVA1 knockdown cells formed significantly fewer colonies compared to control and NOVA1 rescue cells ($p < 0.05$, Figure 12A). NOVA1 knockdown H1299 cells did not invade as efficiently as H1299 shRNA control cells or the NOVA1 rescue H1299 cells in Boyden chamber assays ($p < 0.05$, Figure 12B). Finally, we observed that NOVA1 depleted H1299 cells formed fewer colonies compared to control shRNA H1299 cells and rescue cells in colony formation assays ($p < 0.05$, Supplemental Figure 6). We also assayed cancer growth phenotypes in a second cell line, H920, and both anchorage independent growth and invasive phenotypes were significantly reduced in NOVA1 depleted cells compared to control shRNA cells (Figure 12 A and B; $p < 0.05$). In Calu6 cells, which lack NOVA1 expression, we observed no

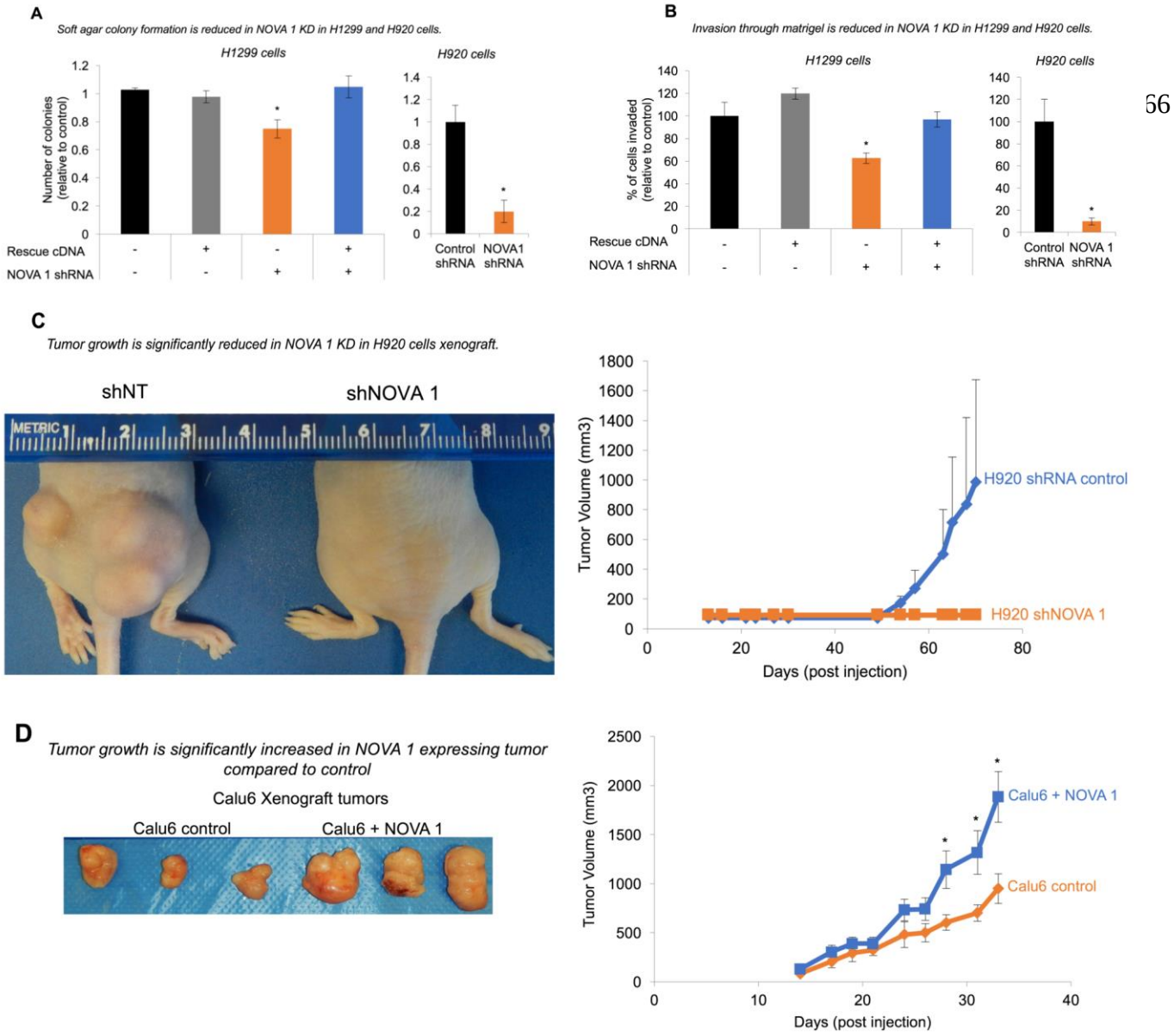

Figure 12. Manipulation of NOVA 1 in cancer cells alters cancer cell growth and xenograft tumor growth characteristics.
A. Knockdown of NOVA 1 in H1299 and H920 cells reduced anchorage independent cell growth compared to controls (n = 3 for each condition and cell line). B. Knockdown of NOVA 1 in H1299 and H920 cells reduced migration through an extracellular matrix (Boyden Chamber assay) compared to controls (n = 3 for each condition and cell line). C. Knockdown of NOVA 1 in H920 cells significantly reduced xenograft growth compared to controls. Left panel shows representative images of the hind quarters of nude mice. Right panel shows growth curves of xenograft tumors during the experiment. (n = 4 injections for each condition, 2 mice per condition) D. Expression of NOVA 1 Calu6 cells results in larger xenograft tumors compared to Calu6 controls (a non-small cell lung cancer cell line lacking NOVA 1 expression). Left panel shows representative images of tumors from Calu6 and Calu6 plus NOVA 1. Right panel shows growth curves of xenograft tumors during the experiment (n = 6 injections for each condition, 3 mice per condition). Data are expressed as means and standard error of the mean where applicable. * = $p < 0.05$

differences in anchorage independent growth, invasion, or colony formation between NOVA1 shRNA cells and control shRNA cells (Supplemental Figure 6).

Xenograft experiments indicate a distinct growth and survival advantage of tumor cells with high levels of NOVA1 and hTERT compared to cells with reduced or lacking NOVA1 and low levels of hTERT

To determine if cell lines with high or low levels of NOVA1 formed tumors in vivo, we injected cells into both hind flanks of immunocompromised mice. We observed 3 out of 4 injections of H920 shRNA control cells formed tumors in vivo while only 1 of 4 injections of H920 NOVA1 knockdown cells formed tumors (Figure 12C). The tumors derived from the control cell lines were significantly larger compared to the single tumor derived from the NOVA1 knockdown cells. It took about seven weeks for H920 control cells to form progressively growing tumors. In the NOVA1 knockdown H920 cells, three of the four tumor cell injections regressed during the course of the experiment. At the time of sacrifice, one very small H920 NOVA1 knockdown tumor was removed. This indicates that NOVA1 knockdown in H920 cells significantly altered the ability of these lung cancer cells to form tumors in vivo and strengthened the potential for future development of a strategy to target NOVA1 in lung cancer cells.

Further, we injected mice with Calu6 control lentiviral vector cells (a cell line that does not express NOVA1) and compared it to Calu6 cells with ectopic expression of NOVA1. We

monitored the tumors for a period of four weeks and observed that NOVA1 expressing cells formed bigger tumors compared to the control Calu6 cells (Figure 12D). Similar to the knockdown experiment, the NOVA1 expression conferred a growth advantage in vivo over cells that lacked NOVA1.

NOVA1 binds to hTERT pre-mRNA in a highly conserved deep intronic region to enhance the inclusion of exons 7 and 8 and promote the production of FL hTERT

We propose the following model for how the NOVA1 protein is interacting with TERT pre-mRNAs (Figure 13). In our series of experiments, we have provided substantial evidence that a deep intronic element in intron 8 of hTERT has a large influence on the choice of the alternative splicing machinery to include or skip hTERT exons 7 and 8, thus producing active or inactive hTERT mRNAs respectively. In our first model which only considers NOVA1's influence on hTERT pre-mRNAs, we propose that NOVA1 binds to DR8, recruiting the basal exon junction recognition machinery which promotes the use of the exon 8 5' splice donor with the exon 9 3' splice acceptor site to generate hTERT mRNAs, including exons 7 and 8 (Figure 13A). In a second model, we consider both pre-mRNA secondary structures and NOVA1. We previously defined a role for RNA:RNA pairing in regulating minus beta splice choice (Wong, Shay, et al., 2014). In our previous report we showed that RNA:RNA pairing may be occurring between a region in intron 6 and a region in intron 8 of hTERT pre-mRNAs. In the RNA:RNA pairing model (Figure 13B), we propose that RNA:RNA pairing of a VNTR in intron 6 of hTERT creates an RNA secondary structure that brings the exon 6

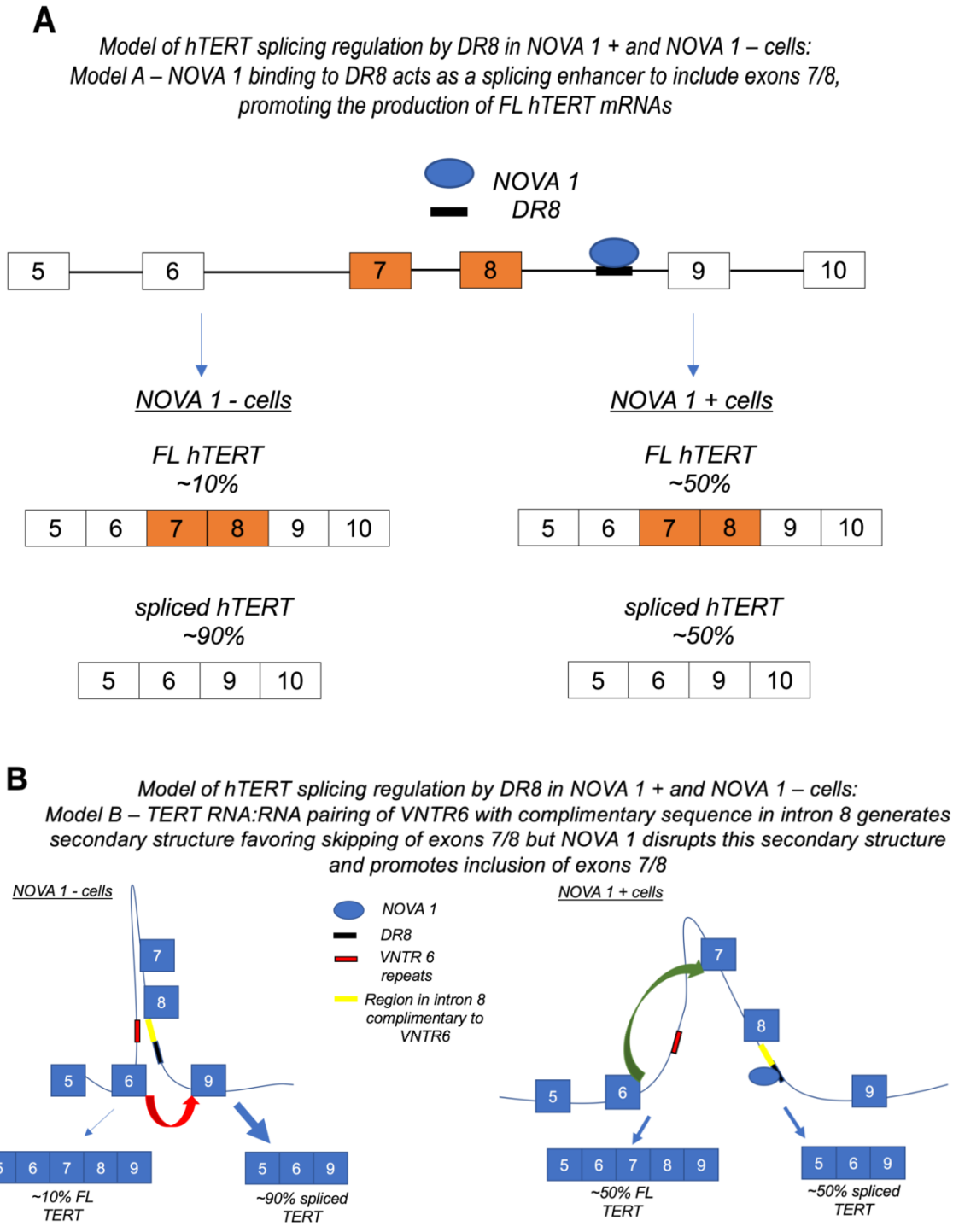

Figure 13. NOVA 1 binds to a deep intronic sequence in hTERT DR8 and acts as a splicing enhancer to promote the inclusion of exons 7/8 and greater levels of fulllength hTERT mRNAs.
A. Linear model depicting NOVA 1 binding to TERT DR8 promoting the inclusion of exons 7/8. B. Alternative model of NOVA 1 promoting full-length TERT considering RNA:RNA pairing/RNA secondary structure of TERT pre-mRNAs.

5’ splice site in close proximity to the exon 9 3’splice site, generating a scenario where joining of exon 6 to exon 9 is favored over exon 6 to exon 7, producing the minus beta splice variant over FL mRNAs. The VNTR in intron 6 and the region in intron 8 are close to the binding site of NOVA1 in hTERT pre-mRNAs. Finally, we propose that when NOVA1 is present and bound to DR8, this binding event disrupts the secondary structure created by RNA:RNA pairing between intron 6 and intron 8 and brings the exon 8 5’ splice site closer to the exon 9 3’ splice site, thus producing more FL molecules of hTERT compared to cells lacking NOVA1 (Figure 13B).

Discussion

Our splicing factor screen and the work of others point to the importance of hnRNP proteins in telomere biology and in the regulation of hTERT pre-mRNA splicing (Ford, Suh, Wright, & Shay, 2000; Le et al., 2013; Q. S. Zhang et al., 2006). Our screen and bioinformatics approaches confirmed factors that were previously reported to directly bind to hTERT pre-mRNA and regulate minus beta splice choice (SRSF11, minigene; hnRNPH2, minigene and bioinformatics, hnRNPL, minigene), providing strength to our current dataset (Figure 7). Our screen and bioinformatics approaches confirmed factors that were previously reported to directly bind to hTERT pre-mRNA and regulate minus beta splice choice (SRSF11, minigene; hnRNPH2, minigene and bioinformatics, hnRNPL, minigene), providing strength to our current dataset (Listerman et al., 2013). We also observed significant changes in the minigene reporter assay for hnRNPH1, hnRNPF, and hnRNPM, which were all observed to

potentially bind hTERT pre-mRNAs in CLIP experiments (Mallinjoud et al., 2014). Future studies of these proteins and their interactions with hTERT are clearly warranted.

In addition to the broad potential of our screen and bioinformatics analyses, we confirmed and detailed the extensive effects of one "hit" from our analyses on cancer biology. We demonstrated that in certain cancers, NOVA1 expression promotes the inclusion of exons 7 and 8, located in the reverse transcriptase domain of hTERT, to produce enzymatically active telomerase and regulate telomere length (Figure 13). Further, NOVA1 may be a regulator of growth and invasion related signaling in cancer cells, coupling telomere length maintenance to other cancer cell hallmarks (Figure 12). How NOVA1 and telomerase are integrated into a biological network associated with splicing and cell survival during development and in cancer progression remains to be more fully studied. We also show that manipulation of the splicing machinery (i.e., reduction in NOVA1 protein) may represent a vulnerability in NOVA1 positive cancers that could be exploited for therapeutic purposes.

CHAPTER FIVE
PTBP1 Results

PTBP1: POLYPYRIMIDINE TRACT BINDING PROTEIN 1

Background

Our group previously identified highly conserved sequences in old world primates and humans that regulate TERT splicing choice (Wong et al., 2013; Wong, Shay, et al., 2014). A sequence in intron 8 (following exon 8) was identified that when blocked with an oligonucleotide induced exon exclusion (skipping) of exons 7 and 8, thus promoting the production of the TERT minus beta transcript (Wong et al., 2013). We termed this region direct repeat 8 (DR8) as it had 85% homology to another region in intron 6 that we called direct repeat 6 (DR6). A cursory glance over the DR8 region revealed a large polypyrimidine tract located nearby. A closer examination revealed a number of potential binding sites for polypyrimidine tract binding proteins (Figure 14).

Although DR8 was included in the minigene screen, the sequence used in the minigene did not include the large polypyrimidine tract near DR8. Still, knock down of polypyrimidine tract binding protein 1 (PTBP1), polypyrimidine tract binding protein 2 (PTBP2) and polypyrimdine tract binding protein 3 (PTBP3) did have effects on hTERT splicing based on results from the minigene screen. Due to the well documented importance of PTBP1 in cancer, we focused on this member of the PTBP protein family.

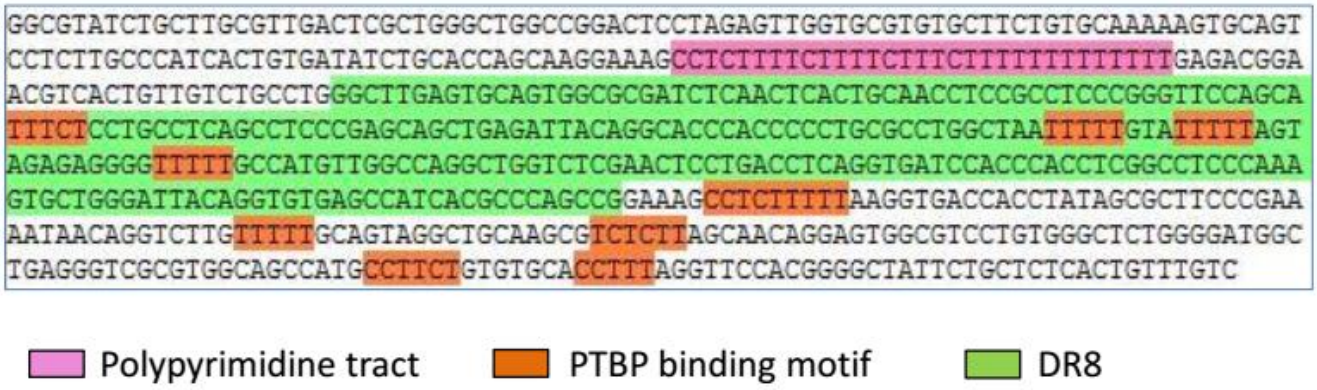

Fig 14. hTERT DR8 region
A long polypyrimidine tract made up of 31 pyrimidines is located near direct repeat 8 (DR8) in intron 8 of hTERT. There are four polypyrimidine tract binding protein binding motifs located within DR8 and five located just outside of DR8.

PTBP1 is expressed in all non-small cell lung cancer cell lines that we tested. We found that stable knockdown of PTBP1 shifted hTERT splicing toward inactive transcripts, reduced telomerase activity, which led to progressively shortened telomeres. Further, we demonstrate via RNA pull down that PTBP1 is able to directly bind hTERT mRNA. These experiments demonstrate another example of a splicing factor that regulates hTERT alternative splicing in cancer cells.

Results

PTBP1 expression in cell lines and tissues

PTBP1 is expressed in all cancer cell lines tested. It has been known to have a number of functions in non-disease contexts including muscle and neural differentiation, viral translation, T cell activation (Ge, Quek, Beemon, & Hogg, 2016; J. C. Lin & Tarn, 2011;

Matus-Nicodemos et al., 2011; Verma, Bhattacharyya, & Das, 2010; Xue et al., 2013). We quantified PTBP1 expression by droplet digital PCR (ddPCR) and found that PTBP1 is expressed at varying levels in all the cells we tested. The panel included normal cells such as IMR90, BJ and primary HBECs, transformed HBEC3KT cells, and a number of non-small cell lung cancer (NSCLC) cell lines (Figure 15). Indeed, the Human Protein Atlas also shows that PTBP1 expression is high or moderate in almost all tissues with the exception of muscle and liver (Figure 16) (Uhlen et al., 2015).

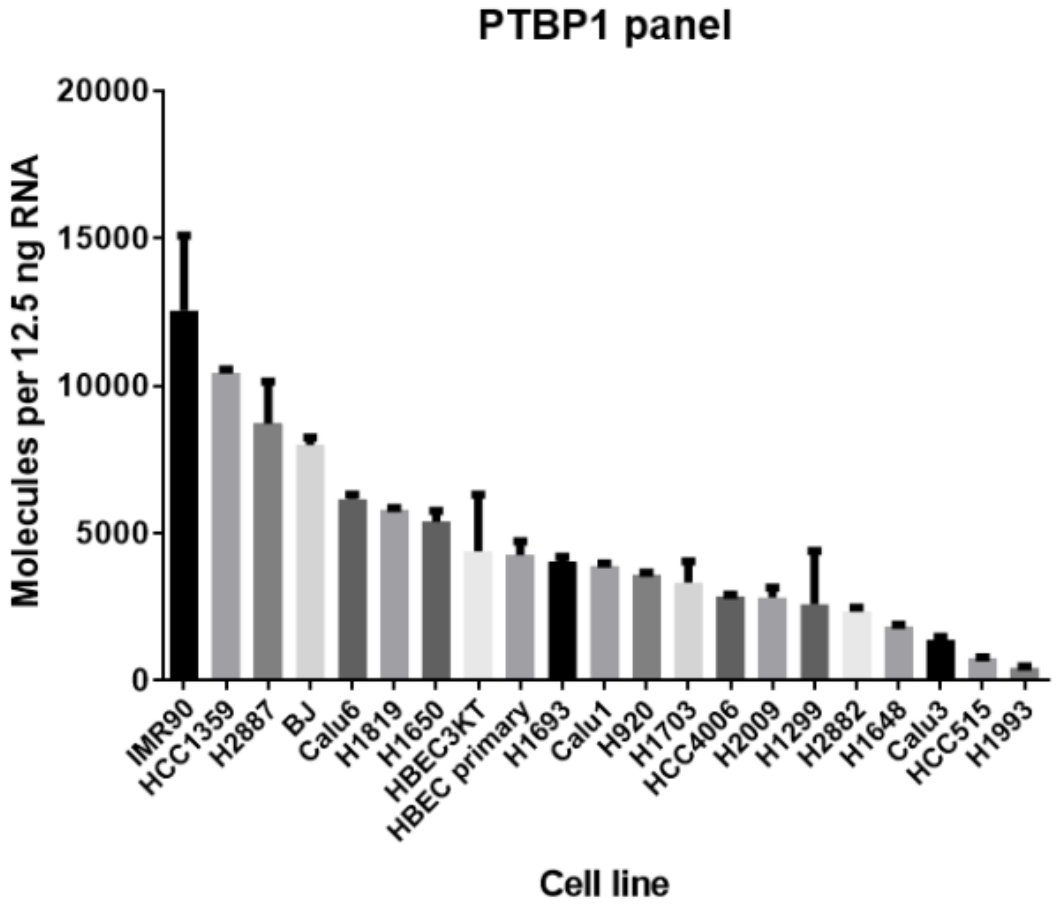

Fig 15. PTBP1 expression in a panel of cancer, transformed and normal cell lines
PTBP1 was measured by ddPCR in a panel of cell lines including non-small cell lung cancer cell lines and a number of normal or transformed cell lines including IMR90, BJ, HBEC primary and HBEC3KT.

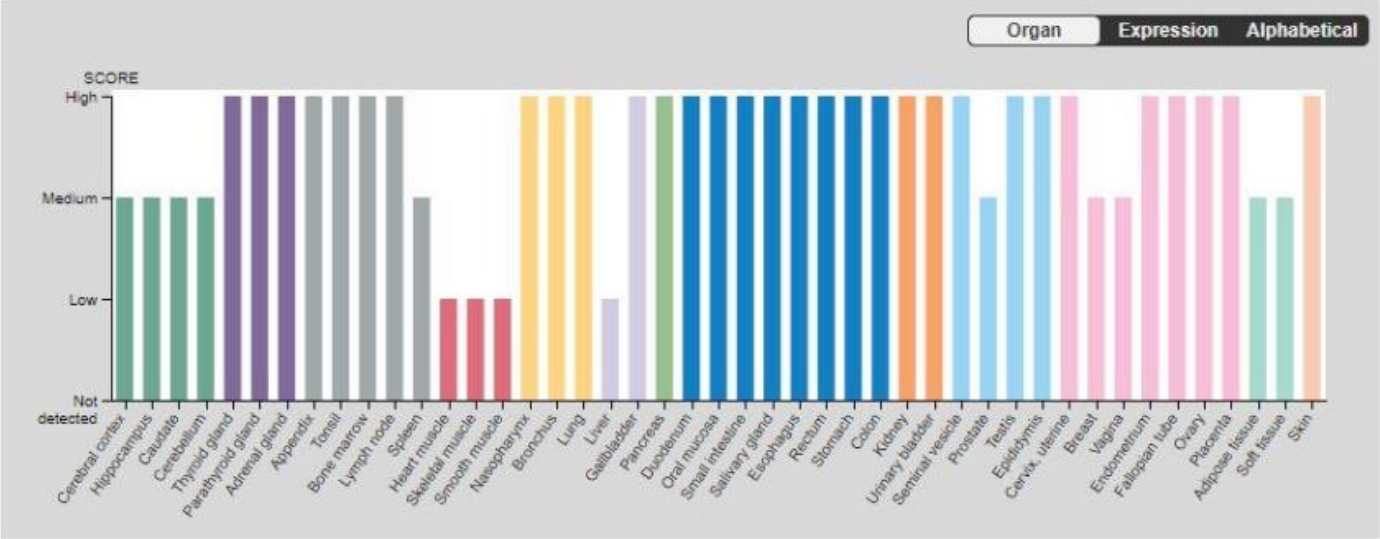

Fig 16. PTBP1 expression from the Human Protein Atlas (v16.1.proteinatlas.org)
PTBP1 protein has moderate to high expression in almost all adult tissues with the exception of various muscle tissues and liver.

Short-term knockdown of PTBP1 decreases telomerase activity in NSCLC cell lines

In order to determine the potential effects of PTBP1 on telomere biology, we performed siRNA knock down experiments. A panel of NSCLC cell lines that expressed varying amounts of PTBP1 was transfected with siRNAs and PTBP1 expression and telomerase activity were measured 72 hours after transfection. The siRNAs against PTBP1 were extremely effective at decreasing PTBP1 mRNA in a panel of NSCLC cell lines as measured by ddPCR (Figure 17). Telomerase activity was differentially affected by PTBP1 knockdown in the NSCLC cell lines. PTBP1 knockdown had no significant effect in some cell lines

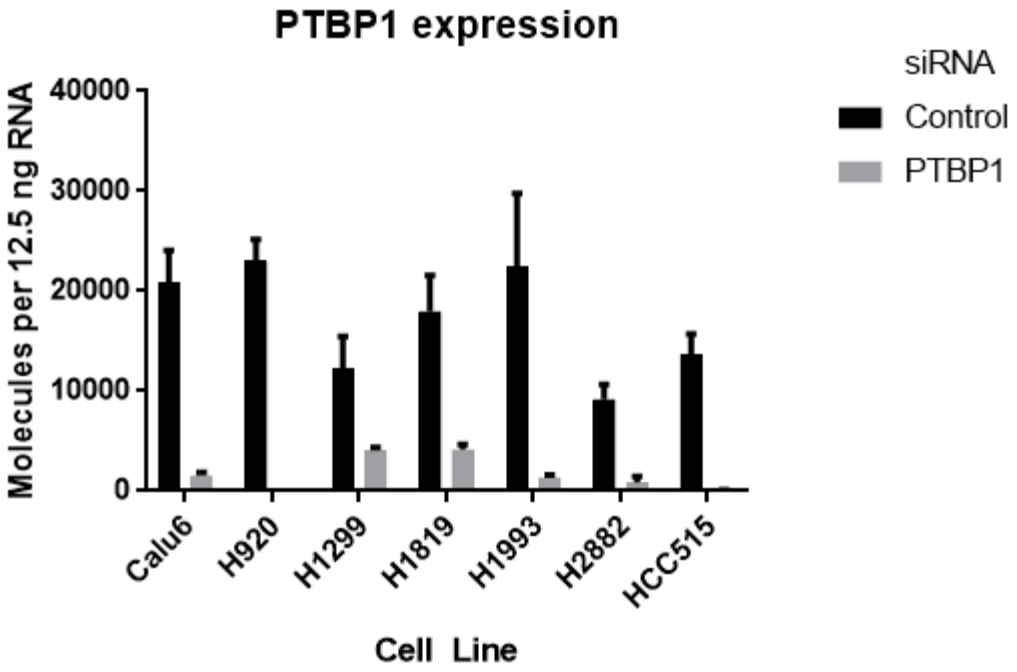

Fig 17. PTBP1 expression in NSCLC cell lines with control siRNA and siRNA targeting PTBP1
PTBP1 expression as measured by ddPCR is dramatically decreased in NSCLC cell lines 72 hours after siRNA transfection targeting PTBP1.

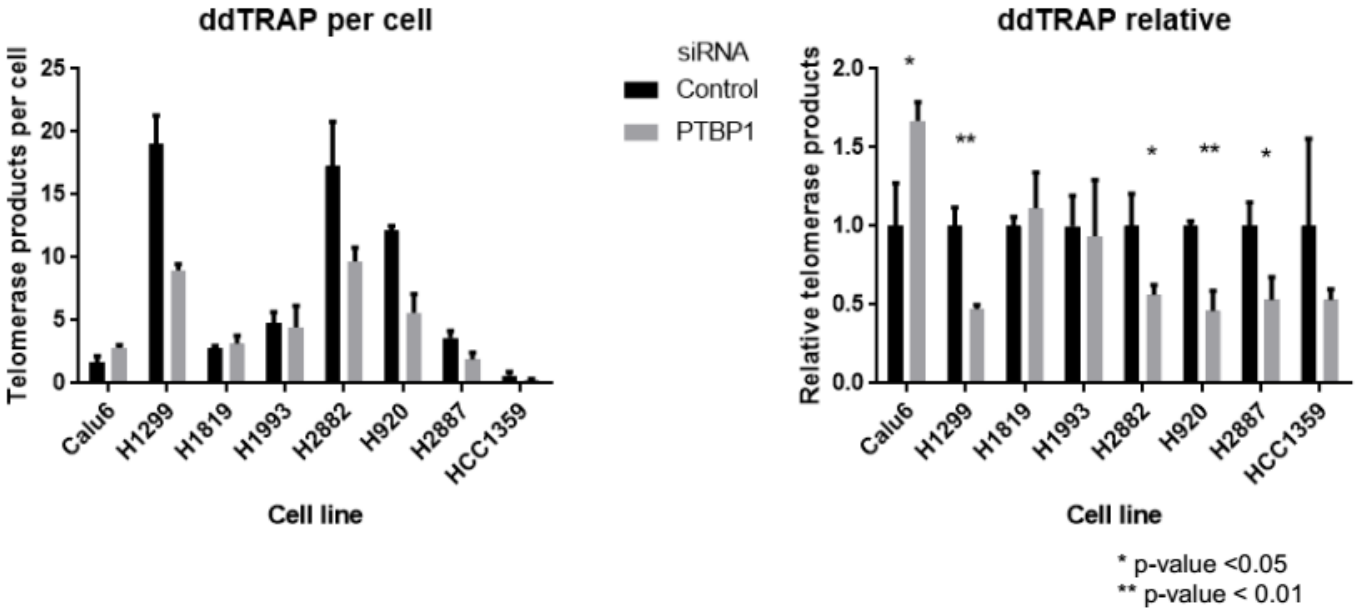

Fig 18. PTBP1 knockdown in NSCLC cell lines altered telomerase activity in some but not all NSCLC cell lines
PTBP1 knockdown resulted in a significant decrease in telomerase activity in H1299, H2882, H920, and H2887 and an increase in telomerase activity in Calu6.

(H1819, H1993, HCC1359), increased telomerase activity in Calu6, and decreased telomerase activity in a number of others (H920, H1299, H2882. H2887)(Figure 18). Short term knockdown of PTBP1 in BJ fibroblasts had no effect on hTERT splicing, telomerase activity, or cell growth over the course of three days (data not shown).

This differential response to PTBP1 knockdown was not associated with level of PTBP1 knockdown since all cancer cell lines had dramatically decreased PTBP1 after siRNA transfection. It is likely that the response of cells to PTBP1 depends on the presence or absence of other factors involved in telomerase activity.

Long-term knockdown of PTBP1 alters hTERT splicing and decreases telomerase activity in NSCLC cells

In order to determine the long-term effects of PTBP1 knockdown in cancer cells, we generated shRNA-mediated stable knockdown cell lines. PTBP1 was knocked down in three cell lines based on our siRNA results-two that were sensitive to PTBP1 knockdown (H1299 and H920) and one that seemed resistant to PTBP1 knockdown (Calu6). Knock down ranged from 70-80% across the tested cell lines (Figure 19). Stable knockdown of PTBP1 shifted hTERT splicing in the two PTBP1 knockdown-sensitive cell lines (H1299 and H920) but had no effect on hTERT splicing in the PTBP1 knockdown-resistant cell line, Calu6 (Figure 20). Concurrently, telomerase activity was significantly decreased in H1299 and H290 cells with PTBP1 knockdown but was not changed in Calu6 after PTBP1 knockdown (Figure 21).

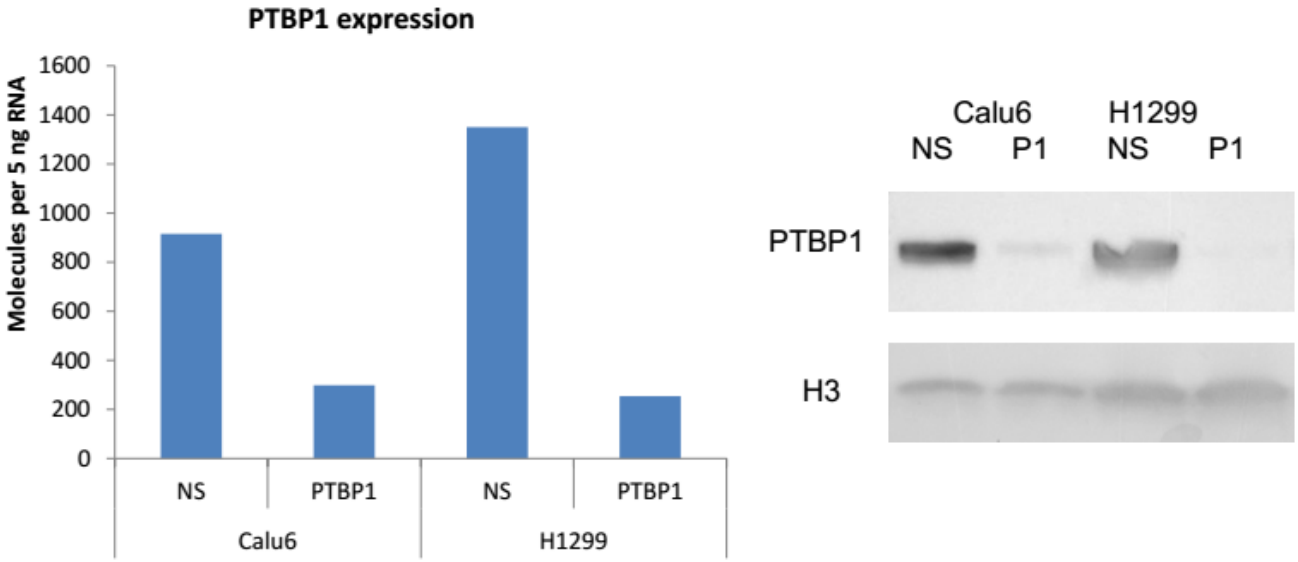

Fig 19. Stable PTBP1 knockdown in NSCLC cell lines
PTBP1 was successfully knocked down in Calu6, H920 and H1299 cell lines as confirmed by ddPCR and western blot.

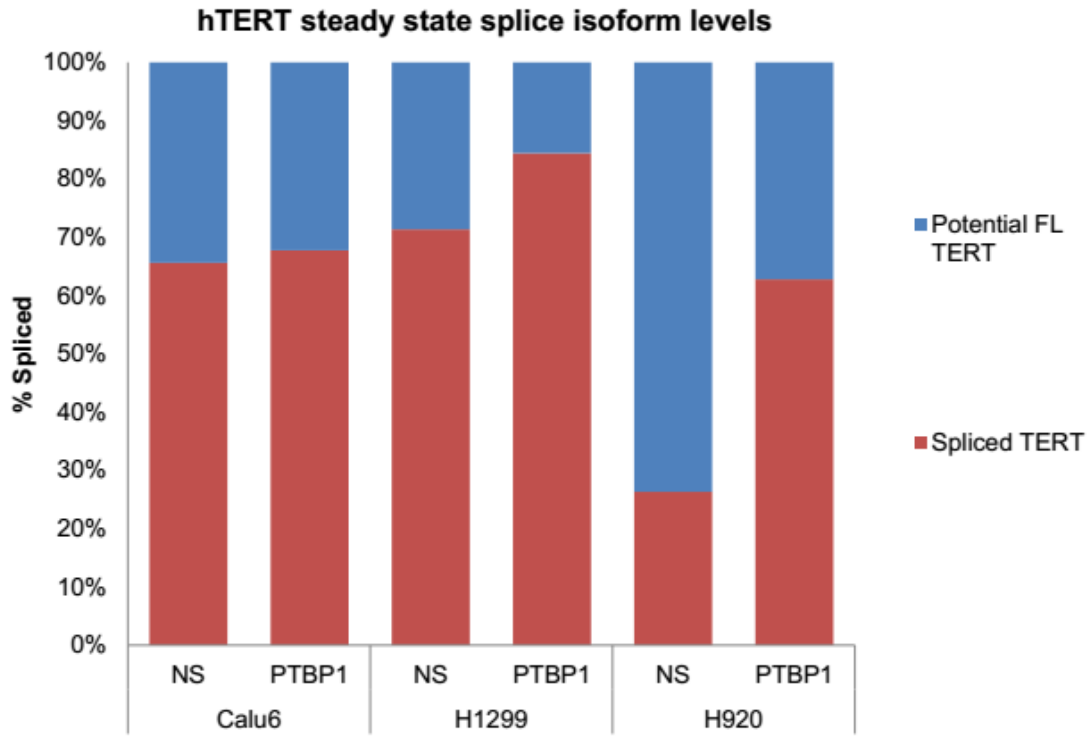

Fig 20. Stable PTBP1 knockdown in NSCLC cell lines altered hTERT splicing in H1299 and H920 but not Calu6.
hTERT splicing as measured by isoform-specific primers show a shift in splicing toward increased spliced or inactive hTERT isoforms in H1299 and H920 cell lines but not in Calu6.

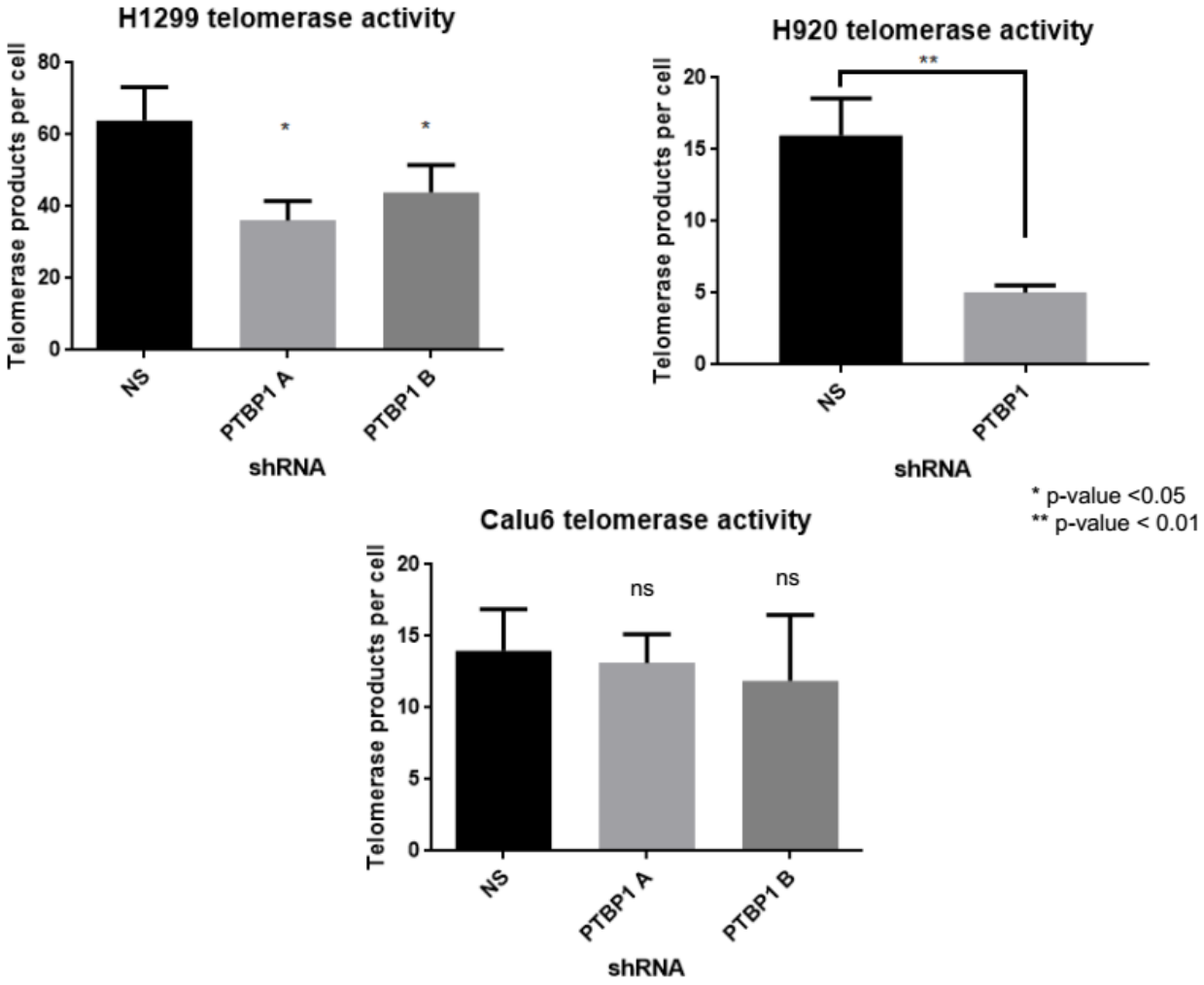

Fig 21. Stable PTBP1 knockdown in NSCLC cell lines decreased telomerase activity in H920, H1299 and Calu6 cells Telomerase activity was measured by ddTRAP and showed a decrease in telomerase activity in H1299 and H920 cell lines and no change in Calu6 cells.

PTBP1 is important for telomere length maintenance

Considering the role of PTBP1 in hTERT splicing and telomerase activity in certain cancer cell lines, it seemed likely that stable knock down of PTBP1 may also result in changes in telomere length maintenance during the long term growth of these cancer cell lines in culture. Our experiments are still currently ongoing but preliminary results suggest that H1299 telomeres are shortening with time in culture.

PTBP1 is able to directly interact with hTERT intron 8

After observing the effects of PTBP1 on telomerase activity and hTERT splicing, we wanted to demonstrate whether PTBP1's effects are due to direct or indirect interaction with hTERT. To answer this question, the same RNA baits from the NOVA1 RNA pulldown experiments were used to determine whether or not the DR8 region of hTERT is able to pulldown PTBP1. Using the same 1 kb sequence containing the DR8 region of hTERT, PTBP1 pulldown was tested for in H1299 and Calu6 cell lysates. The 1 kb RNA bait was able to pull down PTBP1 in the H1299 cell lysate but not in the Calu6 cell lysate even though PTBP1 is present in Calu6 cells, indicating an additional factor in H1299 likely dictates the ability of PTBP1 to bind to DR8 (Figure 22). Furthermore, using the same series of five oligos from our NOVA1 RNA pulldown experiments, we tested to see which oligo would be able to pull down PTBP1 in H1299 and 293 cell lysates. PTBP1 was pulled down strongly by Oligo 3, 4 and 5 and was most strongly pulled down by Oligo 4 in both cell lysates (Figure 23).

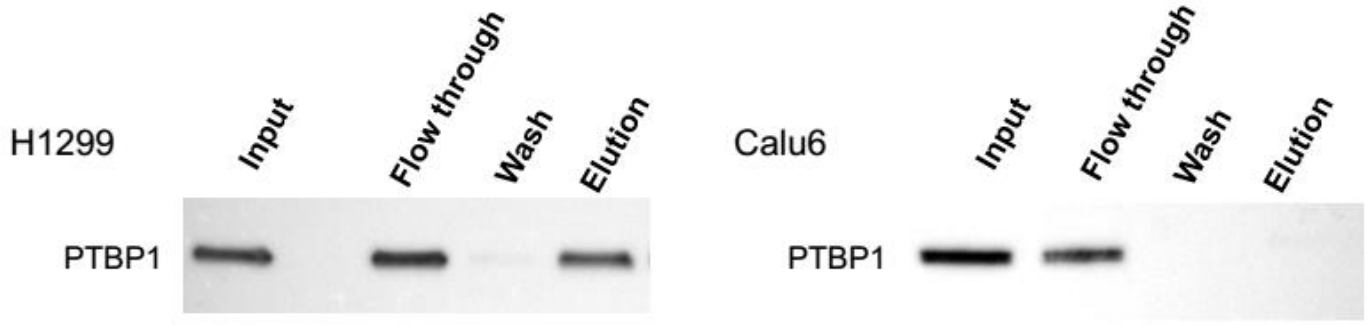

Fig 22. DR8 region pulldown of PTBP1 in H1299 and Calu6 cell lysate
A 1kb biotinylated RNA containing the DR8 region of hTERT is able to pulldown PTBP1 in H1299 cell lysate but not in Calu6 cell lysate.

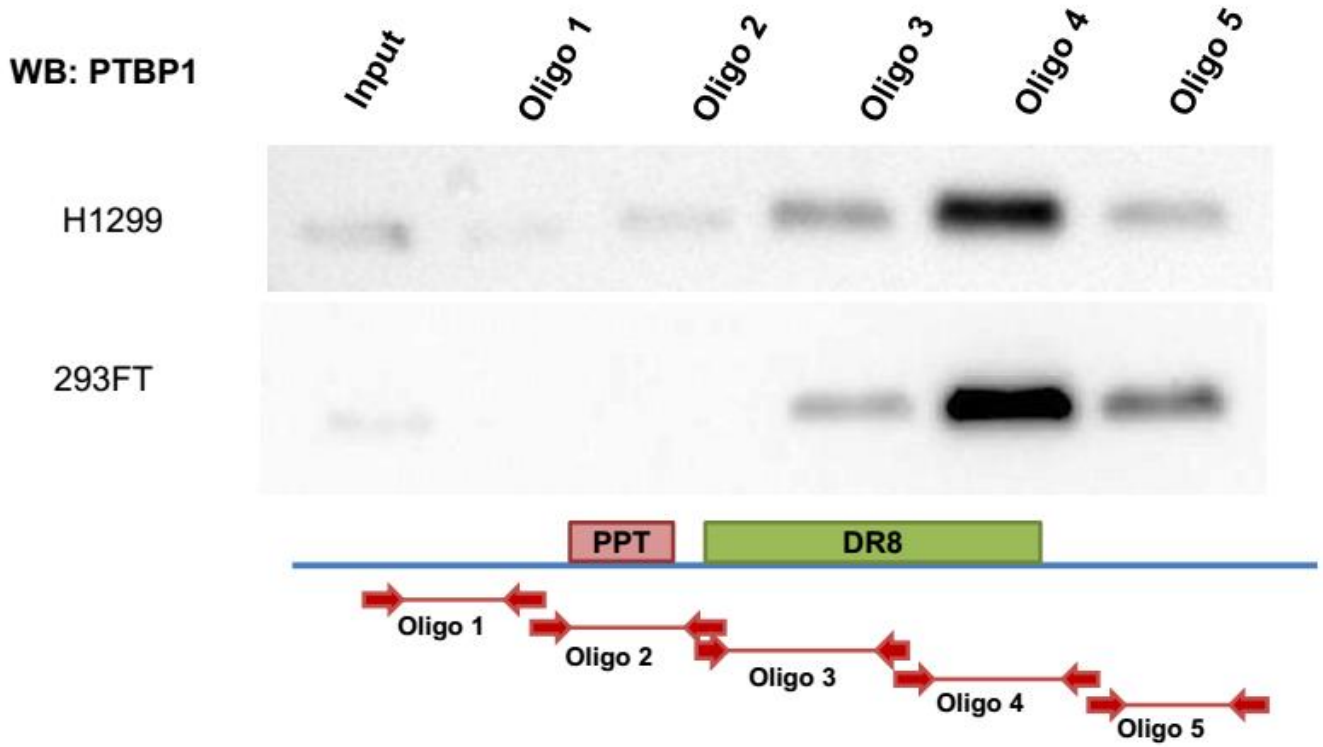

Fig 23. DR8 region pulldown of PTBP1 in H1299 and 293FT cell lysate
A series of 100-180nt RNA oligonucleotides spanning the hTERT DR8 region were generated. PTBP1 was pulled down strongly by Oligo 3, 4 and 5 and most strongly by Oligo 4 in both H1299 and 293 cell lysates.

Discussion

We demonstrated that in certain cancers, PTBP1 expression is important for full length hTERT splicing and telomerase activity. Additionally, PTBP1 is able to directly interact with the DR8 region of hTERT in the same general area where NOVA1 binds. However, unlike NOVA1 which is more tissue specific, the ubiquitous expression of PTBP1coupled with its diverse functions in a wide range of tissues makes it less likely to be targetable as a cancer therapeutic as global knock down might have negative effects in a number of tissues. Still, PTBP1's well documented importance in cancer biology continues to make it interesting

from a basic science perspective. Better understanding of how PTBP1 affects cancer biology could identify interacting factors critical to its functions in cancer that are more targetable.

We are also currently investigating the possibility that PTBP1 and NOVA1 directly interact. Preliminary co-immunoprecipitation experiments do not show a stable interaction between them but it is possible that they are more transiently interacting. If they do rely on each other for their functions on hTERT splicing, targeting their interaction may be a viable option to disrupt full-length hTERT splicing and decrease telomerase activity.

Interestingly, PTBP1 is located less than 1 Mb from the end of chromosome 19p and could potentially be affected by telomere position effects (TPE). Our lab and others have shown that telomere length can alter gene expression of genes located near chromosome ends and that chromosome looping allows telomeres to make contacts with genes up to 10 Mb away from chromosome ends (W. Kim et al., 2016; Robin et al., 2015; Robin et al., 2014). This raises the interesting possibility that PTBP1 could potentially be regulated by telomere length, especially considering the observations that PTBP1 expression as well as PTBP-dependent splicing has been shown to change with disease and age (Santiago & Potashkin, 2015; Tollervey et al., 2011).

CHAPTER SIX
Conclusions and Recommendations

The catalytic protein component of telomerase, hTERT, is spliced into multiple isoforms, but only full-length hTERT mRNA is capable of producing enzymatically active protein that can maintain telomeres (Wong et al., 2013). We demonstrate that in certain cancers, NOVA1 and PTBP1 expression promotes the inclusion of exons 7 and 8, located in the reverse transcriptase domain of hTERT, to produce enzymatically active telomerase and regulate telomere length (Figure 13). Further, NOVA1 may be a regulator of growth and invasion related signaling in cancer cells, coupling telomere length maintenance to other cancer cell hallmarks (Figure 12). We also show that manipulation of the splicing machinery (i.e., reduction in NOVA1 or PTBP1 protein) may represent a vulnerability in NOVA1 positive cancers that could be exploited for therapeutic purposes. Thus, manipulation of telomerase activity and telomere length may be feasible by targeting the alternative splicing machinery with small molecule drugs or by exon skipping/inclusion approaches that may have new implications for cancer therapy.

Considering the recent reports on hTERT promoter mutations and hTERT gene expression (mRNA) levels, we did not find any mutations in the TERT promoter of the lung cancer lines used in this study (Borah et al., 2015; Horn et al., 2013). Thus, we propose that there are several pathways (increased transcription and promoter mutations, altered chromatin and

methylation, and misregulated splicing) that lead to the re-activation of TERT and telomerase in transformed cells. How NOVA1, PTBP1 and telomerase are integrated into a biological network associated with splicing and cell survival during development and cancer progression remains to be more fully studied.

Our work complements and extends earlier findings that RNA metabolism is a critical regulatory component of telomerase activity and telomere length maintenance (L. Y. Chen & Lingner, 2012; Pont, Sadri, Hsiao, Smith, & Schneider, 2012). For instance, the mRNA decay pathway was found to be critical in telomere length maintenance in yeast (Advani, Belew, & Dinman, 2013). Additionally, our splicing factor screen and the work of others point to the importance of hnRNP proteins in telomere biology and in the regulation of hTERT pre-mRNA splicing (Ding et al., 1999; Ford et al., 2000; Jean-Philippe et al., 2013; Q. S. Zhang et al., 2006). We significantly expand this work by providing a list of candidate genes in human cells that are related to full-length mRNA hTERT splicing (Figure 7). Our screen and bioinformatics approaches confirmed factors that were previously reported to directly bind to hTERT pre-mRNA and regulate minus beta splice choice (SRSF11, minigene; hnRNPH2, minigene and bioinformatics, hnRNPL, minigene), providing strength to our current dataset (Listerman et al., 2013). We also observed significant changes in the minigene reporter assay for hnRNPH1, hnRNPF, and hnRNPM, which were all observed to potentially bind hTERT pre-mRNAs in CLIP experiments, further validating our minigene data (Mallinjoud et al., 2014). Future studies of these proteins and their interactions with

hTERT are clearly warranted. These are the first studies to investigate knockdown of splicing factors in human cancer cells with regard to telomere biology and we found that even modest knockdown is compatible with long-term tissue culture studies to investigate changes in the telomere length of cells.

Our work is part of the rapidly evolving field of RNA processing and suggests that underappreciated or unknown mechanisms may be responsible for the regulation of low abundance genes, such as hTERT. The mechanisms of hTERT alternative splicing may involve a combination of RNA:RNA pairing of sequences in two different introns (6 and 8) forming a secondary structure that promotes skipping of exons 7 and 8 (Figure 13) and the binding of splicing factors to these intronic sequences (Wong, Shay, et al., 2014). We found that NOVA1 or PTBP1 binding to DR8 can potentially disrupt the secondary structure of the precursor mRNA, resulting in the inclusion of exons 7 and 8 (Figure 10, 11 and 13). These findings emphasize the potential importance of sequences outside of intron/exon junctions on the regulation of alternative splicing.

Why a system such as this has evolved in humans comes back to the issue of tumor suppression in large, long-lived mammals/primates. When examining the mouse TERT gene, there is no evidence of such sequences that could result in RNA:RNA pairing. This coincides with the observation that the majority of the mouse TERT RNA molecules are full-length activity coding mRNAs. One possibility is that upon the rodent/primate split in evolution, the

larger, longer lived primates required a mechanism to down-regulate TERT and telomerase as a means to prevent pre-cancerous growth from dividing enough times to accumulate mutations and become malignant cancers. The regulation of TERT in primates by alternative splicing could be one of those blocks.

The present studies also indicate that in addition to the roles of NOVA1 in neurons, it also plays a role in the survival of certain cancer cells (i.e., acting as an oncogene) (Figure 12). We provide evidence that the regulation and splicing of NOVA1 is likely different between neurons, stem cells, and cancer cells (Figure 8). We hypothesize that exon 4 of NOVA1 contains regulatory information (i.e., phosphorylation sites or docking sites for other proteins) that in cells with telomerase activity (cancer cells, stem cells, and testis) or telomerase competent cells (stimulated immune cells) allows NOVA1 to interact with hTERT. While in differentiated neurons this regulatory information needed for NOVA1 to interact with hTERT is absent either through the lack of a modification of exon 4 or a missing co-factor that dictates NOVA1's specificity to hTERT. This differential regulation may make NOVA1 more amenable to targeting than previously assumed. It is unlikely that NOVA2 is playing a similar role in cancer biology as it is only expressed in brain cancers and absent in nearly all other human tumors (http://www.proteinatlas.org/ENSG00000104967-NOVA2/cancer).

Our studies also suggest that PTBP1's functions in cancer cells may also involve regulation of hTERT splicing. Interestingly, unlike NOVA1 which has drastically differential expression in cancer cells and in normal tissues, PTBP1 is relatively ubiquitously expressed (Figure 16). Still, its ability to regulate hTERT splicing and telomerase activity was not dependent on its expression. Instead, its function in hTERT splicing seems to be dependent on the presence of other factors, potentially requiring another factor such as NOVA1 to recruit it to the hTERT locus. Indeed, the lines that relied on PTBP1 for hTERT splicing and telomerase activity were the same as the ones that expressed NOVA1 while Calu6, a line that does not express NOVA1, was also not dependent on PTBP1 for hTERT splicing and telomerase activity. Further studies in more cell lines by comparing gene expression between differentially dependent cell lines may provide insights into what is contributing to PTBP1's ability to splice hTERT in certain cancers and not others.

Conclusions and future directions

We provide substantial evidence that hTERT alternative splicing is regulated in non-small cell lung cancer by a deep intronic element in intron 8 that acts as a splicing enhancer when bound by NOVA1 and/or PTBP1. Overall, our results are consistent with the idea that hTERT splicing is malleable in cancer cells by either targeting the intronic DR8 region or by targeting NOVA1 and potentially PTBP1. We observed a stronger phenotype of telomere shortening when NOVA1 was reduced compared to mutation of the NOVA1 binding sites, indicating that NOVA1 likely produces strong telomere maintenance, anti-apoptotic, and

survival signaling pressure to help cancer cells survive. While the splicing of hTERT is complex, we have provided evidence that inclusion of exons 7 and 8 is a major step in the production of FL hTERT mRNA which can then go on to produce hTERT protein and telomerase ribonucleoprotein complexes that can maintain telomeres (Figure 24).

Further studies will be needed to determine if NOVA1 or PTBP1 are targetable, including the identification of proteins that interact with them to promote/repress FL TERT mRNAs in this region and other regions of hTERT that are commonly alternatively spliced (i.e., exons 2, 11, and 14/15). Additionally, the mechanism of utilizing a deep intronic element as a splicing enhancer docking site may not be specific to hTERT and future studies will be needed to determine if other genes utilize these non-canonical splicing regulatory regions. By using hTERT as a model gene and applying the mechanistic methods in this report, we have revealed an entirely underappreciated new regulatory mechanism of alternative splicing. NOVA1, but not NOVA2, is broadly expressed in human cancers and highly expressed in neuroendocrine-like tumors, supporting a more general application of the present findings. Similarly, PTBP1, not PTBP2 or PTBP3, has been linked to the promotion of cancer growth. Thus, if NOVA1 or PTBP1 are targetable, it may have far reaching applications in treating cancers that rely on these splicing factors for growth and survival. We conclude that alternative splicing of hTERT in cancer cells is a highly attractive cancer therapeutic target because it could potentially reduce telomerase enzyme activity, shorten telomeres (limiting

growth potential), and could also kill tumors that have come to depend on the splicing factor for survival.

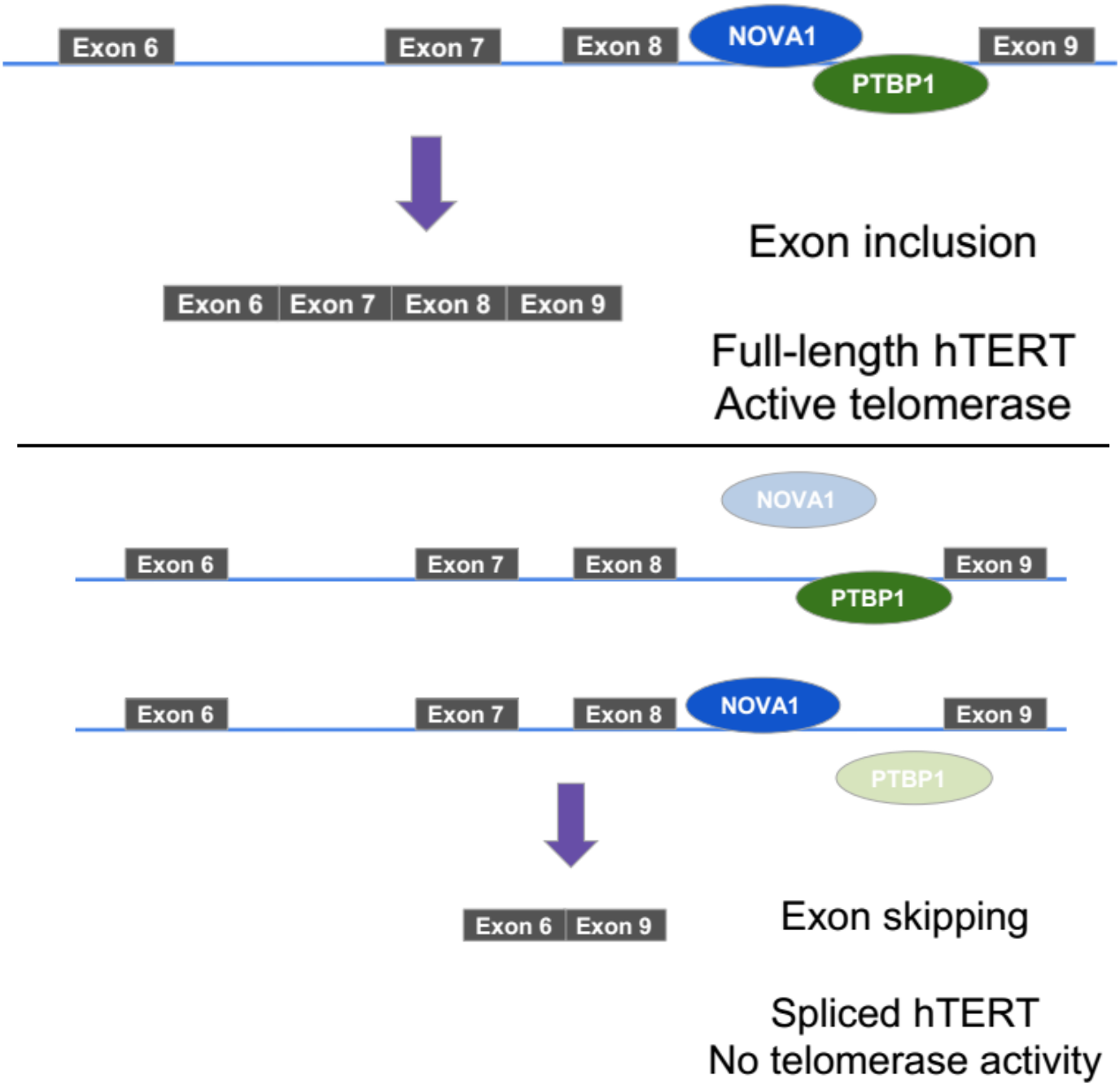

Fig 24. Model of NOVA1 and PTBP1 action on hTERT

A. When NOVA1 and PTBP1 are present, inclusion of exons 7 and 8 is promoted. This produces full length hTERT which can then be translated into active telomerase, producing telomerase activity and maintaining telomeres.
B. When NOVA1 or PTBP1have reduced expression, skipping of exons 7 and 8 is promoted over inclusion. This produces spliced hTERT, which does not produce active telomerase.

APPENDIX A
Supplemental Figures and Tables

Telomerase-based therapy	Mechanism	Source
Imetelstat (GRN163L)	Oligonucleotide, telomerase competitive inhibitor	Marian, Cho et al. 2010; Tefferi, Lasho et al. 2015;Frink, Peyton et al. 2016
6-thio-2'-deoxyguanosine (6-thio-dG)	Nucleoside analogue precursor	Mender, Gryaznov et al. 2015; Mender, Gryaznov et al. 2015a
GRNVAC1/AST-VAC1	Autologous dendritic cell vaccine	Nair, Heiser et al. 2000;Frolkis, Fischer et al. 2003
GV1001, Vx-001	Telomerase peptide vaccine	Brunsvig, Aamdal et al. 2006; Staff, Mozaffari et al. 2014; Kotsakis, Papadimitraki et al. 2014

Supplemental Table 1. Table of telomerase-based therapies and mechanisms of action
A number of types of therapies targeting telomerase have been studied and found to be effective in different contexts. Imetelstat is a competitive inhibitor and has been studied in a number of clinical trials but has consistently shown hematological toxicity. 6-thio-dG is a novel nucleoside analogue precursor which is converted into a nucleotide and incorporated into telomeres, resulting in telomerase-dependent telomere uncapping. GRNVAC1, now known as AST-VAC1, is an autologous dendritic cell vaccine against hTERT that has been studied in clinical trials for AML. GV1001 and Vx-001 are telomerase peptide vaccines that are being studied in NSCLC and pancreatic cancers.

Supplemental Table 2. Primers and probes				
Primer pairs	Primer name	Exon location	Primer sequence (5'-3')	Probe – UPL or custom
1	hTERT 3' F	15/16 boundary	GGGTCACTCAGGACAGCCCAG	37
1	hTERT 3' F	16	GGGCGGGTGGCCATCAGT	
2	hTERT RT F	7/8 boundary	ACAGTTCGTGGCTCACCTG	52
2	hTERT RT R	8	GCGTAGGAAGACGTCGAAGA	
3	hTERT 5' F	2	AAGCATGCCAAGCTCTCG	17
3	hTERT 5' R	3	CAGGATCTCCTCACGCAGAC	
4	Minus Beta F	6/9 boundary	CAAGAGCCACGTCCTACGTC	58
4	Minus Beta R	9	CAAGAAATCATCCACCAAACG	
5	Minus Alpha F	5	GTTCAGCGTGCTCAACTACG	custom
5	Minus Alpha R	6	GTTCTGGGGTTTGATGATGC	
6	delta4-13 F	3	CCGGAAGAGTGTCTGGAGCAAGTTGCAAAGC	24
6	delta4-13 R	14	GATGGAGTAGCAGAGGGAGGCCGTGTCAGAG	
7	INS3 F	Intron 14	AGAGATGGAGCCACCCCGCA	custom
7	INS3 R	Intron 14/exon 15 boundary	AGCGACATCCCTGGGGGAAAAC	
8	INS4 F	Exon 14	TGAAAGCCAAGAACGCAGGTAT	custom
8	INS4 R	Intron 14	TAAGCCCAGATTCACTCAGTCTCC	
9	MS2 T1 F	linear RNA	GTCGCGGTAATTGGCGC	custom
9	MS2 T1 R	linear RNA	GGCCACGTGTTTTGATCGA	
10	MS2 T2 F	linear RNA	CCTCAGCAATCGCAGCAAA	custom
10	MS2 T2 R	linear RNA	GGAAGATCAATACATAAAGAGTTGAACTTC	

Note: UPL – Universal Probe Library (Roche)

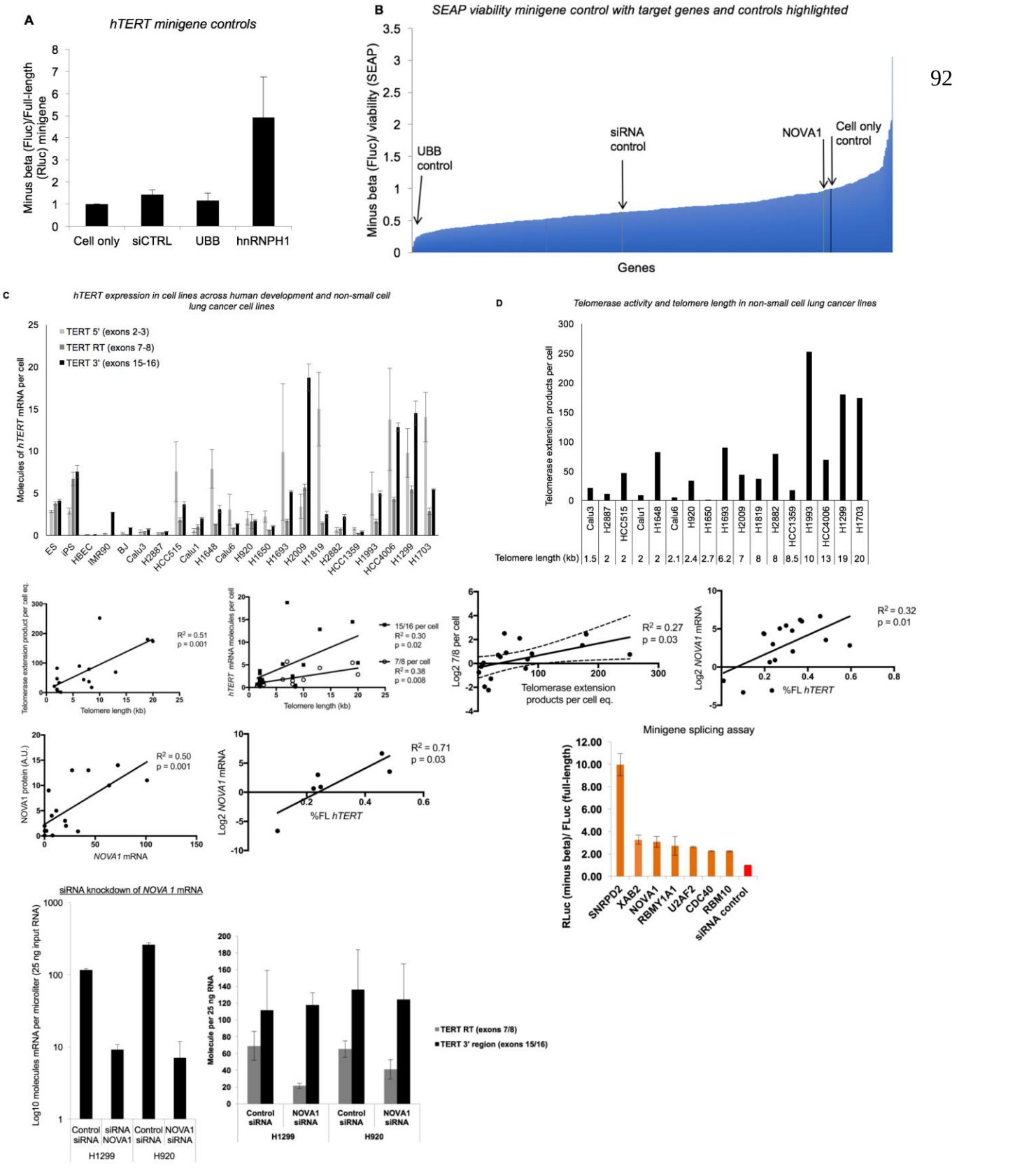

Figure S1A. hTERT minigene luciferase assay of controls used in the screen. 'Cell only' contains on cells and media. 'siCTRL' is the siRNA scrambled control. 'UBB' is a pool of four siRNAs targeting ubiquitin. hnRNPH1 is a pool of four siRNAs targeting the splicing factor hnRNPH1. B. Secreted embryonic alkaline phosphatase viability assay. Viability for the 'cell only', siRNA scrambled control (siRNA control), NOVA 1, and UBB siRNA pools are pointed out. C. Steady state expression of hTERT mRNAs across the hTERT locus showing general expression patterns in cell lines with and without telomerase activity. D. Telomerase enzyme activity as determined by droplet digital TRAP (ddTRAP) and telomere length (terminal restriction fragment lengths from Frink et al. 2016, Oncotarget). E-J. Correlation analysis. K. Minigene targets that overlapped with "Pathway" analysis of microarray data. L. Validation of NOVA 1 siRNA mediated knockdown at the mRNA level in H1299 and H920 cells. M. mRNA expression of hTERT following siRNA mediated knockdown of NOVA 1 in H1299 and H920 cells.

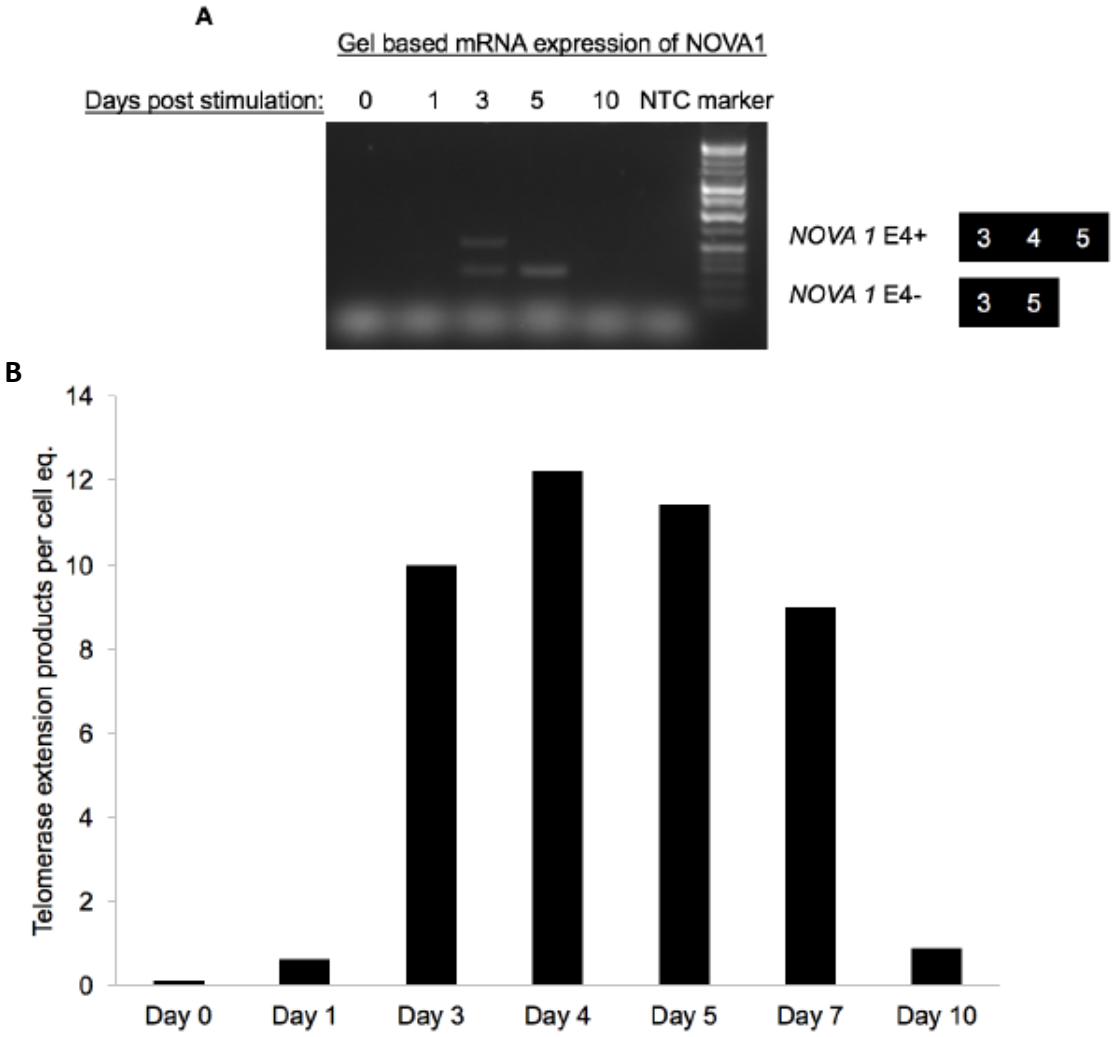

Figure S2
A. RT-PCR gel of NOVA 1 expression in stimulated human lymphocytes at days 0, 1, 3, 5, and 10. Primers for NOVA 1 were in exons 3 and 5.
B. Telomerase enzyme activity in stimulated human lymphocytes at days 0, 1, 3, 4, 5, 7, and 10 as determined by droplet digital TRAP.

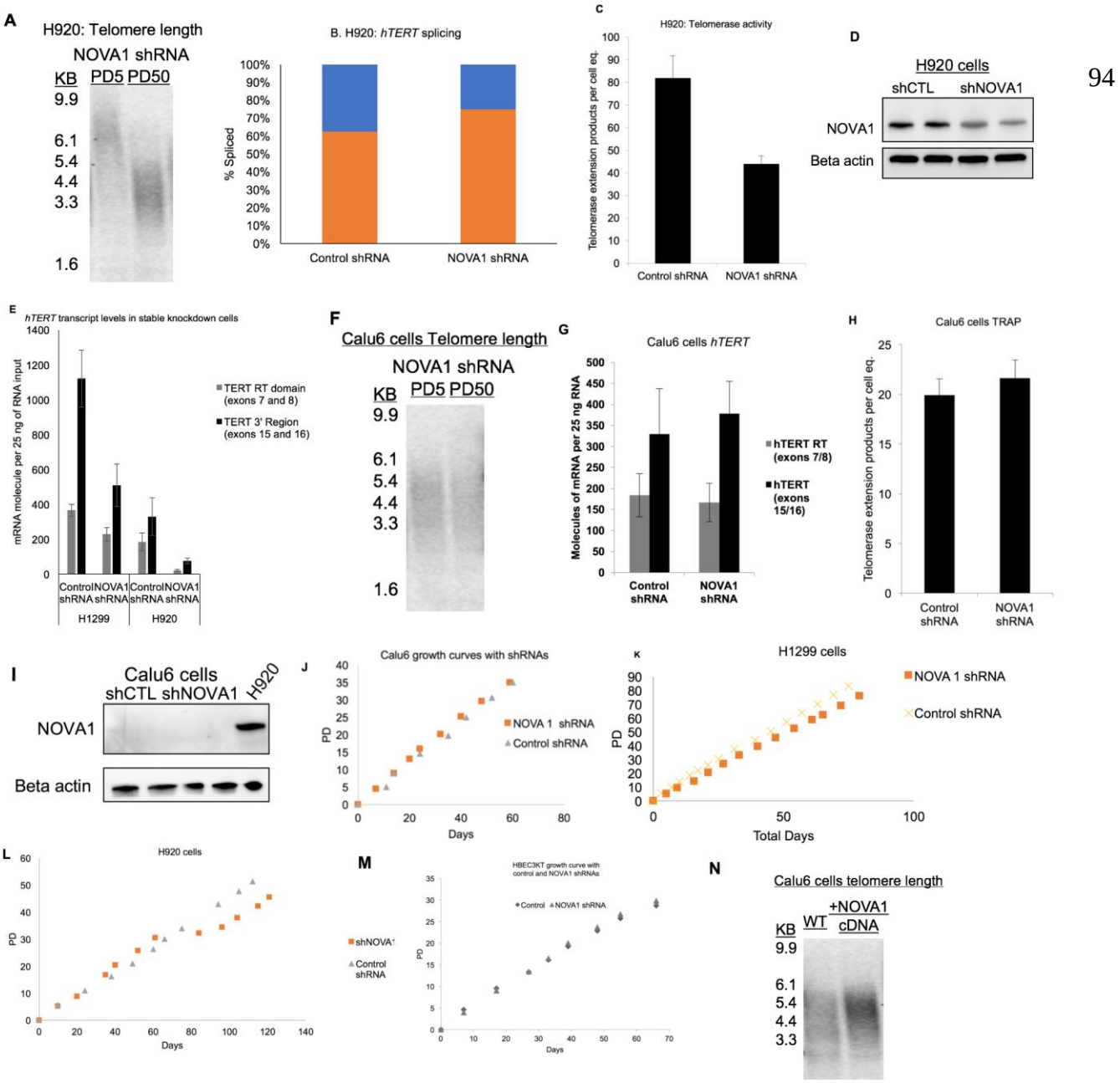

Figure S3

A. TRF analysis of H920 cells with NOVA 1 shRNA media knockdown at population doubling (PD) 5 and 50.

B. hTERT RT-ddPCR analysis of H920 cells with NOVA 1 shRNA media knockdown over the course of the experiment.

C. Telomerase activity determined by ddTRAP in H920 cells with NOVA 1 shRNA media knockdown over the course of the experiment.

D. Western blot showing NOVA 1 protein levels in H920 cells with NOVA 1 shRNA media knockdown.

E. H1299 and H920 steady state hTERT transcript levels in stable shRNA knockdown of NOVA 1 cells.

F. TRF analysis of Calu6 cells with NOVA 1 shRNA media knockdown at population doubling (PD) 5 and 50. Calu6 cells lack NOVA 1 protein.

G. Calu6 steady state hTERT transcript levels in stable shRNA knockdown of NOVA 1 cells. Calu6 cells lack NOVA 1 protein.

H. Telomerase activity determined by ddTRAP in Calu6 cells with NOVA 1 shRNA media knockdown over the course of the experiment. Calu6 cells lack NOVA 1 protein.

I. Western blot of Calu6 cells with shRNA control (CTL) and NOVA1 knockdown. H920 protein lysate was used as a positive control for NOVA 1 antibody. Calu6 growth curve with shRNAs.

J. H1299 cells growth curves with control and NOVA 1 shRNAs.

K. H920 cells growth curves with control and NOVA 1 shRNAs.

L. TRF analysis of Calu6 cells without NOVA 1 (WT) and with a V5-Tagged NOVA 1 cDNA.

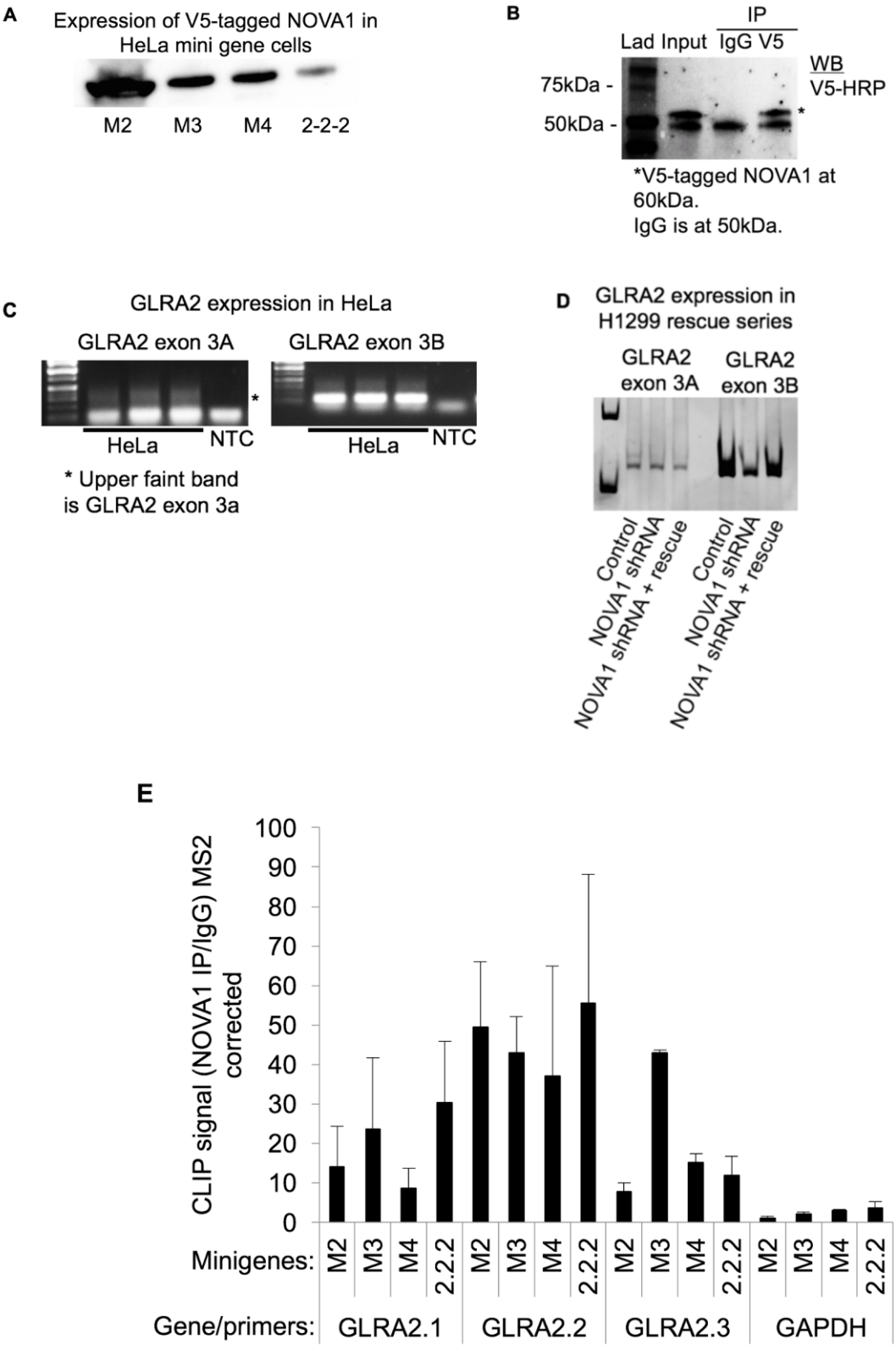

Figure S4

A. Expression of V5-Tagged NOVA 1 in HeLa cells containing hTERT minigenes M2, M3, M4, and 2-2-2.

B. Representative immunoprecipitation of V5-tagged NOVA 1 from HeLa cells with hTERT minigenes.

C. GLRA2 mRNA expression of alternatively spliced exons 3A and 3B in HeLa cells with hTERT minigenes.

D. GLRA2 mRNA expression of alternatively spliced exons 3A and 3B in H1299 cells with shRNA control, shRNA against NOVA 1, or with shRNA against NOVA 1 rescued with a NOVA 1 cDNA resistant to NOVA 1 shRNA.

E. Compiled CLIP data showing successful pulldown of NOVA 1 target gene GLRA2 and a negative control RNA, GAPDH.

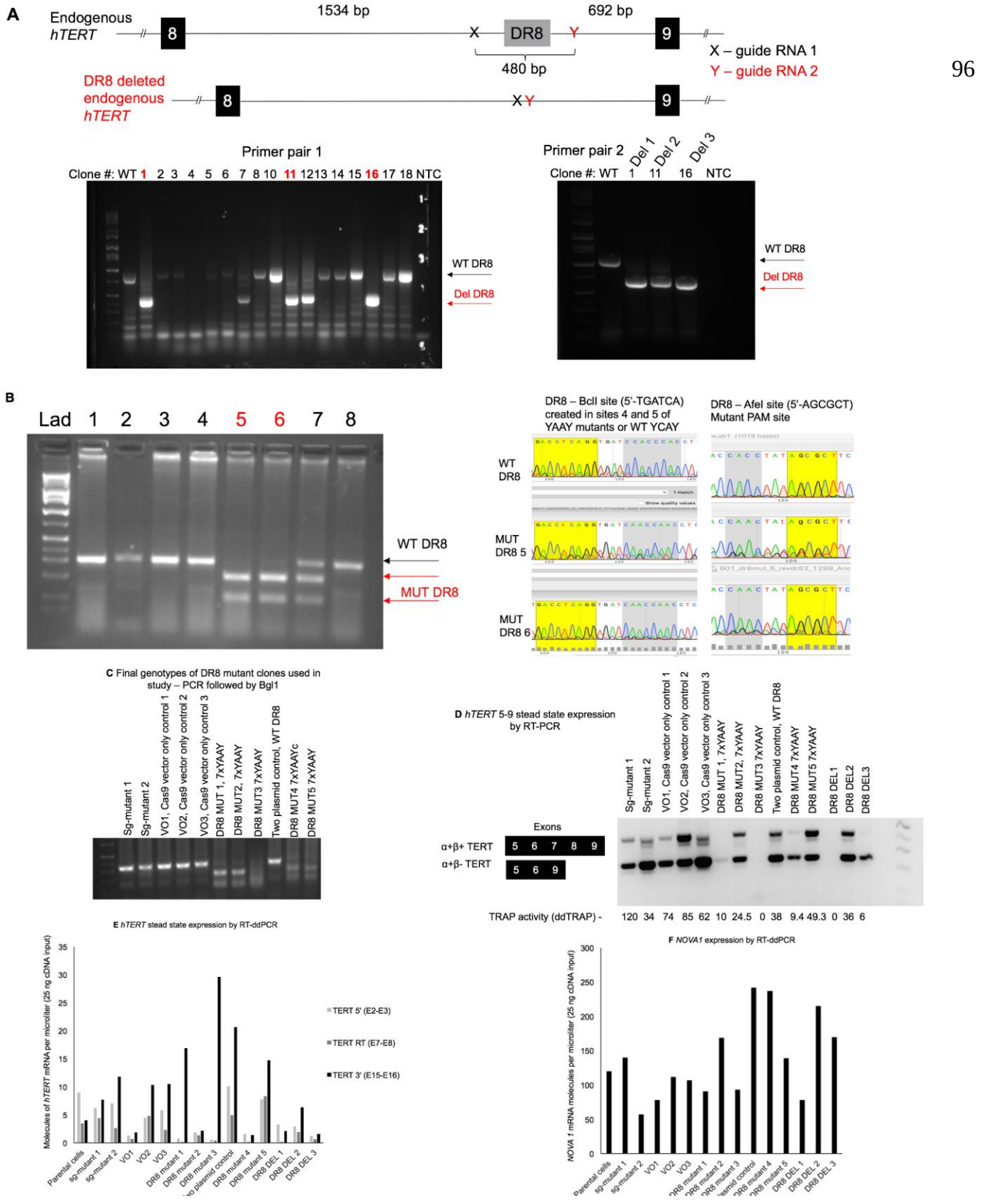

Figure S5

A. Cartoon of hTERT locus showing DR8 in intron 8 deletion scheme and primer locations for genotyping. Agarose gels following PCR with primer pair 1. Agarose gels following PCR with primer pair 2 in WT and mutants 1, 11 and 16.

B. Agarose gel of digested PCR product of WT (lane 1) and attempted CRISPR/Cas9 manipulation of intron 8 to be NOVA 1 resistant. Sanger sequencing confirm gel analysis.

C. Agarose gel of digested PCR products of controls and 7x YAAY NOVA1 resistant clones.

D. hTERT 5-9 gel PCR analysis of steady state mRNA levels of mutant H1299 cells. TRAP quantification (telomerase extension products per cells equivalent) shown below each clone's lane.

E. hTERT ddPCR analysis of steady state mRNA levels in mutant H1299 cells.

F. NOVA 1 mRNA expression (exon 4 containing) levels in mutant H1299 cells.

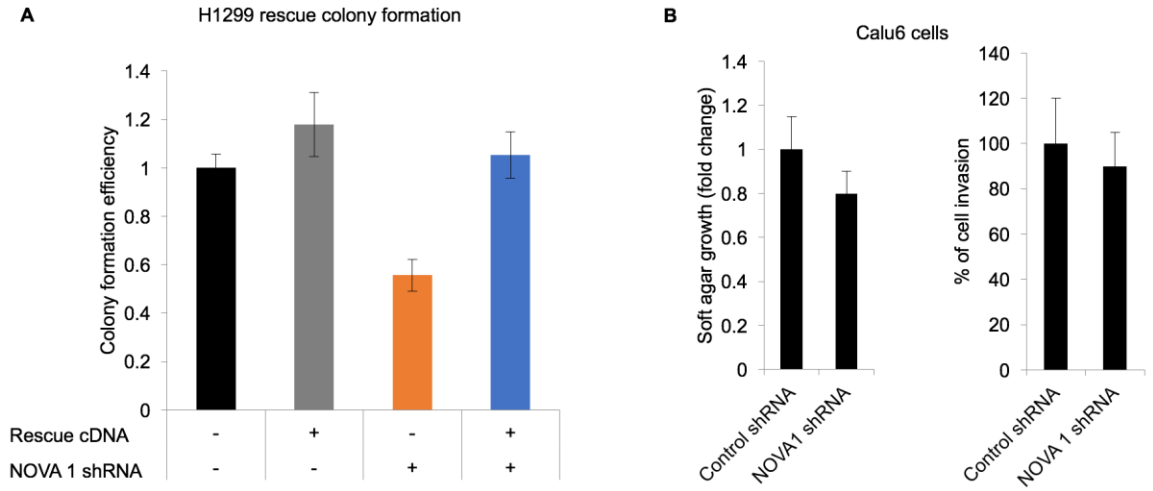

Figure S6
A. Colony formation assay in H1299 rescue series cells.
B. Tumeroginicty analysis in Calu6 cells with control and NOVA 1 shRNAs. Growth on soft agar and invasion through matrigel (Boyden Chamber) analysis was performed in biological triplicate and technical duplicate.

CHAPTER SEVEN
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