CONSEQUENCES OF TELOMERASE INHIBITION AND TELOMERE DYSFUNCTION IN BRCA1 MUTANT CANCER CELLS

Elizabeth Ann Phipps

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Brittney-Shea Herbert, PhD, Chair

Doctoral Committee

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Brenda R. Grimes, PhD

June 6, 2013

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George W. Sledge, MD

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John J. Turchi, PhD

_____

Kenneth E. White, PhD

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ABSTRACT

Elizabeth Ann Phipps

CONSEQUENCES OF TELOMERASE INHIBITION AND TELOMERE DYSFUNCTION IN BRCA1 MUTANT CANCER CELLS

Telomere maintenance is a critical component of genomic stability. An increasing body of evidence suggests *BRCA1*, a tumor suppressor gene with a variety of functions including DNA repair and cell cycle regulation, plays a role in telomere maintenance. Mutations in *BRCA1* account for approximately half of all hereditary breast and ovarian cancers, and the gene is silenced via promoter methylation and loss of heterozygosity in a proportion of sporadic breast and ovarian cancers. The objective of this study was to determine whether GRN163L, a telomerase inhibitor, currently in clinical trials for the treatment of cancer, has enhanced anti-cancer activity in *BRCA1* mutant breast/ovarian cancer cell lines compared to wild-type cancer cells. *BRCA1* mutant cancer cells were observed to have shorter telomeres and increased sensitivity to telomerase inhibition, compared to cell lines with wild-type *BRCA1*. Importantly, GRN163L treatment was synergistic with DNA-damaging drugs, suggesting potential synthetic lethality of the BRCA1 cancer subtype and telomerase inhibition In a related study to examine the roles of BRCA1/2 in telomere maintenance, DNA and RNA extracted from peripheral blood were used to investigate the age-adjusted telomere lengths and telomere-related gene expression profiles of BRCA1 and BRCA2 individuals compared to individuals who developed sporadic cancer and healthy

controls. BRCA1 mutation carriers and breast cancer patients showed the shortest average telomere lengths compared to the other groups. In addition, distinct genomic profiles of BRCA mutation carriers were obtained regarding overexpression of telomere-related genes compared to individuals who developed sporadic or familial breast cancer. In summary, telomerase inhibition may be a viable treatment option in BRCA1 mutant breast or ovarian cancers. These data also provides insights into further investigations on the role of BRCA1 in the biology underlying telomere dysfunction in cancer development.

Brittney-Shea Herbert, PhD, Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

53BP1	Tumor suppressor p53-binding protein 1
ALT	Alternative Lengthening of Telomeres
altNHEJ	Alternative Non-Homologous End Joining
ANOVA	Analysis of variance
A-T	Ataxia-telangiectasia
ATCC	American Type Culture Collection
ATM	Ataxia telangiectasia mutated
ATR	Ataxia Telangiectasia and Rad3 related
BAP1	BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)
BARD1	BRCA1-associated RING domain protein 1
BASC	BRCA1-associated genome surveillance complex
BER	Base excision repair
BMI	Body mass index
bp	Base pair
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
BRCT	BRCA1 C Terminus domain
BSA	Bovine serum albumin
CCDP	cis-diamminedichloroplatinum(II)
CGH	Comparative genomic hybridization
CI	Combination index
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma in situ
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle Medium
dox	Doxorubicin
ER	Estrogen receptor
FBS	Fetal bovine serum
GEO	Gene Expression Omnibus
Gy	Gray
HBSS	Hank's balanced salt solution
HER2	Human epidermal growth factor receptor 2
HMECs	Human mammary epithelial cells
HR	Homologous recombination
hTERT	see TERT
hTR	Human telomerase RNA component
IR	Ionizing radiation
Kb	Kilobase
LB	Lysis buffer
IC	Internal control
LOH	Loss of heterozygosity
MEFs	Mouse embryonic fibroblasts
MEM	Minimum essential medium
MM	Mismatch

MRN	Mre11, Rad50, NBS1
mTerc	Mouse telomerase RNA component
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NP40	Nonyl phenoxypolyethoxylethanol
pBp	pBABEpuro
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with tween 20
PCR	Polymerase chain reaction
PR	Progesterone receptor
RTA	Relative telomerase activity
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNPs	Single nucleotide polymorphism
SSC	Saline-sodium citrate
TERT	Telomerase reverse transcriptase (hTERT in humans, mTERT in mice)
TIFs	Telomere dysfunction-induced focus
TNBC	Triple negative breast cancer
TRAP	Telomeric repeat amplification protocol
TRF	Telomere restriction fragment
UT	Untreated
UV	Ultraviolet

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Cancer Morbidity and Mortality

Despite advances in diagnosis and treatment, cancer remains the second leading cause of death in the United States. The annual economic impact of healthcare for the disease is estimated at over $200 billion according to the National Institutes of Health. Approximately 500,000 people in the United States will die of cancer this year. Though 5-year survival rates for many cancers have improved dramatically since the 1970s, treatment of metastatic disease poses significant challenges (American Cancer Society, www.cancer.org).

Breast cancer is the most commonly diagnosed cancer in women and the second leading cause of cancer deaths in women each year, accounting for 14% of overall cancer fatalities. Breast cancer outcomes vary widely depending upon disease stage and subtype. Five-year survival for ductal carcinoma in situ (DCIS), the earliest stage of breast cancer (Stage 0), is approximately 93%. Survival rates go down if cancer has spread to one or more lymph nodes at time of detection and, if cancer is detected in distant organs or lymph nodes (Stage IV disease), 5-year survival is estimated at 15% (American Cancer Society, www.cancer.org).

For well over ten years, breast cancer treatment has been determined by tumor expression of estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2). Though expression of hormone receptors remains central to breast cancer classification and treatment, work by Perou and colleagues in 2000 led to breakthroughs in our understanding of the complexity and heterogeneity of the disease.

Using cDNA microarray analysis of 65 breast cancer surgical specimens, Perou and collaborators revealed five distinct molecular subtypes of disease: basal-like, HER2, normal breast-like, luminal A, and luminal B (Perou, Sorlie et al. 2000). The intrinsic subtypes were subsequently demonstrated to be associated with patient outcome, with basal-like cancers having the worst overall survival (Sorlie, Perou et al. 2001). Most basal-like cancers lack expression of ER, PR, and HER2 receptors and, as a consequence, this subtype does not respond to endocrine therapies and Herceptin®. In addition, many basal-like cancers exhibit germline mutation or sporadic dysfunction in the *BRCA1* (breast cancer 1, early onset) tumor suppressor gene. (Schnitt 2010).

The development of breast cancer is as complex as the varied pathology of the disease itself. Lifestyle factors such as weight, alcohol use, contraceptive use, hormone replacement therapy, and pregnancy history have all been linked to an increased risk of breast cancer. Other risk modifiers include age, race, breast tissue density, and family history of breast cancer. Rates of breast and ovarian cancer are higher among women with germline mutations in *BRCA1* or *BRCA2* tumor suppressor genes (American Cancer Society, www.cancer.org), and hereditary breast cancers that develop in BRCA1 carriers are often early-onset, particularly aggressive, basal-like, and lack targeted treatment options (Holstege, Horlings et al. 2010).

The design of therapies specific for hereditary breast cancer is aided by an understanding of the molecular contributions of BRCA1 and BRCA2 to maintenance of genomic stability. Biallelic loss of either tumor suppressor facilitates selection of additional mutations favoring tumor evolution, and recent evidence suggests loss of a single allele of BRCA1 also propagates this process (Konishi, Mohseni et al. 2011). This

work tests the hypothesis that genomic instability after BRCA1 loss occurs, in part, through alterations to telomere length and expression of telomere and telomerase-associated genes. Targeted therapies must take aim at features, or "hallmarks of cancer" tumors depend upon to develop and thrive, and limitless replicative potential fueled by telomerase reactivation is one such hallmark (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Telomerase is reactivated and functions to maintain telomere length in nearly all cancers, and this thesis examines inhibition of telomerase activity in the context of BRCA1 deficiency and telomere dysfunction in BRCA mutation carriers.

Telomeres and Cancer

Telomeres are nucleoprotein structures that cap and protect the ends of linear chromosomes in most eukaryotic organisms. Human telomeres are comprised of TTAGGG repeats ending in a single-stranded 3' G-overhang of approximately18-600 nucleotides (Moyzis, Buckingham et al. 1988; Zhao, Hoshiyama et al. 2008). The single-stranded 3' overhang loops into the double-stranded DNA forming a T-loop structure (Griffith, Comeau et al. 1999). The "cap" formed at telomeres is comprised of six protein shelterin components that interact with DNA and function to prevent telomeres from being recognized by cellular DNA repair machinery as double-stranded breaks. The shelterin components TRF1, TRF2, TIN2, TPP1, and RAP1 bind double-stranded telomeric DNA, while POT1 binds the 3' single-stranded overhang. TRF1, TRF2, and POT1 show specificity for binding TTAGGG repetitive DNA (de Lange 2005) (Figure 1.1).

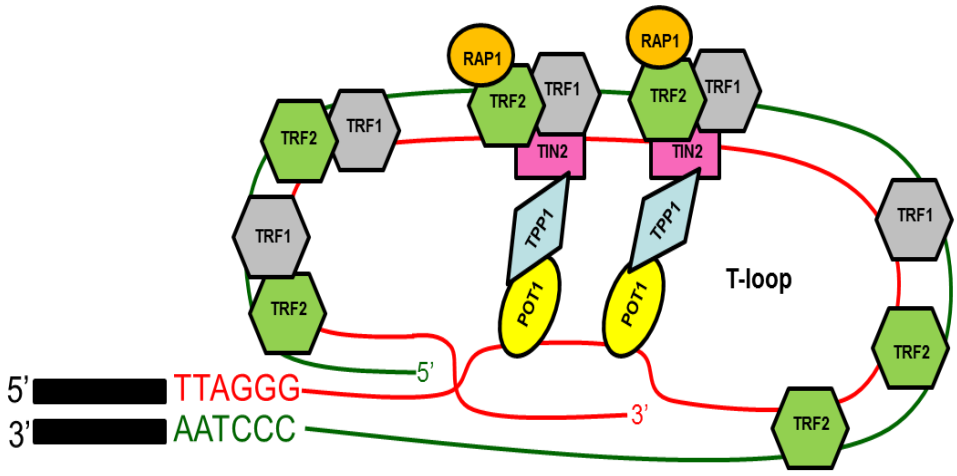


Figure 1.1 Telomeres cap and protect chromosome ends.
Telomeres are comprised of TTAGGG repeats ending in a single-stranded 3' G overhang that forms a t-loop structure. Telomeres, together with shelterin components TRF1, TRF2, TIN2, TPP1, RAP1 and POT1, cap the ends of chromosomes and prevent them from being recognized as double-strand breaks by cellular repair machinery. Adapted from (Denchi 2009; Gu, Bessler et al. 2009)

Murine models using conditional deletion of *TRF1* and *TRF2* demonstrate these proteins prevent ATM (ataxia telangiectasia-mutated) and ATR (ATM and Rad3-related) signaling, DNA resection, HR (homologous recombination), and both classical (cNHEJ) and alternative (altNHEJ) NHEJ (non-homologous end joining) (Sfeir and de Lange 2012). Shelterin components are also integral to the formation of telomeric structure. Both dominant negative inhibition of TRF2 and RNA interference-mediated knockdown of POT1 result in loss of the 3' G-overhang (van Steensel, Smogorzewska et al. 1998; Hockemeyer, Sfeir et al. 2005). In addition, POT1 is thought to play a role in sequence determination at the 5' end of telomeric DNA (Hockemeyer, Sfeir et al. 2005).

Due to an inability of standard DNA polymerases to remove the last primer on the lagging DNA strand after replication, a phenomenon known as the "end-replication problem", erosion of DNA occurs at chromosome ends with each cell division (Harley, Futcher et al. 1990). Telomeric loss in human leukocytes is estimated to be approximately 25 base pairs per year, though higher rates of loss have been reported (Muezzinler, Zaineddin et al. 2013). As such, the repetitive, non-coding DNA at telomeres protects critical genes from being lost during replication (Moyzis, Buckingham et al. 1988; Levy, Allsopp et al. 1992). Regardless of the protective barrier telomeres provide, the shortening that occurs with each cell division eventually limits the replicative lifespan in cells lacking telomerase, the enzyme that synthesizes telomeres, and acts as a mitotic clock. This correlation between telomere length and replicative potential is known as the telomere hypothesis (Harley 1991; Harley and Villeponteau 1995). Ultimately, telomeric erosion triggers a DNA damage response, growth arrest, or apoptosis (Shay and Wright 2005).

A DNA damage response at the telomere can also be triggered independently of telomere length through loss of shelterin components (Harrington and Robinson 2002). With TRF2 disruption, telomere dysfunction and uncapping occurs rapidly and without concurrent loss of telomeric DNA (Karlseder, Broccoli et al. 1999). Conversely, inhibition of telomerase, the enzyme responsible for synthesizing telomeric DNA, leads to gradual telomere shortening. The DNA damage response triggered by disruption of shelterin components differs from the damage response triggered by progressive telomere shortening not only in terms of timing, but also in terms of molecular response. For instance, NHEJ following TRF2 deletion is Ligase 4 (Lig4)-dependent, while telomere shortening triggers Lig4-independent NHEJ (Rai, Zheng et al. 2010).

Both p53-dependent and p53-independent mechanisms of telomere dysfuction have been demonstrated using inhibition of shelterin components or depletion of telomerase. The DNA damage response and subsequent growth arrest and apoptosis triggered by dominant-negative disruption of endogenous TRF2 are p53 dependent (Karlseder, Broccoli et al. 1999), though a p53-independent mechanism has been demonstrated in a p53 mutant background (van Steensel, Smogorzewska et al. 1998). Both responses result in rapid chromosomal fusions and growth arrest (Karlseder, Broccoli et al. 1999; van Steensel, Smogorzewska et al. 1998). Mice deficient in mTerc, the protein component of the telomerase enzyme, show defects in cell proliferation, wound healing, and p53-dependent apoptotic loss of germ cells resulting in sterility (Chin, Artandi et al. 1999). Though depletion of p53 restores fertility (Chin, Artandi et al. 1999), late generation *mTerc/p53* -/-mice show an increased incidence of lymphomas and teratocarcinomas compared to *mTerc* -/- mice, providing the first direct link between

telomere dysfunction and cancer (Chin, Artandi et al. 1999; Artandi, Chang et al. 2000; Rudolph, Millard et al. 2001).

Short, dysfunctional telomeres are a feature of cancer cells (Maser and DePinho 2002). Telomere shortening or uncapping contributes to genomic instability and tumorigenesis through promoting chromosomal fusions and breakages at unprotected chromosome ends (Artandi, Chang et al. 2000; Gunes and Rudolph 2013). These rearrangements may ultimately result in deregulation of critical genes that directly drive cancer, with subsequent selection of alterations that favor growth and metastasis (Gunes and Rudolph 2013). Evidence from telomerase-deficient mice and from studies of human disease suggest telomere dysfunction plays a role in the early stages of carcinogenesis (Chin, Artandi et al. 1999; Rudolph, Chang et al. 1999; Artandi, Chang et al. 2000; Rudolph, Millard et al. 2001; Feldser, Hackett et al. 2003; Meeker and Argani 2004; Tanaka, Abe et al. 2012). In support of this notion, mutations in hTERC cause autosomal dominant Dyskeratosis Congenita (DKC), a disease characterized by bone marrow failure, telomere shortening and increased incidence of cancer (Vulliamy, Marrone et al. 2001). Moreover, telomere dysfunction followed by reactivation of telomerase promotes tumor development in mice, providing further support for the role of telomere dysfunction early in tumorigenesis (Begus-Nahrmann, Hartmann et al. 2012).

Telomerase and Cancer

Telomerase is an enzyme comprised of a C-rich RNA (termed hTR for humans and encoded by the *TERC* gene) with a template region for use by the catalytic reverse

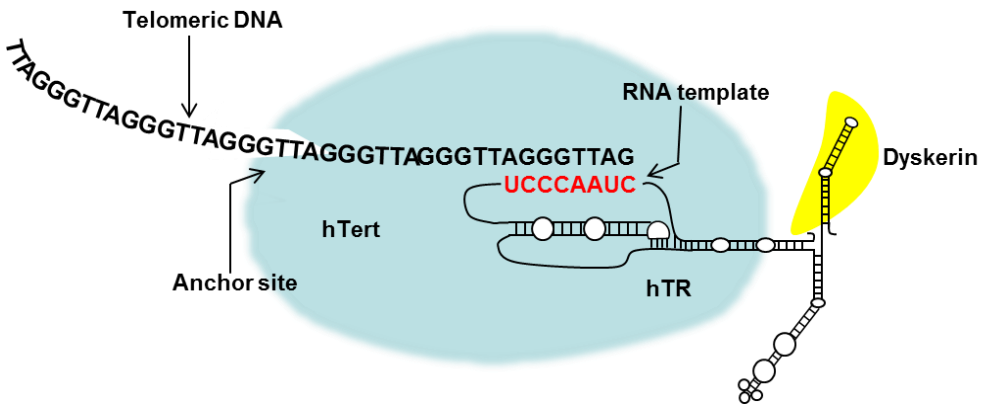


Figure 1.2. The telomerase enzyme complex.
With its C-rich RNA template component and catalytic subunit containing reverse transcriptase activity, the telomerase enzyme is specifically equipped for synthesizing telomeres. Dyskerin, a highly conserved nucleolar protein that modifies newly synthesized ribosomal RNAs, associates with telomerase and is a critical component of telomere maintenance. Mutations in the gene that encodes dyskerin, *DKC1*, lead to X-linked Dyskeratosis Congenita (DC), while mutations in *TERC*, *TERT*, and *TIN2* [not shown] have been identified in the autosomal dominant form of the DC disease. Adapted from (Dokal 2011; Goldblatt 2009; Harley 2008).

transcriptase protein component (termed hTERT and encoded by the *TERT* gene located on the short arm of chromosome 5) (Feng, Funk et al. 1995; Nakamura, Morin et al. 1997). Unlike standard replicative DNA polymerases, telomerase is specifically equipped for synthesizing telomeres. The C-rich RNA component acts as a template for addition of telomeric repeats using the hTERT reverse transcriptase (Chan and Blackburn 2004) (Figure 1.2). The first clues of a link between telomerase expression, telomere length maintenance, and cellular immortalization came from studies of SV40-transformed human embryonic kidney cells. While most cells showed genomic instability and died after a finite number of population doublings, approximately 1 in $1x10^7$ cells acquired telomerase activity and was able to divide indefinitely (Counter, Avilion et al. 1992). Others subsequently demonstrated that ectopically introducing telomerase in normal, telomerase-negative cells extends telomeres and allows cells to grow indefinitely (Bodnar, Ouellette et al. 1998). Despite conferring limited replicative capacity, the addition of telomerase does not cause tumorigenicity. To become tumorigenic, additional factors are required, specifically large SV40 T-antigen and oncogenic *RAS* (Hahn, Counter et al. 1999).

Telomerase is tightly regulated, with expression levels maintained low, and its activity off, in most normal cells. Telomerase is expressed at low levels in stem cells and germ cells to allow continued proliferation of these tissues. This finding holds significance in that stem cells are hypothesized to play a role in cancer initiation and maintenance (Gunes and Rudolph 2013). Without telomerase, fibroblasts are limited to approximately 50-70 population doublings, an observation first described by Leonard Hayflick and termed the Hayflick Limit (Hayflick 1979). After this finite period of

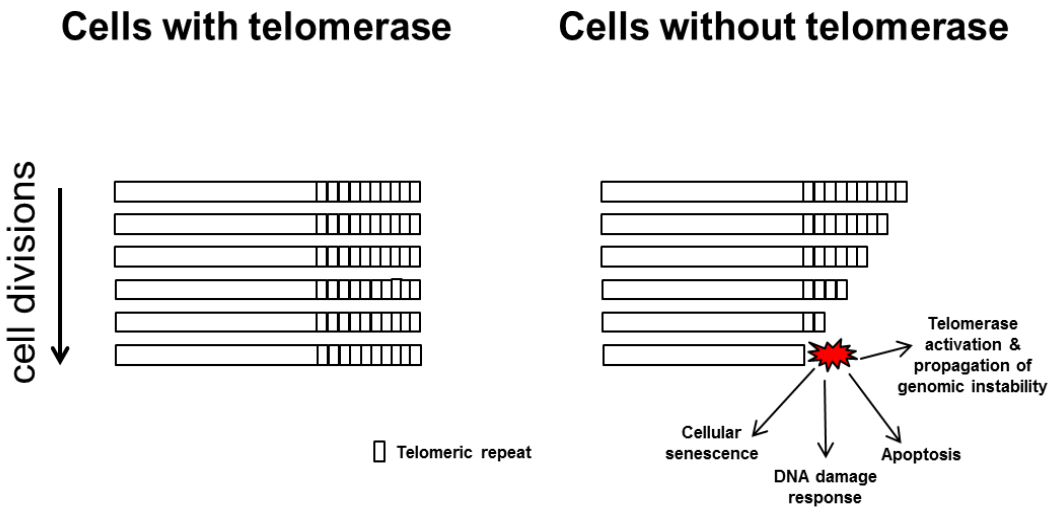


Figure 1.3. Telomerase functions to maintain telomere length.
Cells without telomerase undergo a finite number of population doublings (approximately 50-70 population doublings in fibroblasts) before reaching replicative (cellular) senescence. In cells without telomerase, once telomeres reach a critically short level DNA damage or apoptotic responses may be triggered. Cells with telomerase activity, such as germ cells and stem cells, can continue growing and dividing indefinitely allowing for continued repopulation of these tissues. In cancer development, cells are thought to undergo a number of population doublings in the absence of telomerase, leading to telomere shortening. Ultimately, a rare cell acquires telomerase activity and is able to continue growing and dividing indefinitely despite having short, dysfunctional telomeres. The vast majority of cancers utilize telomerase to achieve unlimited replicative potential. Adapted from (Goldblatt 2009; Harley 2008).

growth, cells reach replicative senescence; this cessation of growth correlates with telomere shortening (Allsopp, Vaziri et al. 1992) (Figure 1.3). Restriction and tight regulation of telomerase expression acts as a tumor suppressive mechanism by preventing uncontrolled growth and division. The importance of telomerase in cancer development is illustrated by the observation that tumor suppressors and oncogenes deregulated in cancer affect telomerase expression in ways that cooperatively promote tumorigenesis. For example, oncogene c-Myc is often amplified in cancer and is an activator of *hTERT* (Greenberg, O'Hagan et al. 1999). Aurora A kinase, a G2/M cell cycle regulator commonly amplified in epithelial malignancies (Fu, Bian et al. 2007), positively regulates c-Myc, and thus stimulates telomerase activity (Yang, Ou et al. 2004). BRCA1, a tumor suppressor gene lost in a proportion of hereditary breast and ovarian cancers, inhibits *hTERT* transcription through negative regulation of c-Myc (Li, Lee et al. 2002; Zhou and Liu 2003). The tradeoff of tight regulation of telomerase activity in normal cells is telomere shortening and, ultimately, telomere dysfunction, both of which are known to contribute to aging and malignancy (Harley 2008).

Approximately 90% of cancers utilize telomerase to achieve unlimited replicative potential (Shay and Bacchetti 1997), and expression of telomerase is linked to tumor aggressiveness and poor outcome (Sanders, Drissi et al. 2004; Lamy, Goetz et al. 2012). The remaining 10% of cancers, typically soft tissue sarcomas (Henson, Neumann et al. 2002), activate an alternative lengthening of telomeres (ALT) pathway thought to occur via homologous recombination (Greenberg 2005). When telomerase is reactivated in the context of telomere dysfunction in cancer cells, it contributes not only to limitless replicative potential and maintenance of telomeres, but also to prevention of apoptosis in

cells harboring genomic instability. hTERT regulates a number of genes responsible for DNA repair and cell cycle regulation, and recruits initiation factors to sites of DNA damage (Cao, Li et al. 2002; Sharma, Gupta et al. 2003; Lamy, Goetz et al. 2012). Through facilitating DNA repair and preventing apoptosis, hTERT can contribute to chemotherapy resistance (Sharma, Gupta et al. 2003; Lamy, Goetz et al. 2012).

Targeting Telomerase as a Therapeutic Strategy in Cancer

Telomerase is an attractive target in cancer for a variety of reasons. Namely, telomerase activity is required for almost all tumors to achieve limitless replicative potential. The genes that encode telomerase and its associated factors are non-redundant, and this has significant therapeutic implications in that cells are less likely to develop resistance to inhibitors. In addition, there are differences in telomerase expression and telomere length between most normal and cancer cells. These factors contribute to what has been described as a broad therapeutic window for targeting telomerase (Harley 2008). Various strategies have been employed to target telomerase, including gene therapy, active G-quadraplex stabilizers, telomerase immunotherapy, and direct enzyme inhibition of telomerase (Harley 2008). Using a rational oligonucleotide approach, Geron Corporation developed a thio-phosphoramidate antisense oligonucleotide telomerase inhibitor, termed GRN163, and Herbert et al. subsequently demonstrated that a lipid conjugation improved cellular uptake and efficacy (lipid conjugation designated by an "L", GRN163L) (Figure 1.4) (Herbert, Gellert et al. 2005). GRN163L, also known as imetelstat, is a telomerase template antogonist that binds complementary to the template

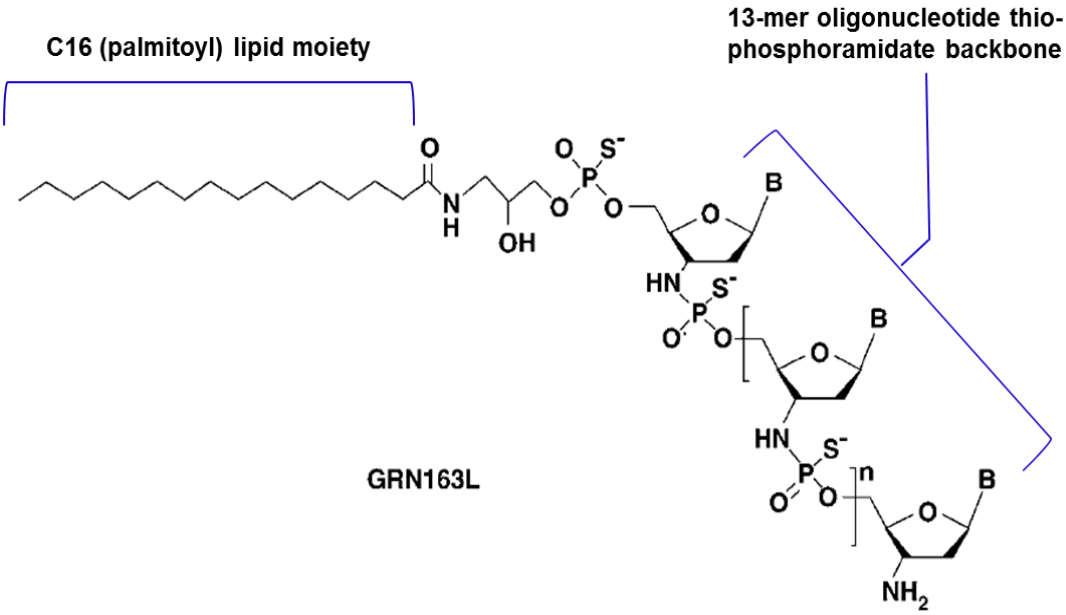


Figure 1.4. GRN163L structure.
GRN163L is a telomerase template (hTR) antagonist, consisting of a 13-mer thio-phosphoramidate oligocucleotide backbone covalently bound to a palmitoyl lipid moiety. The lipid moiety facilitates cellular uptake Adapted from (Roth, Harley et al. 2010; Goldblatt 2009; Harley 2008).

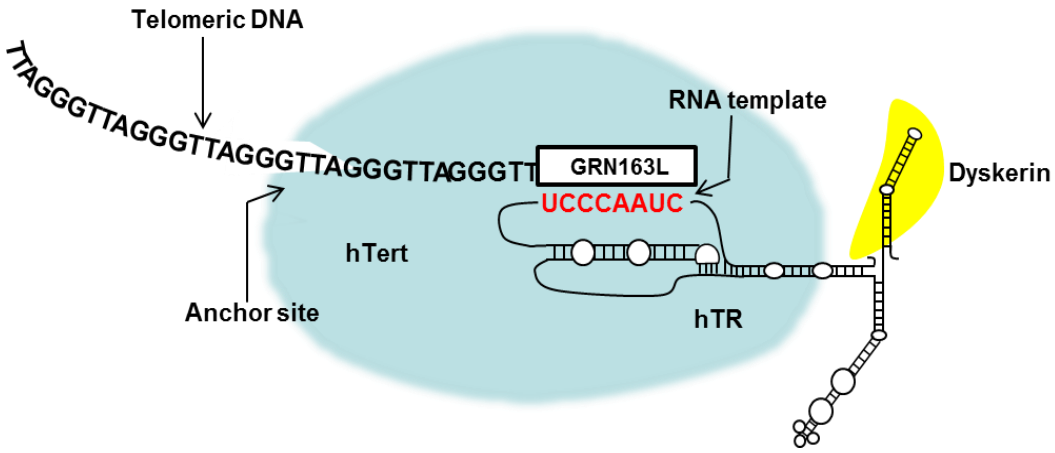


Figure 1.5. GRN163L binds complementary to the RNA template of hTR and prevents telomerase from binding to telomeric DNA.
GRN163L treatment leads to telomerase inhibition, telomere shortening, cellular senescence, and apoptosis. Adapted from (Goldblatt 2009; (Harley 2008)

sequence in hTR, thus preventing telomerase from binding to and extending the telomere (Figure 1.5). One advantage of oliognuclotides is that they are polyanionic, and are therefore less likely to be substrates of multidrug resistance mechanisms (Harley 2008). Work by our laboratory and others has demonstrated GRN163L's efficacy in a wide range of cancer cell lines and in combination with ionizing radiation and chemotherapeutic agents, including paclitaxel, doxorubicin, trastuzumab, and inhibitors of ATM kinase (Djojosubroto, Chin et al. 2005; Agarwal, Pandita et al. 2008; Goldblatt, Erickson et al. 2009; Goldblatt, Gentry et al. 2009; Tamakawa, Fleisig et al. 2010). GRN163L has been in Phase I/II clinical trials for a variety of cancer types such as lung, brain, and breast cancers, including a Phase I trial for refractory HER2 positive breast cancers (clinicaltrials.gov, NCT00732056).

Loss of viability following telomerase inhibitor treatment correlates with initial telomere length. One potential concern with telomerase inhibition is the time required to allow telomeres to reach a critically short level after onset of treatment. Importantly, most cancer cells already have relatively short telomeres, as telomere shortening and dysfunction usually precede telomerase reactivation in cancer. Reactivation of telomerase then serves to maintain, but not necessarily to extend, telomere length, allowing sustained proliferation of cancer cells harboring genomic instability and telomere dysfunction. Nevertheless, finding a good target population is critical for achieving optimal therapeutic response to telomerase inhibition, and cancers with very short telomeres and a high degree of genomic instability might be particularly sensitive. Recent evidence suggests that telomeres in individuals with BRCA1/2 breast cancers are shorter than telomeres in individuals with sporadic breast cancers (Martinez-Delgado,

Yanowsky et al. 2011). Telomere dysfunction has been reported in cells following BRCA1 knockdown, and other links between BRCA1 and telomere maintenance have been reported and are described in the next section. Furthermore, ALT is thought to occur via HR, so *BRCA1* deficient cells, which lack this repair function as described in a subsequent section, may be less inclined to activate ALT and contribute to telomerase inhibitor resistance (Greenberg 2005). Inhibiting telomerase in the context of BRCA1 deficiency may be synergistic due not only to the role of BRCA1 in telomere maintenance, but also due to the reported role of telomerase expression in facilitating double-strand break repair through affecting chromatin remodeling and ATM activation (Masutomi, Possemato et al. 2005).

Hereditary Breast and Ovarian Cancers

Inherited mutations in *BRCA1* or *BRCA2* tumor suppressor genes account for the vast majority of hereditary breast and ovarian cancers with a known genetic cause, and a proportion of the remaining hereditary cases may be attributed to mutations of genes within the same pathway (Ford, Easton et al. 1998; Walsh, Casadei et al. 2006; Kuusisto, Bebel et al. 2011) (Figure 1.6). Mutations in *BRCA1* are inherited in an autosomal dominant fashion and are highly penetrant, with epidemiologic studies suggesting a lifetime risk of breast cancer greater than 80% (Welcsh and King 2001). Mary-Claire King used linkage studies and mapped *BRCA1* to chromosome 17q21 in 1990 (Hall, Lee et al. 1990). The gene was subsequently cloned in 1994 and controversially patented for diagnostic purposes in 1998 by Myriad Genetics (Miki, Swensen et al. 1994; US Patent 5747282).

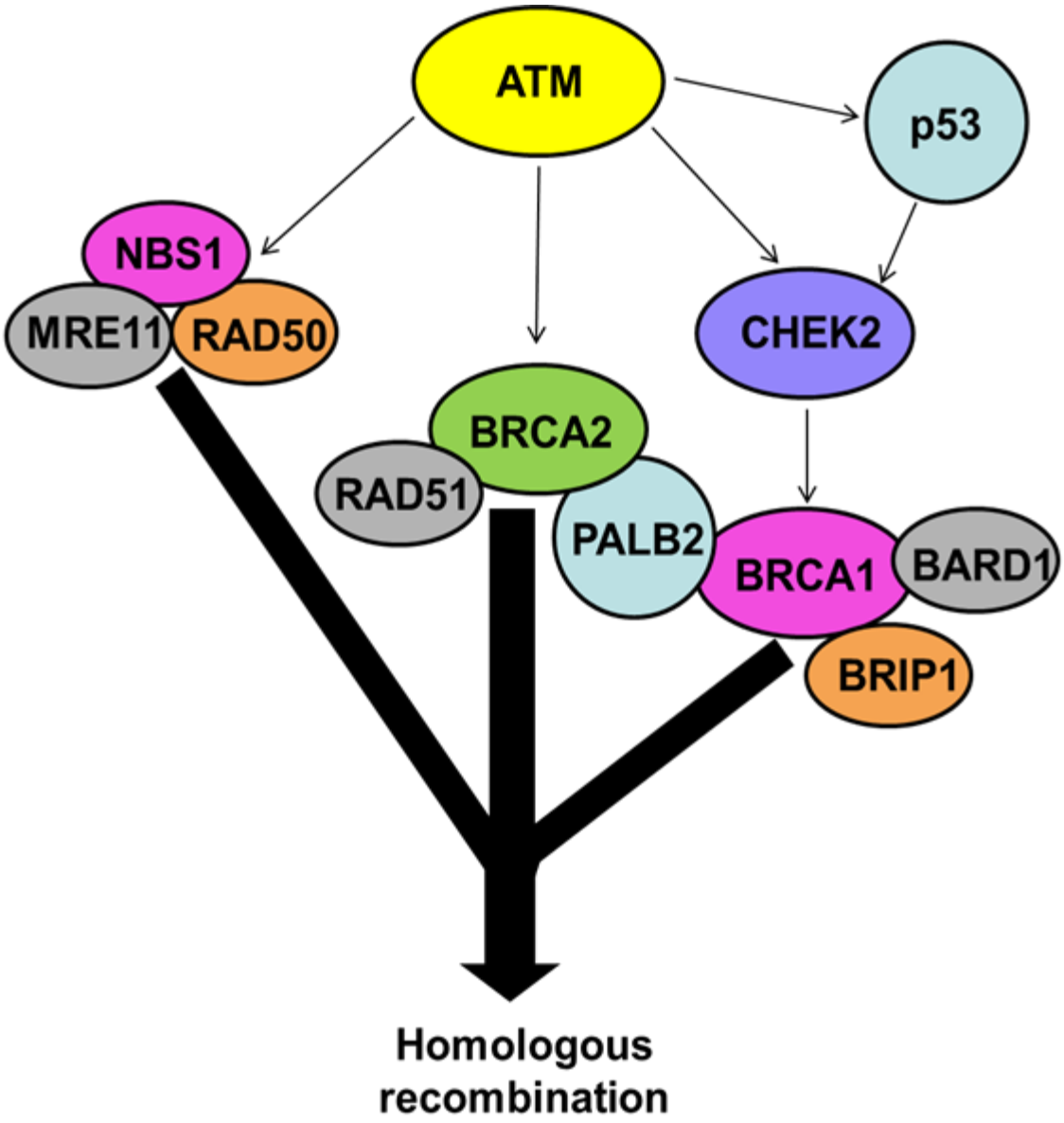
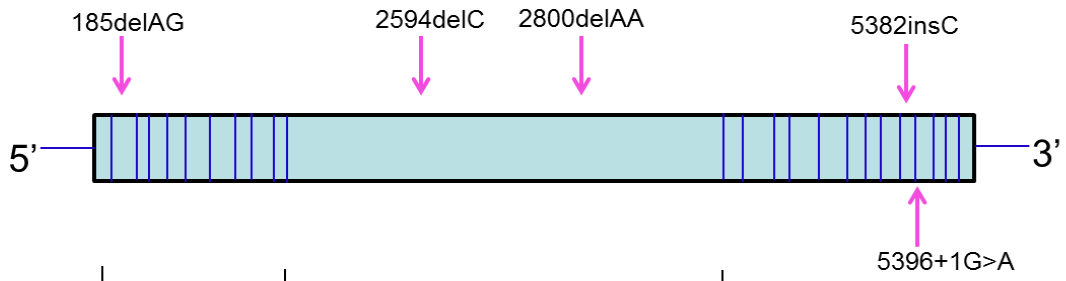


Figure 1.6. Known genetic causes of hereditary breast and ovarian cancer in a convergent DNA repair pathway.
The majority of hereditary breast and ovarian cancers are attributable to inherited mutations in *BRCA1* or *BRCA2*. In both hereditary breast and ovarian cancer (with the exception of ATM mutation in ovarian cancer), germline mutations have also been identified in all genes pictured in the homology-directed double-strand break repair pathway . Additionally, germline mutations in mismatch repair genes (not pictured) have been implicated in hereditary ovarian cancer (2003; Elstrodt, Hollestelle et al. 2006; Walsh and King 2007; Pennington and Swisher 2012).



Domain	Ring finger	Exons 11-13	BRCT
Function	E3 ubiquitin ligase activity	DNA binding, nuclear localization	Phospho-peptide binding, transcriptional activation
Interacting Proteins	BARD1 BAP1	c-MYC Rad50 ATM ATR CHEK2 BRCA2 PALB2	CtIP BACH1 p53

Figure 1.7. Common pathogenic mutations in *BRCA1* and their functional consequences.
Thousands of pathogenic mutations and variants of unknown significance have been identified in the 24 exons of *BRCA1* and within intronic regions, with differing functional consequences. The majority of disease-causing mutations result in frameshifts and protein inactivation. 185delAG and 5382insC are founder mutations in the Ashkenazi Jewish population, while the 2800del AA, 2594delC, and 5396+1G>A are common among northern European populations in Scotland, Ireland, Scandanavia, Denmark, Belgium and the Netherlands. Modified from (Mark, Liao et al. 2005)www.cancer.gov).

The *BRCA1* gene contains 24 exons, and mutations and variants are found a long the entire coding region of *BRCA1* and within intronic sequences (Ferla, Calo et al. 2007), with a range of functional consequences (Figure 1.7). The majority of deleterious mutations are point mutations and small insertions/deletions resulting in frameshifts and protein inactivation, though genomic rearrangements missed by conventional sequencing have also been reported (Mazoyer 2005). Nearly all of the genomic rearrangements reported in *BRCA1* (26 out of 29) are due to unequal homologous recombination, hypothesized to occur frequently due to the high density of Alu repeats within the gene (Welcsh and King 2001; Mazoyer 2005). Thousands of mutations and variants, sometimes rare, and of unknown biological and clinical significance, have been identified in *BRCA1*. Specific pathologic mutations or rearrangements in the gene occur at high frequency, however, due to founder effects in certain ethnic groups, such as Ashkenazi Jews and individuals with ancestors from Iceland, Norway, Sweden and Finland. Among Ashkenazi Jews, it is estimated that 1% of the population harbors the *BRCA1* 185delAG mutation, while 0.13% has a *BRCA1* 5382insC mutation (Roa, Boyd et al. 1996; Ferla, Calo et al. 2007). Both mutations are deleterious, and carry a nearly 70% lifetime risk of breast cancer. Ovarian cancer risk for these particular mutations is lower (14% for the 185delAG mutation and 33% for the 5382insC mutation) (Satagopan, Boyd et al. 2002; Antoniou, Pharoah et al. 2005; Ferla, Calo et al. 2007).

A clinician ordering the BRCA1 diagnostic test employed by Myriad Genetics, Inc. has the option to order a single site mutation test, a test for a panel of specific founder mutations, or a full sequencing test. The full sequencing test also checks for three common rearrangements within the *BRCA1* gene. Myriad classifies a variation or

mutation in BRCA1 as a polymorphism, favor polymorphism, variant of uncertain significance, suspected deleterious or deleterious mutation (Jennifer Saam, genetic counselor at Myriad Genetics, *personal communication*). Clinicians and genetic counselors use results from this test to make recommendations about prevention, though in the event of a "variant of uncertain significance" or "suspected deleterious" result, the guidelines are less straightforward. Nevertheless, mutational testing has significant consequences for individuals of unknown, or moderate to high probability of developing cancer, as current recommendations for risk reduction are prophylactic mastectomy and oophorectomy (Daly, Axilbund et al. 2010). Though deemed worthwhile for high-risk individuals, this preventative strategy has been criticized by some for its lack of clear, consistent guidelines and the potential for overtreatment it invites in lower-risk individuals (Wainberg and Husted 2004; Domchek, Friebel et al. 2010).

Patients diagnosed with BRCA1 cancer currently follow a standard adjuvant chemotherapy regimen, though increased understanding of the role of BRCA1 in homologous recombination has spawned clinical trials incorporating platinum and anthracycline-based chemotherapy and inhibitors of poly (ADP-ribose) polymerase (PARP) (Trainer, Lewis et al. 2010; Tutt, Robson et al. 2010). Not all BRCA patients respond to PARP inhibitors, however, and toxicity to chemotherapy remains a challenge (Maxwell and Domchek 2012). BRCA1 breast cancers are frequently classified as triple negative breast cancer (TNBC) due to their lack of expression on hormone receptors ER, PR, and HER2. Tumors in this subtype are typically basal-like, poorly differentiated, highly aneuploid, aggressive, and carry a poor prognosis (van der Groep, van der Wall et

al. 2011). For these reasons, improved targeted therapies and understanding of the molecular processes central to BRCA1 in tumorigenesis are paramount.

In an effort to understand phenotype and predict clinical outcome among hereditary breast cancers, Price and colleagues used comparative genomic hybridization (CGH) to study a group of early-onset breast cancer patients and found all but one BRCA1 tumor clustered in a group which also included tumors phenotypically similar to BRCA1 tumors, but in which no *BRCA1* mutation was detected. This group was high-grade triple negative, showed a high mitotic rate, gain of 19p, and loss of 5q14-22 and 4q28-32. In contrast, tumors from BRCA2 individuals were not as clearly defined, and were spread all over the six phenotypic groups (Price, Armes et al. 2006). The differences in pathology of BRCA1 compared to BRCA2 tumors may be due to the function of BRCA1 as a gatekeeper and sensor implicated in a wide-range of processes related to safeguarding genomic integrity, while BRCA2 appears to have a more limited and direct role in DNA repair (van der Groep, van der Wall et al. 2011).

BRCA1: Roles in Cancer and Telomere Maintenance

BRCA1 has a wide range of functions related to safeguarding genomic integrity, including DNA repair, cell cycle regulation, transcription, and chromatin remodeling (Figure 1.8). To accomplish these activities, BRCA1 forms a variety of protein complexes, including a heterodimer with BARD1. The BRCA1/BARD1 heterodimer associates with RNA polymerase II, an mRNA synthesizing enzyme. When bound to BARD1, the N-terminus of BRCA1 has ubiquitin ligase activity

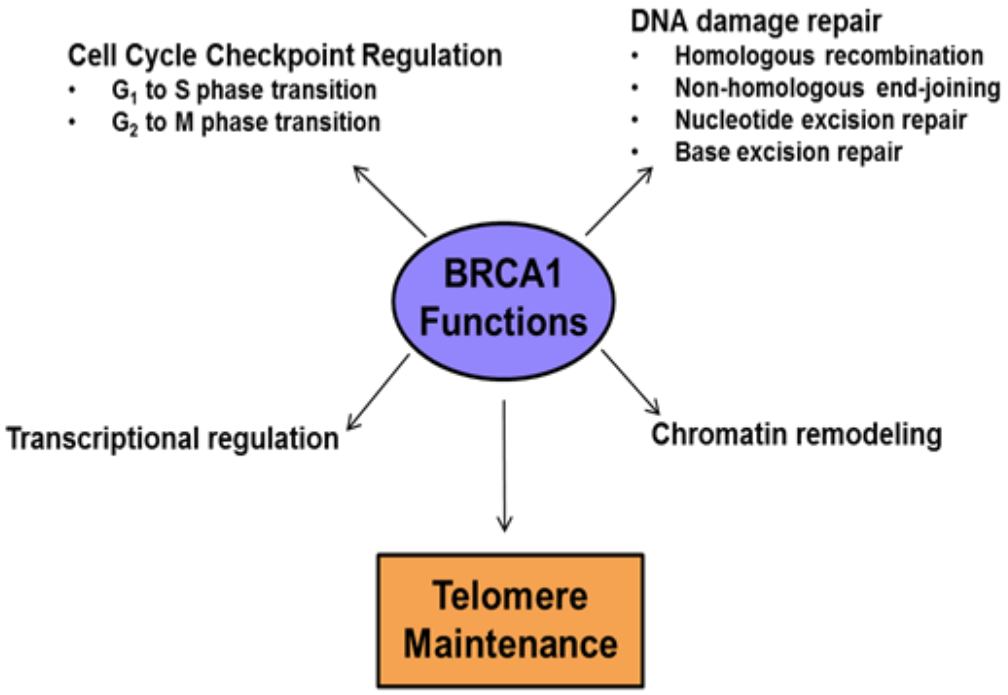


Figure 1.8. BRCA1 is hypothesized to contribute to telomere maintenance through its many functions in maintaining genomic stability.
BRCA1 has been implicated in cell cycle checkpoint regulation, transcriptional regulation, and chromatin remodeling. In addition, BRCA1 is involved either directly or indirectly in homologous recombination, non-homologous end-joining, nucleotide excision repair, and base excision repair. Together, these functions of BRCA1 are hypothesized to contribute to telomere maintenance. Adapted from (Kennedy, Quinn et al. 2004).

(Hashizume, Fukuda et al. 2001), and ubiquinates BRCA1 itself, histone proteins, and FANCD2 and p53, though the biological significance of these reactions is unknown (Parvin 2004). In addition, BRCA1 complexes with a number of repair factors (termed BASC, or BRCA1-associated surveillance complex) and with chromatin remodeling factors. The C-terminus of *BRCA1* contains BRCT (BRCA1 C terminus domain) repeats which function to facilitate interactions between BRCA1 and other repair proteins and cell cycle regulators (Yu, Chini et al. 2003). BRCA1 expression is developmentally regulated and positively correlated with estrogen expression, and this observation is hypothesized to explain why individuals with BRCA1 mutations develop breast and ovarian cancers rather than other cancer types (Welcsh and King 2001). Evidence of this link comes from a recent study demonstrating BRCA1 has a role in nucleotide excision repair (NER) of bulky adducts, which are byproducts of estrogen metabolism (Pathania, Nguyen et al. 2011). In addition, estrogen activates ERK signaling to stimulate rapid proliferation of the breast epithelium during puberty, and this growth is negatively regulated by BRCA1 (Razandi, Pedram et al. 2004). In an individual harboring an inactivating germline mutation in *BRCA1*, the stress and DNA damage produced by this growth likely puts excessive strain on the repair machinery, allowing for accumulation of additional mutations and cancer development (Welcsh and King 2001).

In addition to the aforementioned role of BRCA1 in NER, BRCA1 is also involved in base excision repair (BER) (Saha, Smulson et al. 2010) and repair of double-strand breaks by both error-free homologous recombination (HR) (Moynahan, Chiu et al. 1999) and error-prone non-homologous end joining (NHEJ) (Zhong, Chen et al. 2002) mechanisms. As telomeres function to protect chromosome ends from being recognized

as double-strand breaks, it is perhaps not surprising that many DNA damage response proteins have dual roles in the response to telomere dysfunction. Titia de Lange first described telomere-dysfunction-induced foci (TIFs) containing DNA damage response factors 53BP1, γ-H2AX, Rad17, ATM, and Mre11 (Takai, Smogorzewska et al. 2003). Either HR or NHEJ is activated at dysfunctional telomeres, and each of these pathways has different cellular consequences, with HR leading to activation of ALT and telomere lengthening, and NHEJ leading to chromosomal fusions (Gunes and Rudolph 2013). BRCA1 has a role in each of these repair pathways, and interacts directly or indirectly with the components found in TIFs (Deng and Brodie 2000; Welcsh and King 2001; Rauch, Zhong et al. 2005), though its role in response to telomere dysfunction is unclear at present.

BRCA1 has been described as a "gatekeeper" and is thought to integrate sensors and transducers from multiple repair pathways to facilitate and coordinate the cellular response to genomic insults. In this way, BRCA1 is postulated to mediate the ability of ATM and ATR sensor kinases to phosphorylate downstream target transducers (Foray, Marot et al. 2003). In response to double-strand breaks, H2AX is rapidly phosphorylated by ATM and forms foci at sites of DNA damage. BRCA1 is present at these foci many hours before other DNA repair factors, and is thought to modify chromatin structure to facilitate recruitment of other damage repair proteins to the site (Welcsh and King 2001). BRCA1 also has broad cell cycle influences through regulation of a multitude of cell cycle proteins (Wang, Shao et al. 1997), including induction of p21 leading to G_1/S arrest (Somasundaram, Zhang et al. 1997). In addition, BRCA1 null cells are defective in the G_2/M checkpoint following DNA damage (Larson, Tonkinson et al. 1997).

Numerous repair proteins have overlapping roles not only in the response to telomere dysfunction but also in the prevention of telomere dysfunction (referred to here as telomere maintenance) through interactions with shelterin components and effects on telomere length, some incompletely understood. One study suggests HR directly affects telomere maintenance through generating t-loop deletions of telomeric DNA. The authors demonstrate this effect using a mutant TRF2 that blocked NHEJ. Interestingly, the same t-loop deletions were also seen in normal, unperturbed cells, suggesting a role for HR in normal telomere maintenance (Wang, Smogorzewska et al. 2004). The interplay between telomere maintenance and HR signaling is evident from a study demonstrating that TRF2 binds to and inhibits signaling from ATM (Karlseder, Hoke et al. 2004). ATM was initially found to have a role in telomere maintenance through studies of patients with ataxia telangiectasia (A-T), a rare, neurodegenerative disease caused by inherited mutations in ATM. The disease is characterized by various neurologic and immune problems, accelerated aging, and an increased risk of cancer (Metcalfe, Parkhill et al. 1996). Some of these manifestations may be explained by the observation that A-T individuals have accelerated telomere shortening and extra-chromosomal telomeric DNA (Metcalfe, Parkhill et al. 1996; Hande, Balajee et al. 2001). Mutations in NBS1, another crucial player in double strand break repair, lead to Nijmegen breakage syndrome and short telomeres (Ranganathan, Heine et al. 2001). NBS1, in conjunction with Mre11 and Rad50, form a repair complex (termed the MRN complex) that associates with TRF2 and telomeres in a cell-cycle-regulated manner (Zhu, Kuster et al. 2000). In double-strand break repair, BRCA1 forms a complex with MRN

and another factor, CtIP (C-Terminal Binding Protein Interacting Protein), to facilitate generation of single-stranded DNA needed for HR resection (Chen, Nievera et al. 2008).

Rad51, along with BRCA2, are additional factors with dual roles in double-strand break repair and telomere maintenance. *Rad51* (-/-), *p53* (-/-) mouse embryonic fibroblasts (MEFs) show telomere shortening and telomeric fusions compared to *p53*(-/-) or wild-type MEFs (Tarsounas, Munoz et al. 2004), and BRCA2 has a role in loading Rad51 onto the telomere to facilitate telomere replication and capping (Badie, Escandell et al. 2010). PARP1, a component of the BER pathway, modifies TRF2 and affects its binding to telomeric DNA (Gomez, Wu et al. 2006). Recruitment of PARP1 and modification of TRF2 occur preferentially at eroded telomeres, and are thought to act as a protective mechanism against telomeric fusions (Gomez, Wu et al. 2006).

Overall Objectives and Hypothesis

Evidence of telomere defects in *BRCA1* null cells and patients has been reported in multiple studies (Al-Wahiby and Slijepcevic 2005; Cabuy, Newton et al. 2005; French, Dunn et al. 2006; McPherson, Hande et al. 2006; Martinez-Delgado, Yanowsky et al. 2011), though the mechanism is unknown at present. As BRCA1 is an integral part of the aforementioned pathways through interactions with the repair factors described, it is possible that BRCA1 affects telomere maintenance by acting either directly at the telomere or indirectly through bringing critical factors to sites of telomere dysfunction as it does to sites of DNA damage. In addition, BRCA1 could affect telomere maintenance by halting the cell cycle to allow for repair of dysfunctional telomeres. Thus, BRCA1

may be important both for telomere maintenance and for preventing accumulation of mutations and persistence of genomic instability after telomere dysfunction.

The goals of this project were to better understand the effects of telomerase inhibition in the context of BRCA1 deficiency and the contributions of BRCA1 to telomere maintenance. This thesis specifically addressed the following hypotheses:

1. BRCA1 mutant cell lines are more sensitive to telomerase inhibition compared to BRCA1 wild-type cell lines due shorter telomere lengths at baseline, an increased rate of telomere shortening after telomerase inhibition, and persistence of DNA damage owing to the roles of BRCA1 in DNA repair, cell cycle regulation, and telomere maintenance.
2. BRCA carriers have shorter average telomere lengths at baseline relative to other cancer subtypes, a factor predisposing them to cancer development.
3. BRCA carriers show deregulation of telomere-associated and telomere-proximal genes relative to individuals who developed sporadic or familial breast cancer.

Toward addressing the hypotheses presented in this thesis, a variety of molecular and cellular biology techniques were used to access BRCA1 mutant and BRCA1 wild-type cell lines on the basis of BRCA1 expression levels, baseline telomerase activity levels, and baseline telomere length. In addition, survival assays and immunofluorescence were used to determine functionality of BRCA1 in the two isogenic cell line pairs used for the majority of the cell culture experiments. Telomerase activity was inhibited pharmacologically using GRN163L or a control mismatch (MM) oligonucleotide. Consequences of telomerase inhibition were studied following

GRN163L treatment at various timepoints using telomerase activity, cell survival, cell proliferation, telomere length, and expression of γ-H2AX as endpoints. In addition, telomere length measurements and gene expression profiles were accessed in BRCA1 and BRCA2 carrier individuals to determine contributions of loss of a single copy of BRCA1 or BRCA2 to telomere dysfunction and tumorigenesis.

Significance

Hereditary breast cancers do not have targeted treatment options, largely due to a lack of firm understanding of the molecular features and pathogenesis that differentiate BRCA1 cancers from other cancer subtypes. Determining the contributions of BRCA1 and telomere maintenance to malignant transformation can facilitate the quest for personalized medicine in BRCA patients, and also shed light on the as yet undetermined relationship among BRCA1, telomeres, and shelterin components. As telomerase is activated in the majority of cancers, this work has the potential to impact studies of not only hereditary breast cancer, but of a wide range of malignancies that contribute to the global burden of disease.

CHAPTER TWO

BRCA1 MUTANT CELLS EXHIBIT ENHANCED SENSITIVITY TO GRN163L

Abstract

BRCA1 is a tumor suppressor gene with a variety of functions related to safeguarding genomic integrity. Telomere maintenance is a critical component of genomic stability, and an increasing body of evidence suggests BRCA1 plays a role in this process. The objective of this study was to determine whether GRN163L, a telomerase template antagonist currently in clinical trials, has enhanced activity in BRCA1 mutant breast/ovarian cancer cell lines compared to BRCA1 wild-type breast/ovarian cancer cell lines. We found differences among the cell lines used in this study in terms of baseline telomere length, but not baseline telomerase activity. BRCA1 mutant cell lines showed decreased clonogenic survival capacity following 3-week treatment with GRN163L. In addition, GRN163L caused telomere shortening over a 3- or 6-week period, but no changes in cell cycle distribution. We found increased γ-H2AX protein expression following 3-week GRN163L treatment in UWB1.289 ovarian cancer and HCC1937 pBp breast cancer BRCA1 mutant cell lines relative to their BRCA1wt counterparts. Similarly, immunofluorescence for γ-H2AX revealed an increase in γ-H2AX positive cells in UWB1.289 versus UWB1.289+BRCA1 cells following 1-week GRN163L treatment. Six-week pretreatment and removal of GRN163L, following by addition of cisplatin for 72 hours, augments the action of cisplatin in both UWB1.289 and UWB1.289+BRCA1 cell lines. The combination of GRN163L and doxorubicin added simultaneously was synergistic at the majority of concentration combinations tested in

HCC1937 and HCC1937+BRCA1 cell lines. In summary, this work provides insight into the mechanism behind GRN163L action in BRCA1 mutant and wild-type cells and suggests telomerase inhibition used alone or in combination with DNA damaging agents may be a viable treatment option for this patient population.

Introduction

Telomeres are nucleoprotein structures that cap the ends of linear chromosomes. Telomeres consist of DNA repeat sequences (TTAGGG in humans) and act as sacrificial DNA buffers that are lost with each cell division due to the end replication problem (Moyzis, Buckingham et al. 1988; Levy, Allsopp et al. 1992). Regulation of telomere length is crucial in maintaining genomic stability, with critically short telomeres leading to telomere uncapping, end-to-end fusions, activation of the DNA damage response, and cell cycle arrest (O'Sullivan and Karlseder 2010). Short, dysfunctional telomeres are a feature of cancer cells (Maser and DePinho 2002).

Telomeres are maintained by telomerase, a tightly regulated enzyme with expression levels maintained off in most normal cells but reactivated in cancer cells to maintain telomere length (Knight and Flint 2000). Telomerase activity and telomere maintenance contribute to the unlimited replicative potential of cancer cells, which is a hallmark of cancer (Hanahan and Weinberg 2011). The necessity of telomerase activity for survival of most cancer cells makes it an attractive therapeutic target. Telomerase template antagonist GRN163L is currently in clinical trials for use in combination with chemotherapeutic agents in multiple cancer types, including breast cancer (www.clinicaltrials.gov).

Mutations in the *BRCA1* tumor suppressor gene account for approximately half of all hereditary breast and ovarian cancers, and the gene is silenced via promoter methylation and loss of heterozygosity in a proportion of sporadic breast and ovarian cancers (Honrado, Benitez et al. 2005). BRCA1 functions are still being elucidated, including its potential roles in regulation of cellular senescence and in telomere function. Increased expression of BRCA1 interacting partners has been reported as cell lines become immortalized. In addition, post-stasis (post stress-induced senescence) human mammary epithelial cells (HMECs) with p16 silenced show BRCA1 localization to the nucleus, providing evidence of a role for BRCA1 in the immortalization process (Li, Pan et al. 2007). Current evidence suggests BRCA1 exerts a negative regulatory effect on telomerase activity through inhibition of c-Myc, a proto-oncogene capable of telomerase activation (Wang, Zhang et al. 1998; Greenberg, O'Hagan et al. 1999; Zhou and Liu 2003). This observation is supported by cell culture studies using overexpression of exogenous BRCA1 in human breast and prostate cancer cell lines (Xiong, Fan et al. 2003).

The role of BRCA1 in determining telomere length is not well understood at present. Some evidence suggests knockdown of BRCA1 in cell lines increases average telomere length but may result in more unstable telomeres compared to BRCA1 wild-type cell lines (Ballal, Saha et al. 2009). These findings are supported by work showing a dominant negative *BRCA1* truncation mutant (trBRCA) led to an increase in telomere length in telomerase positive human mammary epithelial cells (French, Dunn et al. 2006). Work from another group, however, reports the opposite relationship between BRCA1 and telomere length, with BRCA1, p53-null murine T cells exhibiting shorter telomeres

as compared to BRCA1 and p53 wild-type cells (McPherson, Hande et al. 2006). In addition, data from patient populations suggests shorter telomeres in *BRCA1* mutation carriers, a feature thought to contribute to their predisposition to cancer development (Martinez-Delgado, Yanowsky et al. 2011). Similarly, the reported reduced telomere length in precancerous gastric lesions compared to normal tissue is correlated with overexpression of telomeric proteins and cytoplasmic export of BRCA1, suggesting mislocalization of BRCA1 may play a role in regulation of telomere length (Hu, Zhang et al. 2010).

The objective of this study was to determine whether GRN163L (imetelstat) has enhanced activity in *BRCA1* mutant breast/ovarian cancer cell lines compared to *BRCA1* wild-type breast/ovarian cancer cell lines. We found differences among the cell lines used in this study in terms of baseline telomere length, and observed enhanced sensitivity to GRN163L in *BRCA1* mutant cell lines compared to their *BRCA1* wild-type counterparts. This sensitivity was coupled with increased expression of DNA damage marker γ-H2AX. In addition, we demonstrated GRN163L treatment acts synergistically with DNA-damaging agents. This work provides insight into the mechanism behind GRN163L action in BRCA1 mutant and wild-type cells and suggests telomerase inhibition used alone or in combination with DNA damaging agents may be a viable treatment option for this patient population.

Results

BRCA1 mutant and wild-type cell line panel exhibits differences in baseline telomere length and BRCA1 levels

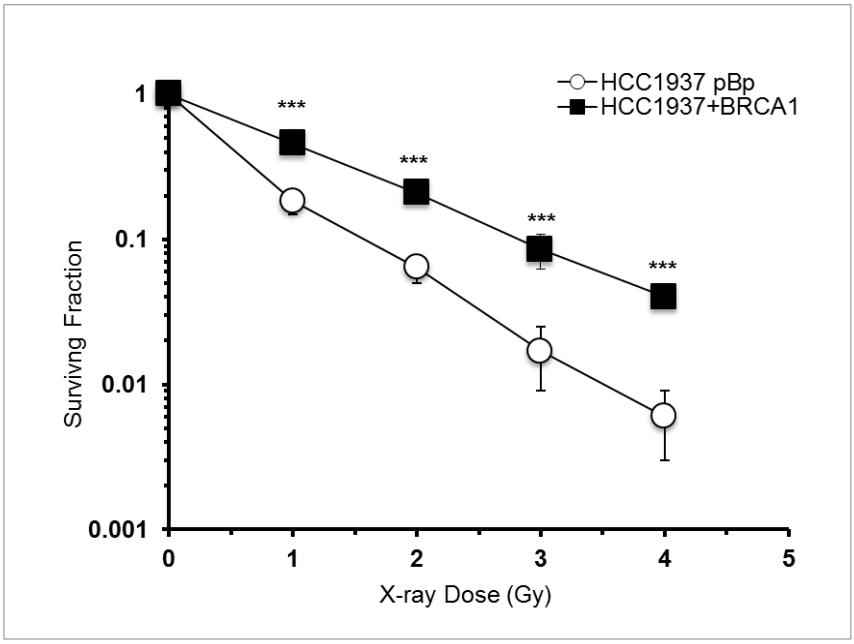
We utilized a panel of breast (n=6) and ovarian (n=2) cancer cell lines containing wild-type *BRCA1* or various somatic or germline mutations from different regions throughout the full-length *BRCA1* gene (Table 2.1). Importantly, HCC1937 pBp and UWB1.289 cell lines were used in conjunction with their isogenic, wild-type BRCA1-reconstituted counterparts. Clonogenic survival assays confirmed the enhanced irradiation sensitivity of the BRCA1 mutant cell line of each isogenic pair relative to the BRCA1 wild-type cell line as reported previously (Figure 2.1) (DelloRusso, Welcsh et al. 2007). In addition, HCC1937+BRCA1 cells, but not BRCA1 mutant HCC1937 pBp cells, showed induction of γ-H2AX following short-term doxorubicin (dox) treatment (Figure 2.2). We first characterized all cell lines used in this study in terms of baseline telomerase activity, BRCA1 protein expression levels, and baseline telomere length, factors that might influence sensitivity to telomerase inhibition. No statistically significant differences were observed (by one-way ANOVA) among the cell lines used, nor among the isogenic BRCA1 mutant and BRCA1 wild-type cell line pairs, in terms of baseline telomerase activity (Figure 2.3). We next performed Western blot analysis using a monoclonal BRCA1 antibody that recognizes the full-length BRCA1 gene product (MS110) (Scully, Ganesan et al. 1996). We found that HCC1937 pBp and HCC1937+BRCA1 cell lines had relatively low levels of BRCA1, as reported previously (S. Elledge, personal communication). UWB1.289+BRCA1 cell lines had relatively high

Table 2.1 BRCA1 mutant and wild-type lines used.

Cell line	Cancer subtype	BRCA1 status
MCF7	Luminal breast	Wild-type
MDA.MB.468	Basal A breast (triple negative)	Wild-type
HCC1937 pBp	Basal A breast (triple negative)	Germline mutation; 5382insC
HCC1937+BRCA1	Basal A breast (triple negative)	Wild-type
UWB1.289	Papillary serous ovarian carcinoma	Germline mutation; 2594delC
UWB1.289+BRCA1	Papillary serous ovarian carcinoma	Wild-type
MDA.MB.436	Basal B breast	Germline mutation; 5396+1 G>A
SUM149PT	Primary inflammatory breast	Somatic mutation; 2288delT

Six breast cancer cell lines and two ovarian cancer cell lines were obtained from ATCC or as described in Materials and Methods. Four cell lines used contained either a germline or a somatic BRCA1 mutation from different regions throughout the full-length BRCA1 gene. Two isogenic cell line pairs (HCC1937 pBp/HCC1937+BRCA1 and UWB1.289/UWB1.289+BRCA1) were included.

A



B

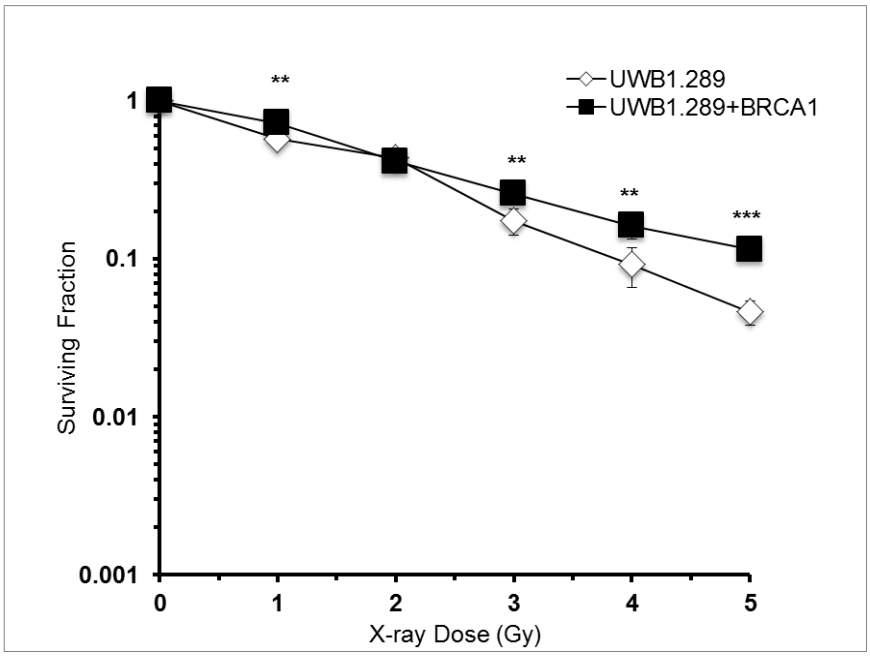


Figure 2.1. BRCA1 mutant cells show enhanced IR sensitivity.
Cells were plated in T25 cm^2 flasks and allowed to attach prior to irradiation at room temperature at 0, 1, 2, 3, 4, or 5 Gy. Following irradiation, cells were incubated at 37°C and 5% CO_2 for 24 hours and then plated in triplicate at low density for clonogenic survival. ** $p < 0.01$, and ***$p< 0.001$.

levels of BRCA1 expression compared to the other cell lines used, with levels appearing higher than that of MCF7 cells, a commonly used positive control for BRCA1 protein expression (Scully, Ganesan et al. 1996) (Figure 2.3). UV-irradiated chronic myelogenous leukemia K-562 cell lysate (Santa Cruz) was also used as a positive control for this assay. Next, we used the TeloTAGGG assay (Roche) to examine baseline telomere lengths. HCC1937 pBp cells had the shortest average telomeres at baseline (3.3 kb), and UWB1.289+BRCA1 cells had the longest (6.7 kb). Interestingly, HCC1937+BRCA1 cells had a slightly longer average telomere length at baseline (3.7 kb) compared to HCC1937 pBp cells (3.3 kb), and this result was statistically significant ($p < 0.05$, two-tailed student's t-test using data from multiple experiments) (Figure 2.4). Similarly, UWB1.289+BRCA1 cells had a longer average telomere length at baseline (6.7 kb) compared to UWB1.289 cells (5.3 kb), and this result was statistically significant ($p < 0.01$, two-tailed student's t-test using data from multiple experiments) (Figure 2.4).

BRCA1 Mutant Cells Show Enhanced Sensitivity to GRN163L

Previous work in our laboratory has demonstrated that HCC1937 pBp cells are exquisitely sensitive to GRN163L treatment as measured by the Telomeric Repeat Amplification Protocol (TRAP) assay relative to other breast cell lines tested (Hochreiter, Xiao et al. 2006). We hypothesized that this sensitivity might be due to the BRCA1 mutant status of these cell lines. To address the importance of BRCA1 in determining sensitivity to telomerase inhibition, we first established a dose response after treatment for 24 hours with GRN163L in the cell lines in our panel (Figure 2.5, isogenic cell line

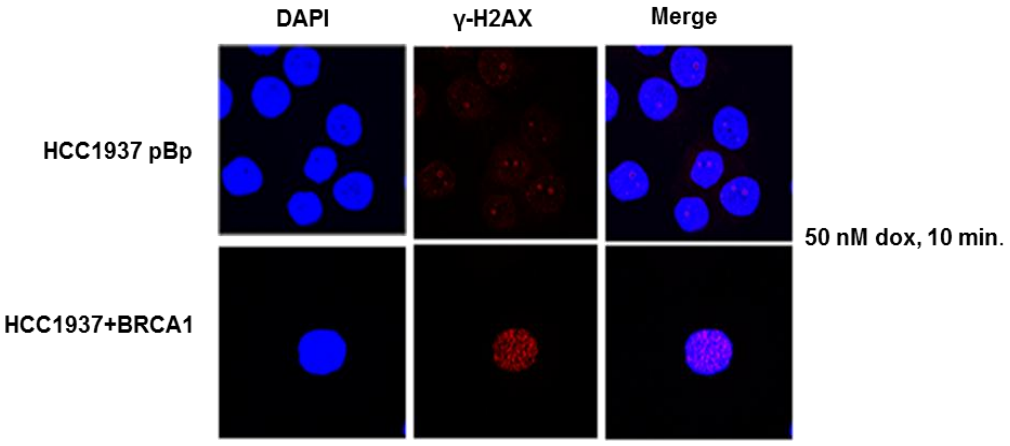


Figure 2.2. BRCA1 wild-type HCC1937+BRCA1 cells, but not BRCA1 mutant HCC1937 pBp cells, show induction of of γ-H2AX following doxorubicin treatment.
Subconfluent cells were plated on 4-well chamber slides and allowed to grow for 1-2 days at 37°C and 5% CO_2 before 10 minute treatment with 50 nM doxorubicin (dox). Doxorubicin treatment was carried out at 37°C and 5% CO_2. Following treatment, cells were washed in ice cold 1 x HBSS, stained for immunofluorescence as described in Materials and Methods, and visualized using confocal microscopy.

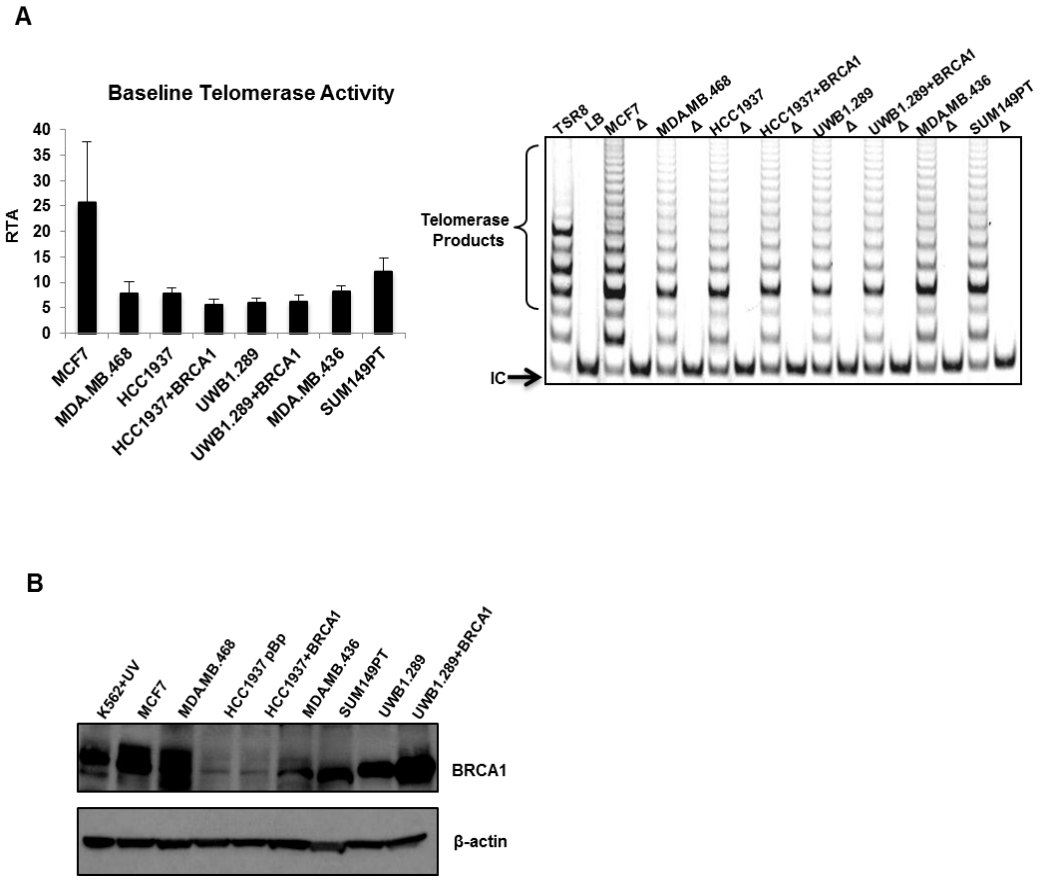


Figure 2.3. BRCA1 mutant and BRCA1 wild-type cell lines show no differences in baseline telomerase activity, but express varying levels of BRCA1 protein.

(A) Baseline relative telomerase activity (RTA) was measured using the TRAP assay as previously described (Hochreiter, Xiao et al. 2006). Cells were lysed in NP-40 lysis buffer (1000 cells/μL). TSR8 was used as a positive control. Lysis buffer alone (LB) and heat inactivated lysate (Δ) served as negative controls. Products were quantified using ImageJ software and presented as the ratio of the telomerase product to the internal control (IC) band. Data was quantified as described in Materials and Methods and is presented as the mean and standard deviation of five independent experiments. (B) BRCA1 expression levels were determined by loading 50 μg protein into 4-15% Bis-Tris SDS-PAGE gradient gels (NuPAGE). Membranes were incubated with MS110 antibody for BRCA1 and blotting for a loading control, β-actin, was included.

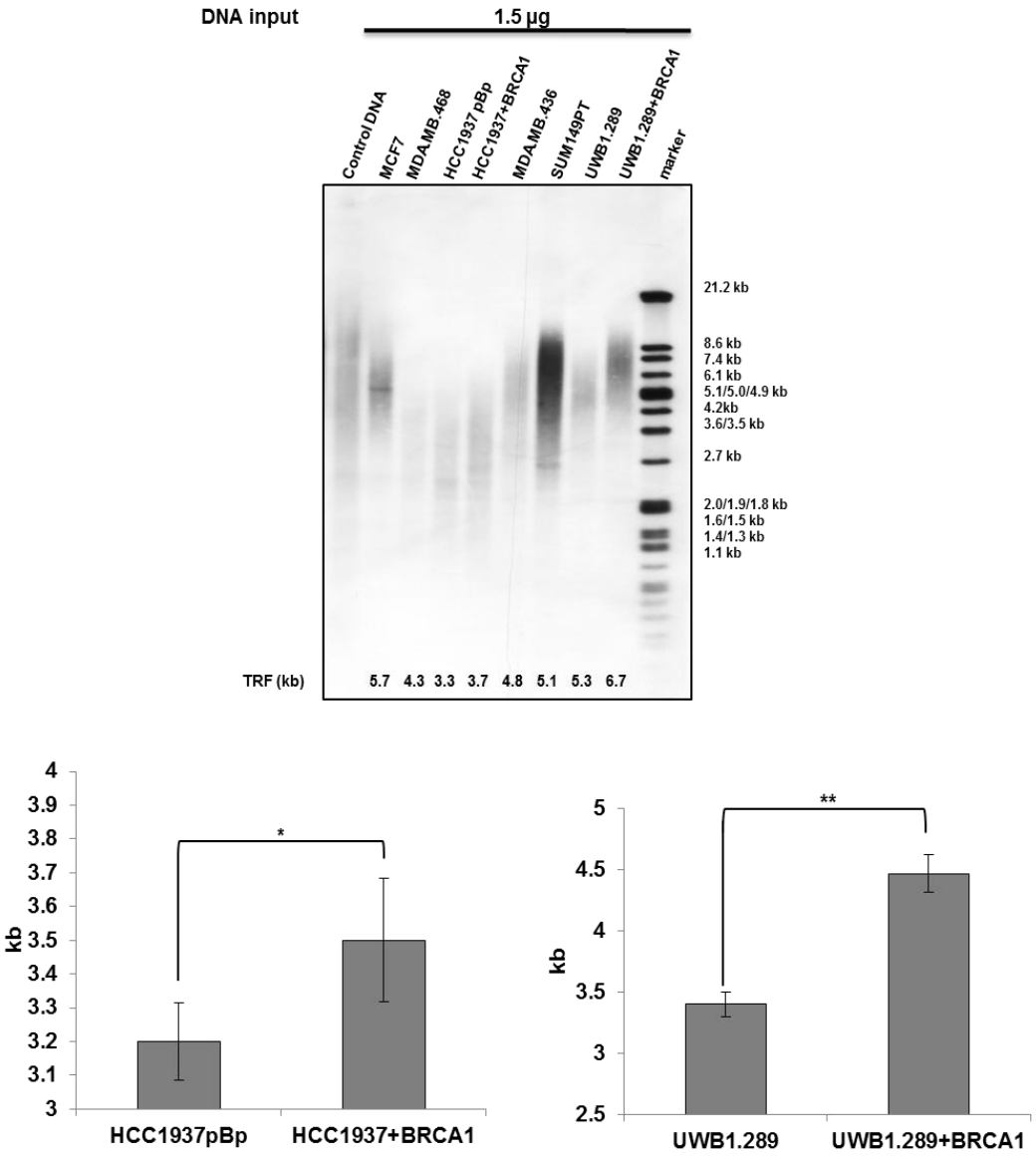


Figure 2.4. BRCA1 mutant and wild-type cell lines exhibit differences in baseline telomere length.

Baseline telomere lengths of cell lines used in this study were determined using the TeloTAGGG assay (Roche) according to the manufacturer's protocol.

* indicates $p < 0.05$, and ** indicates $p < 0.01$, student's t-test using data from multiple experiments.

pairs and SUM149PT cells, as examples). At this timepoint, the UWB1.289 cell line pair appears to be more sensitive to telomerase inhibition compared to the HCC1937 cell line pair (Figure 2.5). HCC1937 pBp cells appear to be slightly more sensitive to GRN163L treatment at this timepoint compared to HCC1937+BRCA1 cells (EC_{50} values for HCC1937 pBp and HCC1937+BRCA1 cells are 0.147 μM and 0.305 μM, respectively) (Figure 2.5). Similarly, UWB1.289 cells appear to be slightly more sensitive than UWB1.289+BRCA1 cells to inhibitor treatment (EC_{50} values for UWB1.289 and UWB1.289+BRCA1 cells are 0.041 μM and 0.104 μM, respectively) (Figure 2.5).

We also tested whether there were differences between late passage (LP = passage 20 for HCC1937pBp cells and LP= passage 19 for HCC1937+BRCA1 cells) and early passage (EP= passage 1 for HCC1937 pBp and HCC1937+BRCA1 cells) HCC1937 pBp and HCC1937+BRCA1 cells. We saw no apparent differences in baseline telomerase activity at LP compared to EP for HCC1937+BRCA1 cells, though HCC1937 pBp cells appear to show slightly increased telomerase activity at LP compared to EP (Figure 2.6). We next examined response to GRN163L in the cell line panel using 12, 24 or 48 hour treatment timepoints, and either treatment at plating (simultaneous treatment) or the day after plating (next-day treatment). 24 hour next-day treatment showed telomerase inhibition ranging from 77% inhibition (MCF7) to 98% inhibition (UWB1.289) with four cell lines (MDA.MB.468, UWB1.289, UWB1.289+BRCA1, and SUM149PT) showing over 90% inhibition (Figure 2.7A). No difference was seen between the BRCA1 mutant and BRCA1 wild-type cell lines when compared as groups using the 24 hour next-day treatment (92+/-6% for BRCA1 mutant versus 85+/-9% inhibition for BRCA1 wild-type cell lines, $p > 0.05$, 2-tailed student's t-test). 48 hour next-day treatment inhibited

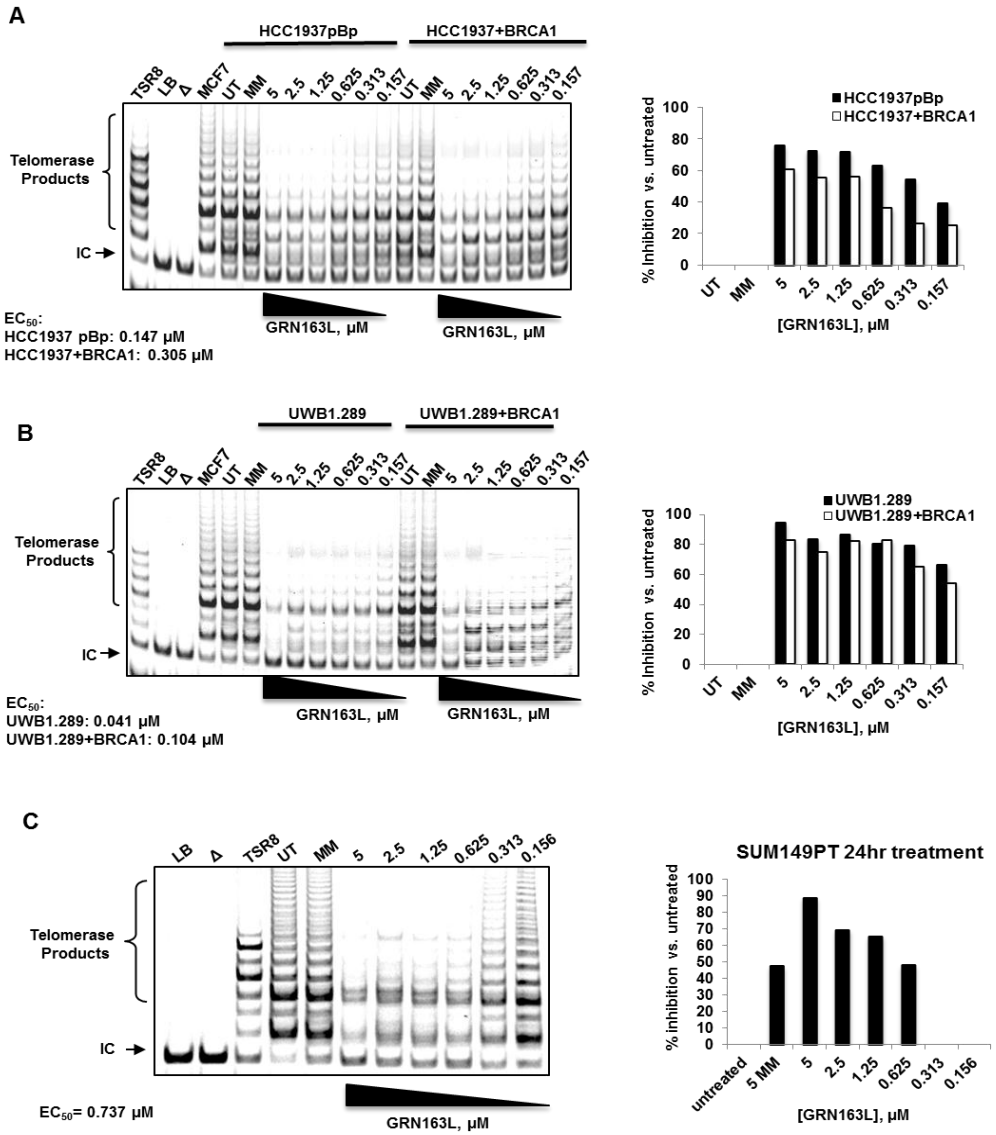


Figure 2.5. Isogenic cell line pairs and SUM149PT cells exhibit a dose-dependent response to GRN163L in TRAP assays.
Cells were plated in 12-well plates and allowed to attach overnight. The next day, media was removed and replaced with fresh media containing GRN163L or MM oligonucleotide. Cells were lysed in NP-40 lysis buffer (1000 cells/μL), and processed (TRAP assay) and analyzed as described in Materials and Methods. Panel A shows the dose-dependent response to GRN163L in HCC1937 pBp and HCC1937+BRCA1 cell lines, and Panel B shows the response to GRN163L in UWB1.289 and UWB1.289+BRCA1 cell lines. Panel C shows the effects of GRN163L in SUM149PT cells.

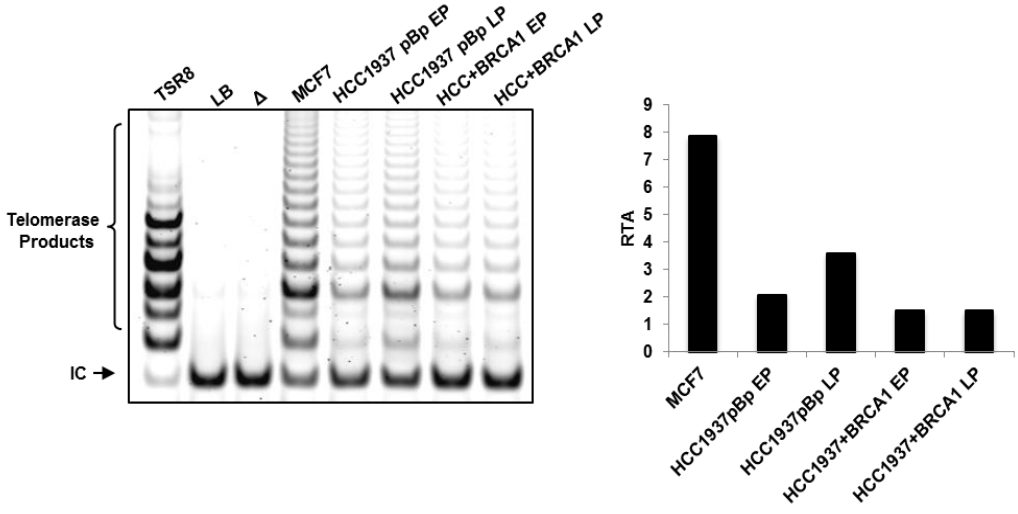


Figure 2.6. Effects of passage number on telomerase activity.
Baseline telomerase activity levels were compared in early passage (p x+1 for HCC1937 pBp and p1 for HCC1937+BRCA1) versus late passage (p19 for HCC1937 pBp and p20 for HCC1937+BRCA1) cell lines. Telomerase activity levels are normalized to the positive control (MCF7) and presented as percent activity of MCF7 cells (Relative Telomerase Activity, or RTA).

telomerase activity in all cell lines tested, ranging from 80% inhibition (HCC1937 pBp) to approximately 99% inhibition (UWB1.289), with 4 cell lines showing over 90% inhibition of telomerase activity (Figure 2.7B). Using 12-hour simultaneous treatment, telomerase inhibition ranged from 74% inhibition (MCF7) to 100% inhibition (UWB1.289 and UWB1.289+BRCA1), with 7 cell lines showing over 90% inhibition (Figure 2.8A). 24 hour simultaneous treatment yielded similar results, with telomerase inhibition ranging from approximately 85% (MCF7) to 100% (SUM149PT), with 6 cell lines showing over 90% inhibition (Figure 2.8B). At all treatment timepoints, regardless of whether treatment was simultaneous or next-day, we saw no significant differences in telomerase inhibition comparing BRCA1 mutant and BRCA1 wild-type cell lines as groups. We observed morphological changes in most of the cell lines tested as early as 12 hours following simultaneous treatment as previously reported (Goldblatt, Gentry et al. 2009) (Table 2.2 and Figure 2.9, isogenic cell line pairs as examples). No notable differences were seen in terms of morphology between BRCA1 mutant and BRCA1 wild-type cell lines at these timepoints.

To better understand the efficacy of GRN163L in BRCA1 mutant versus BRCA1 wild-type cell lines, we focused on longer treatment timepoints using the two isogenic cell line pairs (HCC1937 and UWB1.289 +/- BRCA1). Clonogenic survival assays after 3-week continuous treatment with GRN163L demonstrate the BRCA1 mutant cell line in each isogenic pair shows a statistically significant reduction in clonogenic survival relative to the BRCA1 wild-type cell line (Figure 2.10). To verify that the reduction in clonogenic survival was due to the telomere shortening effects of GRN163L, we also performed clonogenic survival assays at both shorter and longer timepoints. After 1-

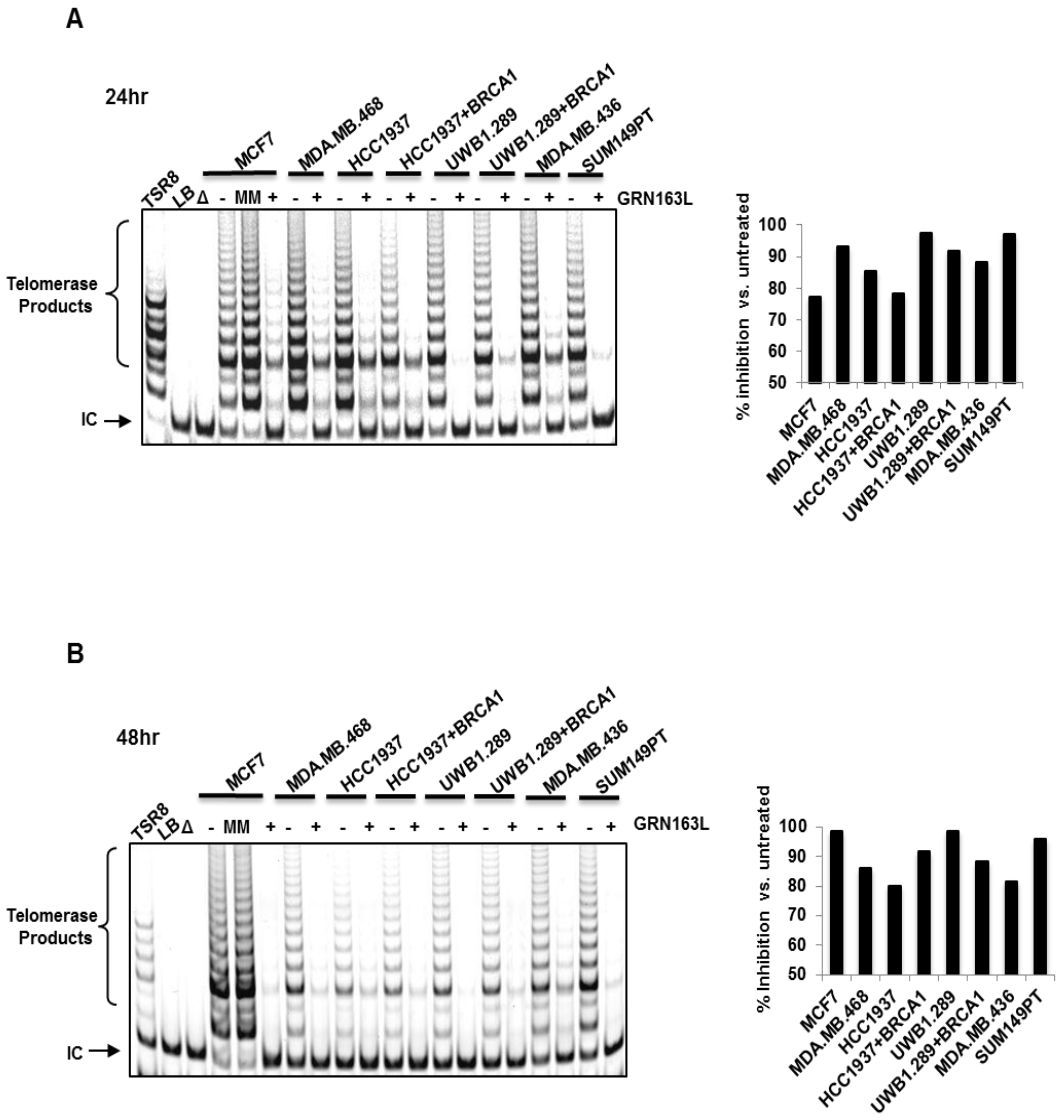


Figure 2.7. All cell lines show telomerase inhibition following next-day treatment f 24 (A) or 48 (B) hr with a clinically relevant concentration of GRN163L.
Cells were treated the day after plating with 1.7 μM GRN163L (+). Pellets were lysed in NP-40 lysis buffer at 1000 cells/μL and used in the TRAP assay as described previously (Hochreiter, Xiao et al. 2006; Clark, Rodriguez et al. 2012; Roy, Chun et al. 2012). TSR8 and MCF7 cells were used as positive controls, and heat-inactivated (Δ) or MM-treated cells were used as negative controls. Products were quantified using ImageJ software and presented as the ratio of the telomerase product to the internal control (IC) band. Data was quantified as described in Materials and Methods, normalized to the untreated telomerase activity level for each cell line, and presented as percent inhibition.

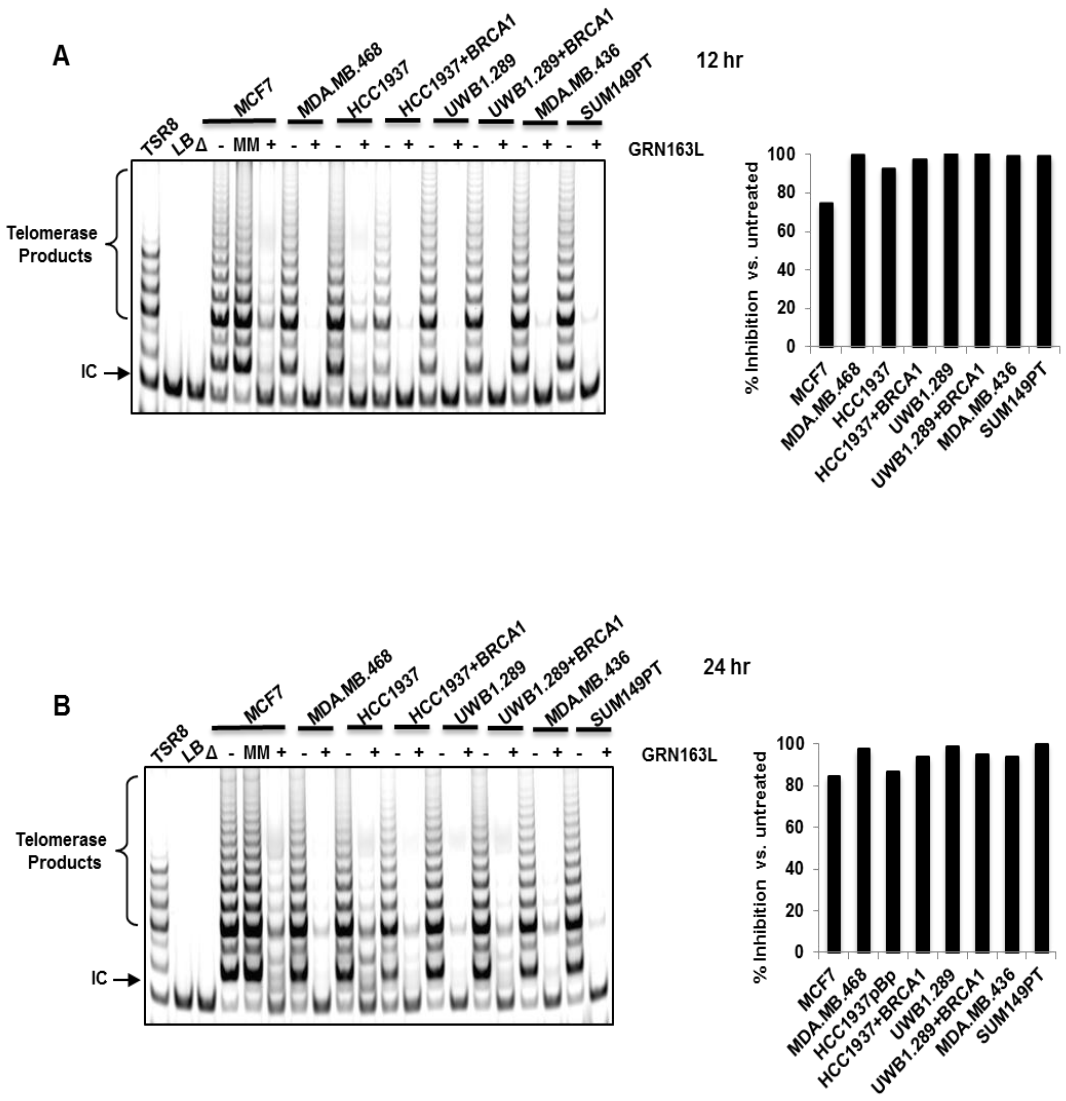


Figure 2.8. All cell lines show telomerase inhibition following 12 (A) or 24 (B) hour simultaneous treatment with a clinically relevant concentration of GRN163L.

Cells were treated at plating with 1.7 μM GRN163L (+). Pellets were lysed in NP-40 lysis buffer at 1000 cells/μL and used in the TRAP assay as described previously (Hochreiter, Xiao et al. 2006). TSR8 and MCF7 cells were used as positive controls, and heat-inactivated (Δ) or MM-treated cells were used as negative controls. Products were quantified using ImageJ software and presented as the ratio of the telomerase product to the internal control (IC) band. Data was quantified as described in Materials and Methods, normalized to the untreated telomerase activity level for each cell line, and presented as percent inhibition.

Table 2.2. Morphologic observations following 12-hour simultaneous treatment.

Cell line	Morphologic changes at 12hr post-treatment
MCF7	Majority attached and in large clumps
MDA.MB.468	Clumped; few floating
HCC1937 pBp	Slightly rounded; most attached
HCC1937+BRCA1	Slightly rounded; 60% attached
UWB1.289	Rounded; 40% attached
UWB1.289+BRCA1	Rounded; 40% attached
MDA.MB.436	Majority floating and in large clumps
SUM149PT	Rounded and dark; majority floating

Cells were plated in 12-well dished and treated at plating. After 12-hour incubation at 37°C and 5% CO_2, cells were imaged and observations were recorded prior to collection for TRAP assays.

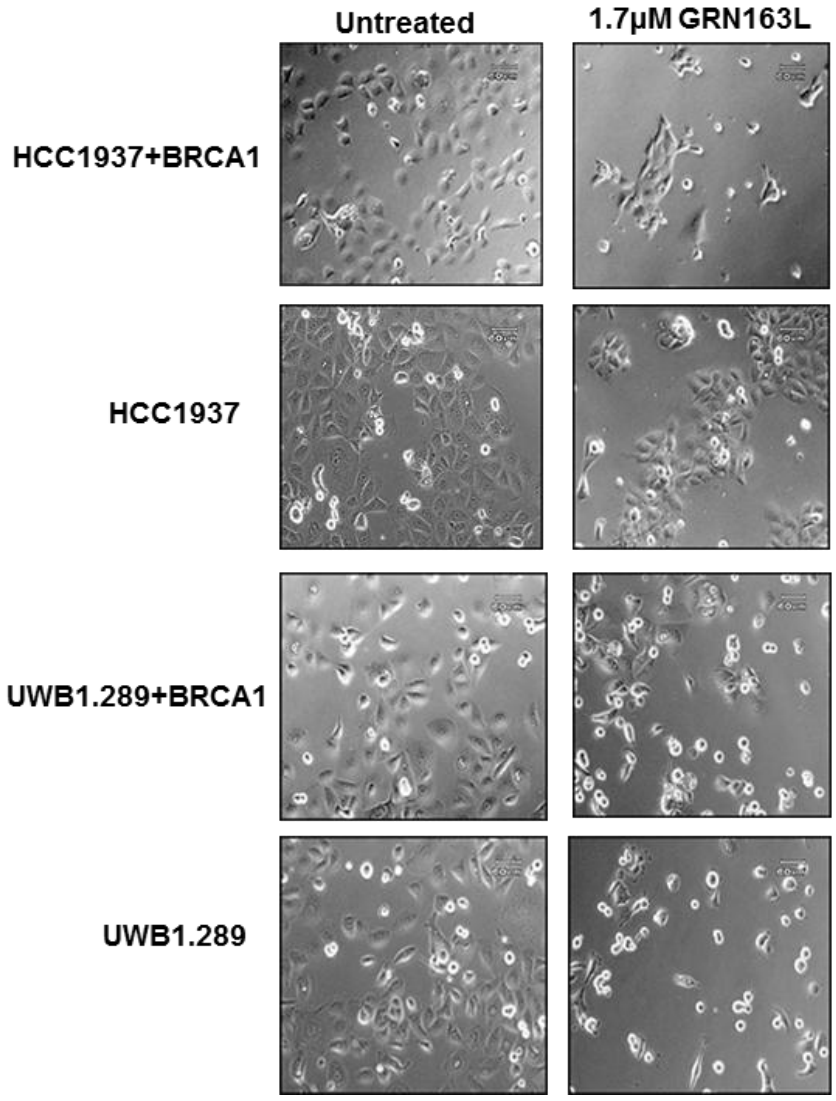


Figure 2.9. 12-hour Simultaneous treatment with GRN163L induces morphologic changes in the majority of cell lines tested.
Cells were treated at time of plating in 12-well dishes and imaged 12 hours later before collection for the TRAP assay.

week continuous treatment, we observed a statistically significant reduction in clonogenic survival among UWB1.289+BRCA1 cells treated with 3.4 μM GRN163L as compared to UWB1.289 cells, but this result is difficult to interpret as 3.4 μM MM also had more of an effect on UWB.1289+BRCA1 cells versus UWB1.289 cells at this timepoint (Figure 2.11A). Following 2-week continuous treatment with GRN163L, we saw a statistically significant reduction in clonogenic survival at 3.4 and 1.7 μM treatment concentrations in UWB1.289 versus UWB1.289+BRCA1 cells (Figure 2.11B). Of note, the fraction of UWB1.289 cells still alive at 2 weeks was greater than at 3 weeks. Furthermore, the differences in sensitivity between UWB1.289 and UWB1.289+BRCA1 cells were more apparent at 3 weeks versus 2 weeks ($p<0.05$ vs $p<0.001$ at 2 weeks and 3 weeks, respectively, for 3.4 μM GRN163L; $p<0.01$ vs $p<0.001$ at 2 weeks and 3 weeks, respectively, for 1.7 μM GRN163L; and not signficant vs $p<0.05$ at 2 weeks and 3 weeks, respectively, for 0.85 μM GRN163L). By 12 weeks, no differences in response were seen between UWB1.289 and UWB1.289+BRCA1 cell lines, but surviving fraction was much less than at 1, 2, or 3 weeks of treatment (Figure 2.11C).

Cumulative population doublings were calculated over longer time periods of drug treatment in both isogenic cell line pairs until one or both cell lines of the pair were completely depleted. After approximately 5 weeks of continuous treatment, we saw a statistically significant reduction in all treatment groups compared to the MM-treated group in HCC1937 pBp cells ($p < 0.001$ for MM vs 3.4, 1.7, or 0.85 μM GRN163L, one-way ANOVA), and this trend continued until all cells were completely dead at approximately 10 weeks (Figure 2.12A). After 7 weeks of continuous treatment, we saw

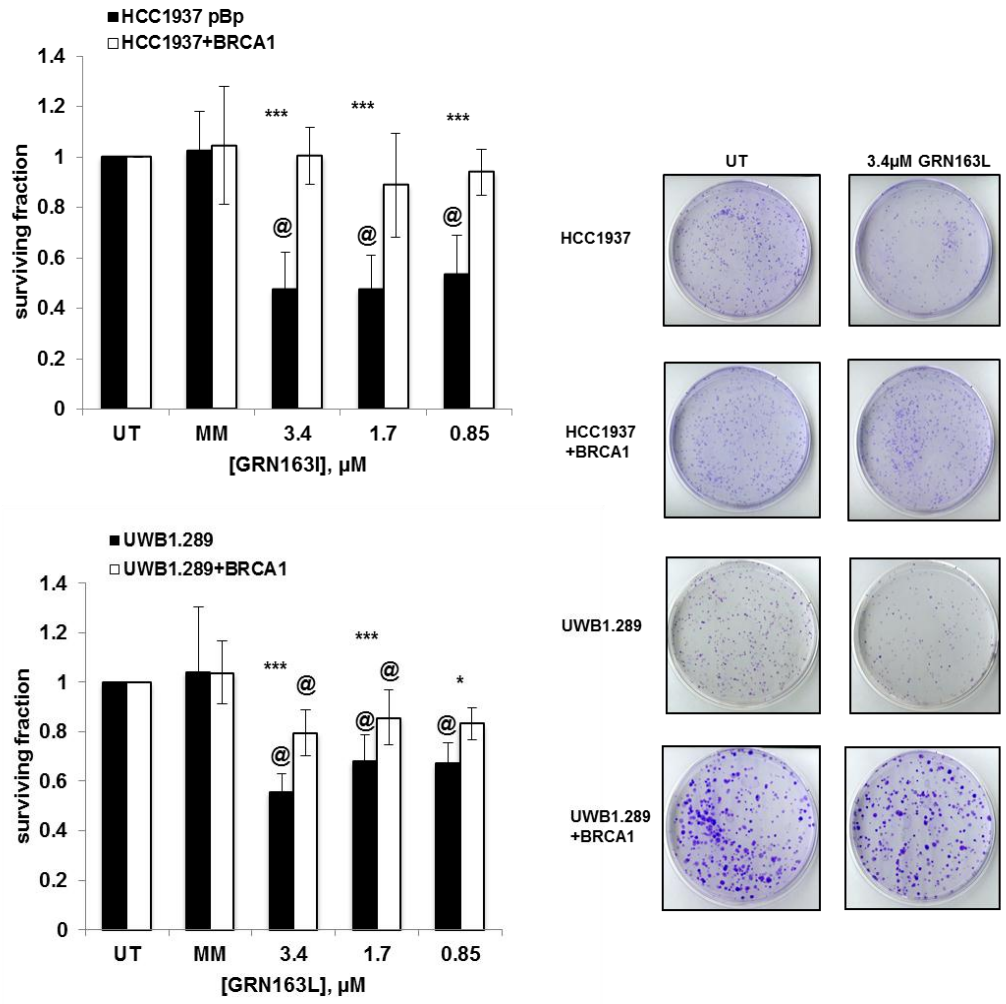


Figure 2.10. Cells with mutant BRCA1 exhibit decreased clonogenic survival following 3-week continuous treatment with GRN163L.
Following a 3-week continuous treatment with GRN163L or MM, 750-1000 cells were seeded on 10 cm^2 plates and grown undisturbed and untreated for 14-18 days. Media was then aspirated and plates were washed in 1 x HBSS before fixing cells with ice cold 100 % methanol. Plates were stained with 0.5% crystal violet in 25% methanol for 10 minutes at room temperature, rinsed in double-distilled water, and allowed to dry overnight. Colonies consisting of at least 50 cells were scored and data is recorded as surviving fraction relative to the corresponding untreated cell line. * $p < 0.05$, HCC1937 pBp vs HCC1937+BRCA1; *** $p < 0.001$, HCC1937 pBp vs HCC1937+BRCA1; @ $p < 0.01$ vs MM, one-way ANOVA.

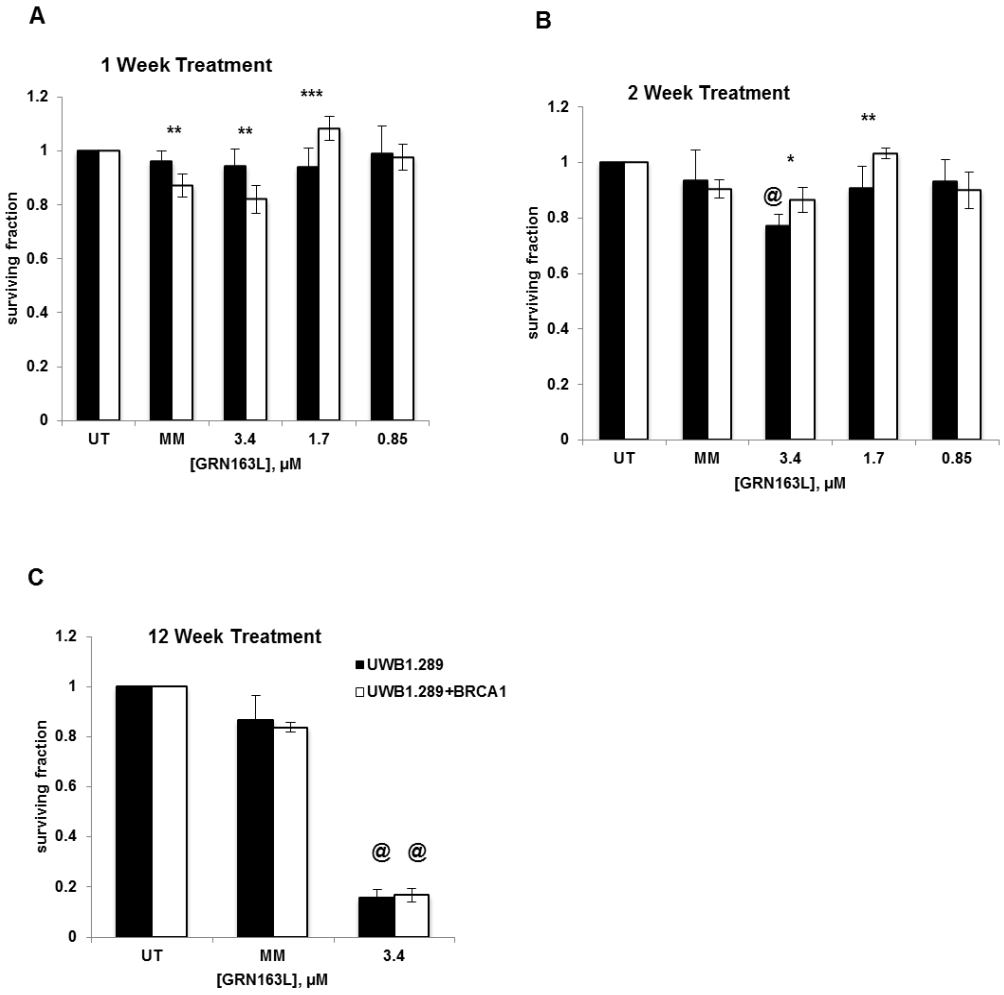


Figure 2.11. Clonogenic survival capacity progressively decreases with increased GRN163L treatment duration.
Following a 1, 2 or 12-week treatment with GRN163L or MM oligonucleotide, cells were seeded at low density on 10 cm^2 plates and grown undisturbed and untreated for 14-18 days. Media was then aspirated and plates were washed in 1 x HBSS before fixing cells with ice cold 100% methanol. Plates were stained with 0.5% crystal violet in 25% methanol for ten minutes at room temperature, rinsed in double-distilled water, and allowed to dry overnight. Colonies consisting of at least 50 cells were scored and data is recorded as surviving fraction relative to the corresponding untreated cell line. *$p < 0.05$ UWB1.289 vs UWB1.289+BRCA1; ** $p < 0.01$ UWB1.289 vs UWB1.289+BRCA1; *** $p < 0.001$ UWB1.289 vs UWB1.289+BRCA1; @ $p < 0.05$ vs MM, one-way ANOVA.

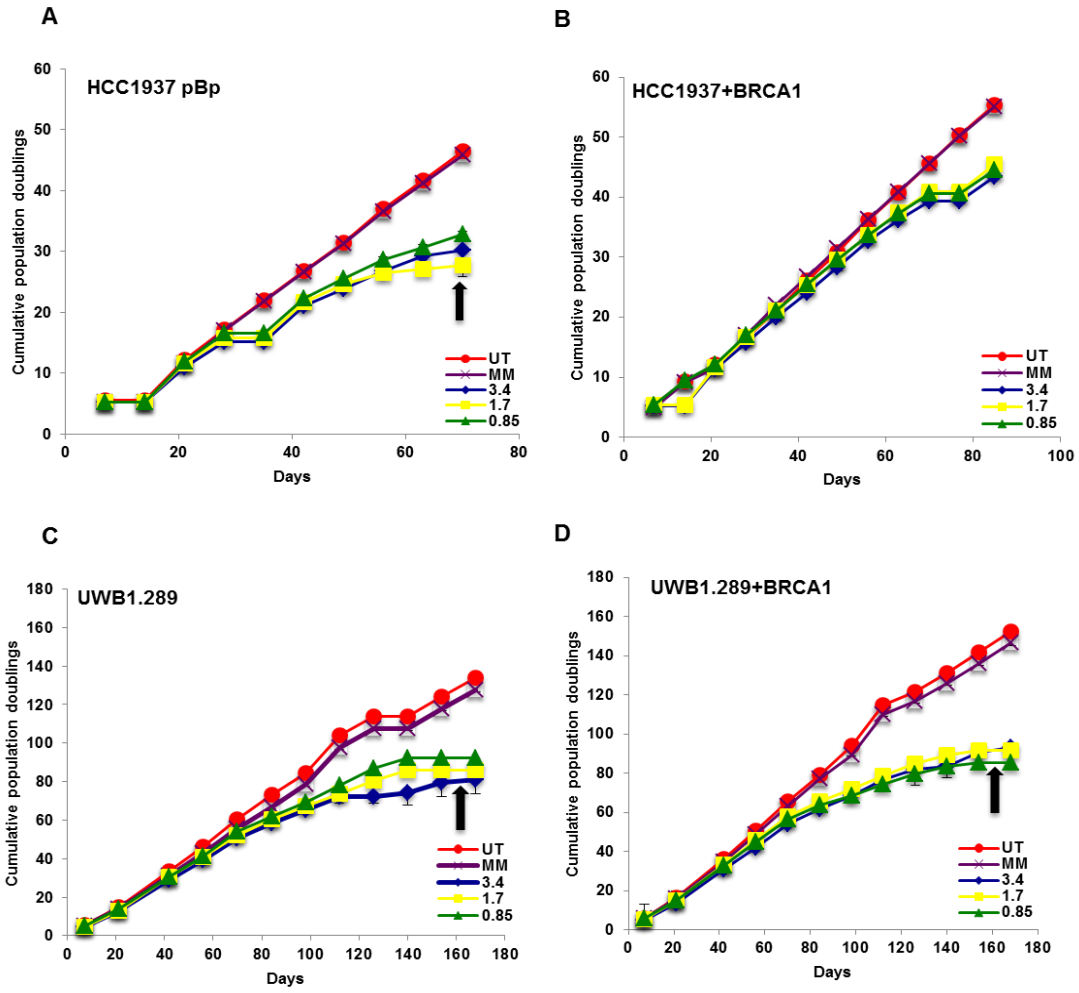


Figure 2.12. GRN163L preferentially induces complete cell death in HCC1937 pBp BRCA1 mutant cells and abolishes UWB1.289 and UWB1.289+BRCA1 cell populations at approximately 24 weeks.
Cells were plated in triplicate at uniform density in 6-well dishes and passaged approximately once per week as needed. Cells were counted at each passage using a counter (Beckman Coulter Zm) and replated at uniform density. Cells were continually treated for the duration of the experiment and GRN163L or MM was replenished every 3-4 days. Population doublings were calculated as the log [(number of cells collected)/(number of cells initially plated)]/log 2 for each passage. Data is presented as cumulative population doubling level for each treatment group. Arrow denotes approximate time at which all cells were dead.

a statistically significant reduction in all treatment groups compared to the MM-treated group in HCC1937+BRCA1 cells ($p < 0.001$ for MM vs 3.4 μM GRN163L, and $p < 0.05$ for MM vs 1.7, or 0.85 μM GRN163L, one-way ANOVA). Although growth was significantly slowed for HCC1937+BRCA1 GRN163L-treated cells, they continued to proliferate throughout the duration of the experiment (Figure 2.12B). UWB1.289 and UWB1.289+BRCA1 cell lines took much longer to be affected by telomerase inhibitor treatment. By approximately 10 weeks, we saw a statistically significant reduction in all treatment groups compared to the MM-treated group in UWB1.289 and UWB1.289+BRCA1 cells (Figure 2.12 C and D). This trend continued for both cell lines until approximately 22 weeks when all cells were depleted. No differences were observed between the UWB1.289 and UWB1.289+BRCA1 cell lines in terms of cumulative population doublings after treatment with GRN163L (Figure 2.12 C and D). Next, TeloTAGGG assays were performed to access telomere length following drug treatment. This experiment confirmed that telomere shortening occurred over a 3- or 6-week period following treatment with GRN163L, but not with MM, in both cell line pairs tested (Figure 2.13). UWB1.289 and UWB1.289+BRCA1 cells shortened approximately twice as quickly as HCC1937 pBp and HCC1937+BRCA1 cells (Figure 2.14).

GRN163L treatment induces γ-H2AX expression in BRCA1 mutant cells, but does not alter cell cycle dynamics

Previous work from our laboratory has demonstrated an increase in population doubling time with increasing lengths of exposure to GRN163L and an increase in the percentage of cells in G_2 phase after 9 and 20 days of treatment

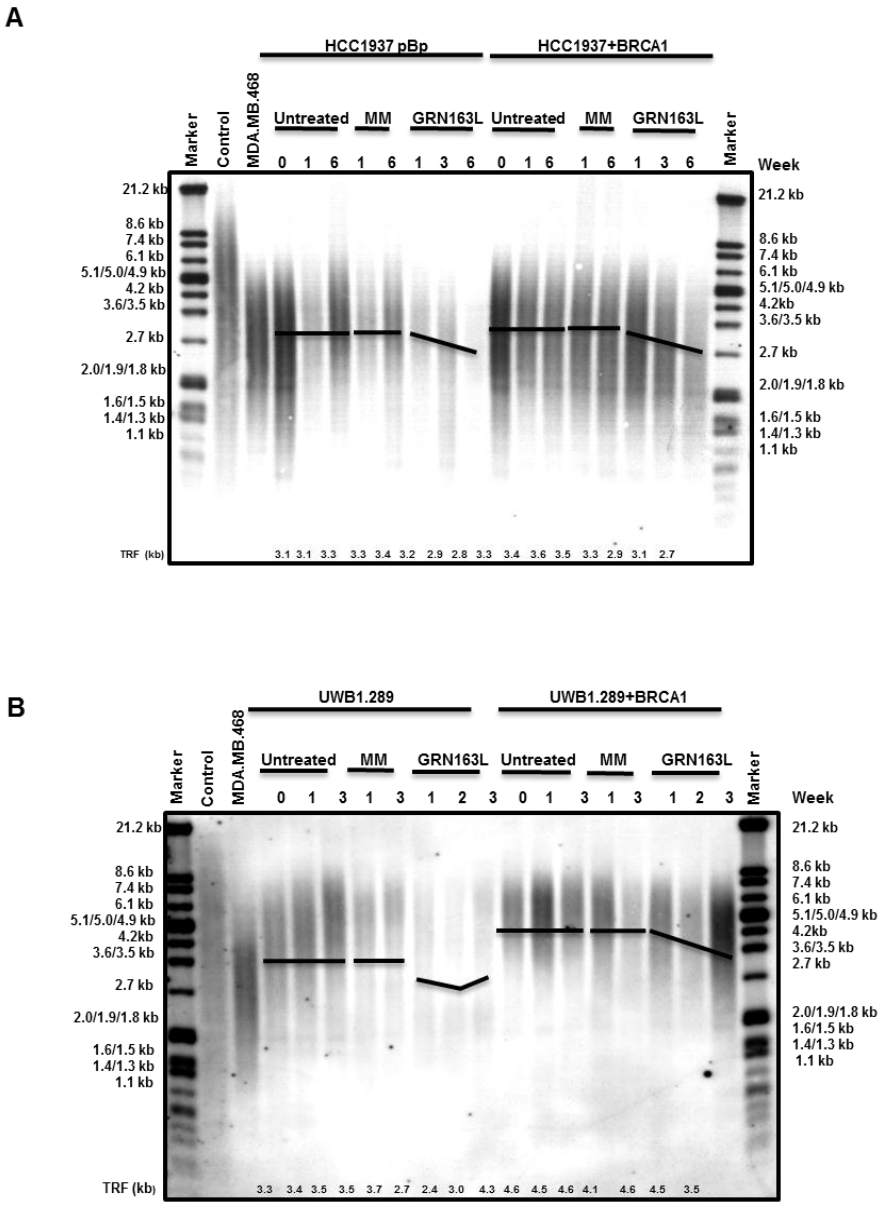


Figure 2.13. Telomere shortening occurs over a 3 or 6-week period of treatment with GRN163L in isogenic cell line pairs.
Cells were grown untreated or in the presence of GRN163L or mismatch over a 6-week (A) or 3-week (B) period. At the specified timepoint, attached and floating cells were washed, pelleted, and collected for subsequent use in the TeloTAGGG assay to measure telomere restriction fragments (TRFs) as described in Materials and Methods. Average TRF (in kb) is listed at bottom of blots.

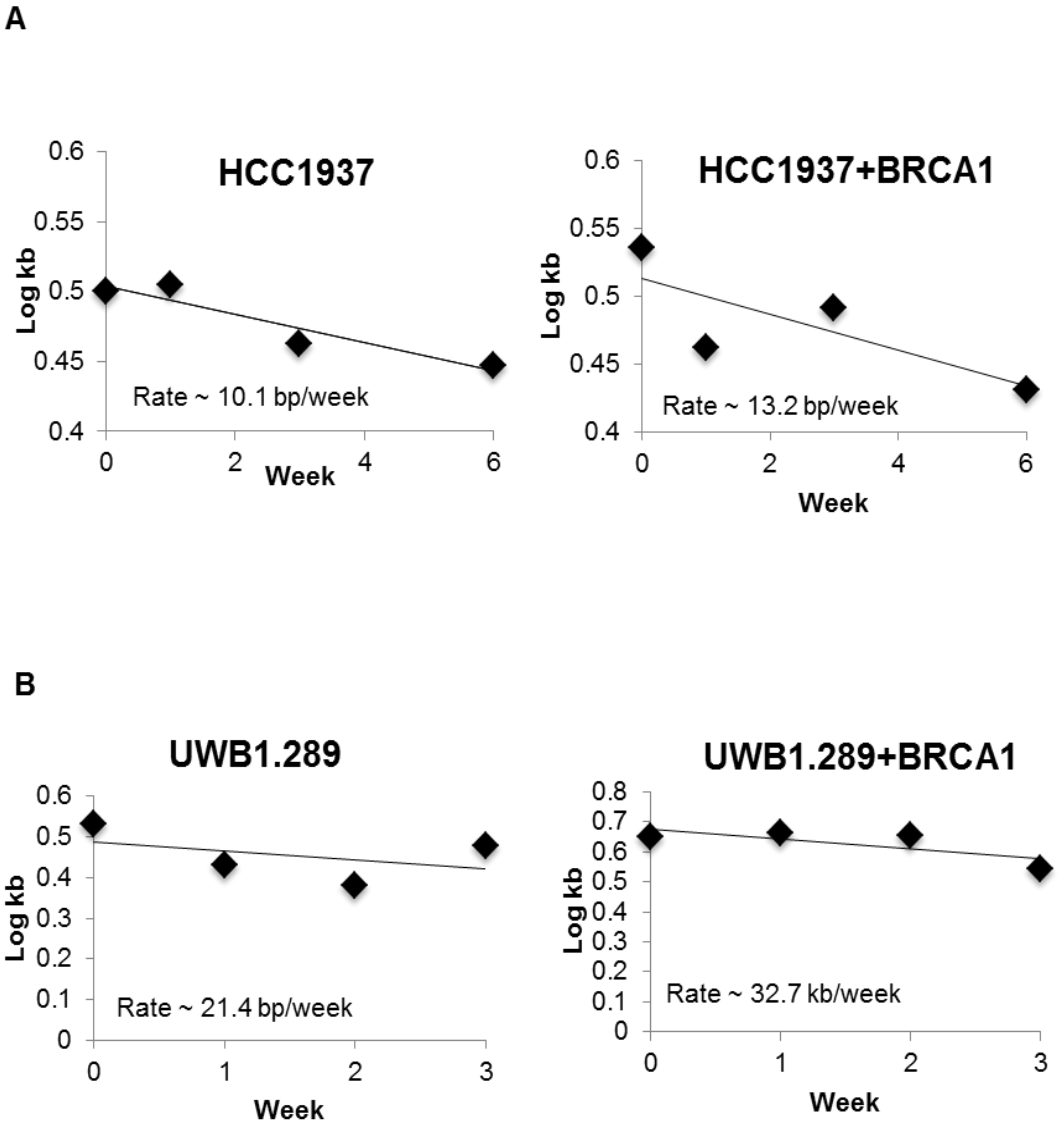


Figure 2.14. Rate of telomere shortening following 3 or 6-week period of treatment with GRN163L in isogenic cell line pairs.
Rate of telomere shortening was calculated by plotting log[kb] as a function of time.

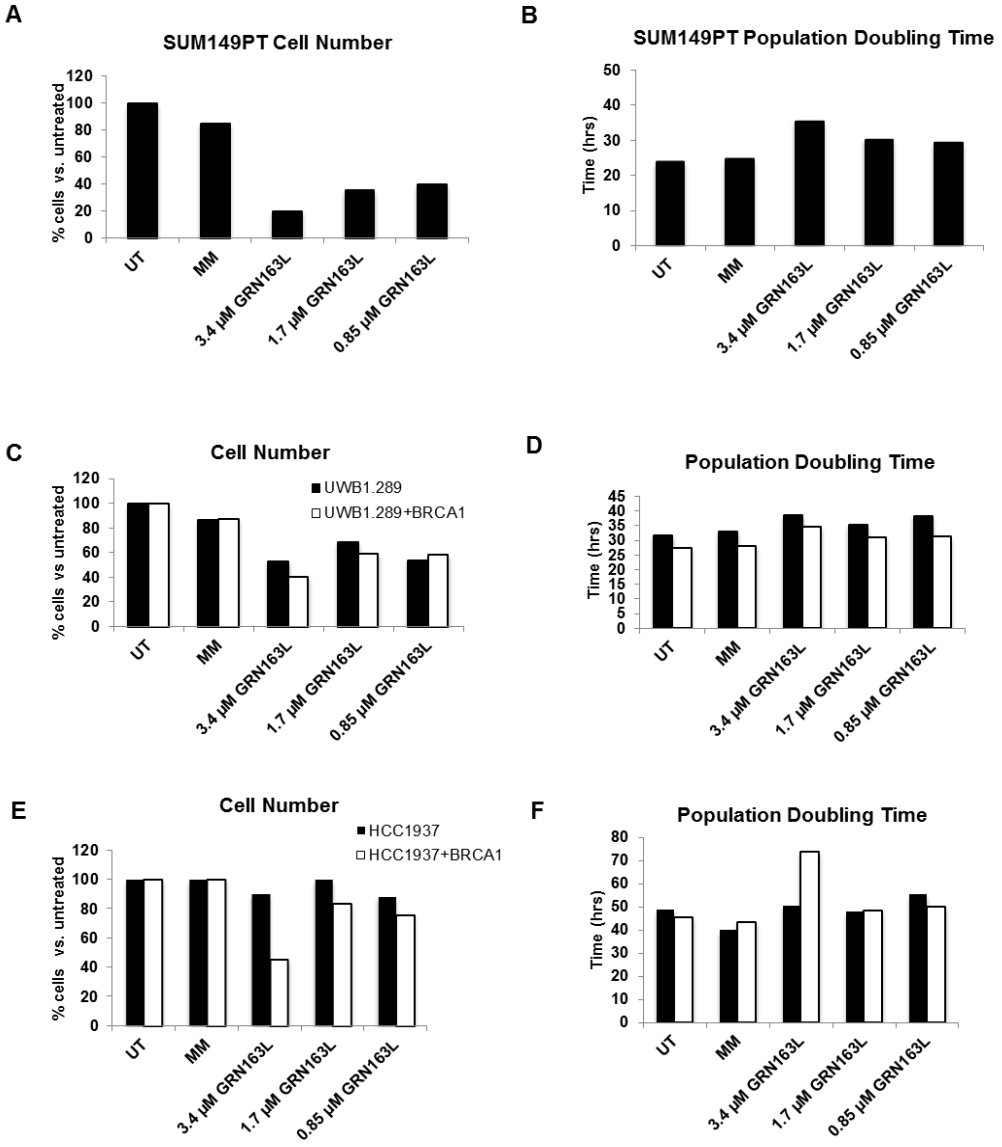


Figure 2.15. Simultaneous treatment with GRN163L hinders cell growth and increases population doubling time.

Cells were treated at time of plating and continually treated for the duration of the experiment. GRN163L or MM was replenished every 3-4 days by spiking the media. Cells were counted after 7 days using a counter (Beckman Coulter Zm) and population doubling time was calculated as the doubling time= experiment duration*log(2)/(log[(number of cells collected)-(log[number of cells initially plated]).

(Gomez-Millan, Goldblatt et al. 2007). Similarly, we observed decreased cell numbers and increased population doubling times as early as 7 days after simultaneous treatment (Figure 2.15). To address the question of whether treatment with GRN163L alters the cell cycle dynamics of BRCA1 wild-type versus BRCA1 mutant cell lines, we used flow cytometry and propidium idodide staining. We hypothesized that long-term (1 week or greater) treatment with GRN163L would lead to irreparable DNA damage in BRCA1 mutant cell lines, resulting in changes in cell cycle distribution. Doxorubicin was included as a positive control for these assays. We examined UWB1.289 and UWB1.289+BRCA1 cell lines after 1, 3 and 6 weeks of treatment with GRN163L or MM. No significant differences were observed in cell cycle distribution at these time points following treatment with either GRN163L or MM oligonucleotide (Figure 2.16A and B, week 6 data). Treatment with 1μM doxorubicin led to a significant decrease in the percentage of cells in G_1 ($p < 0.01$, one-way ANOVA) in UWB1.289 and UWB1.289+BRCA1 cell lines, and this was coupled with a trend toward an increase in the percentage of cells in G_2, though this did not reach statistical significance for all treatment groups ($p > 0.05$ for increase in G_2 cells in doxorubicin vs GRN163L treated groups in both cell lines) (Figure 2.16A and B). Similarly, we saw no changes in cell cycle distribution in HCC1937 pBp and HCC1937+BRCA1 cells after 1, 3, or 6-week treatment with GRN163L or MM (data not shown).

To further investigate the mechanistic effects of GRN163L on BRCA1 mutant and wild-type cells, we employed Western blot analysis and immunofluorescence to look at DNA repair proteins. Upon induction of DNA damage, histone H2A becomes

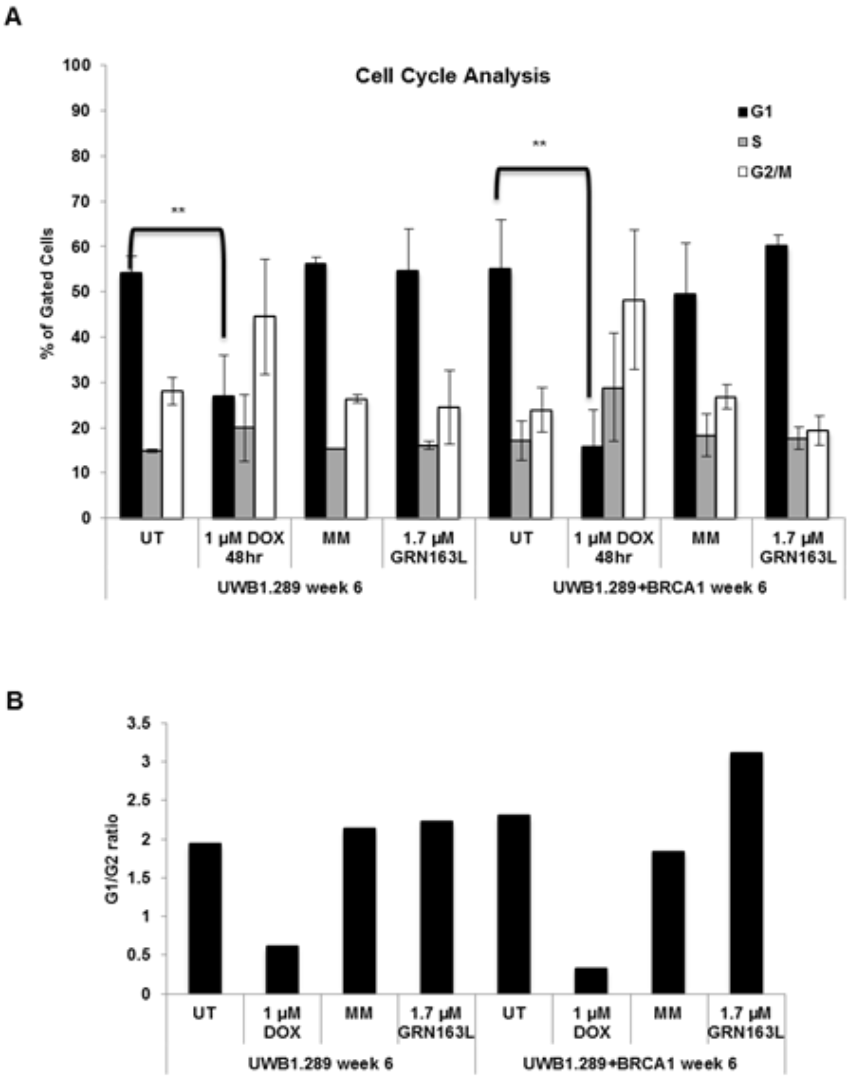


Figure 2.16. 6-week continuous GRN163L treatment does not induce cell cycle changes in UWB1.289 or UWB1.289+BRCA1 cells.
Cells were plated at uniform density to permit passage once per week for the duration of the experiment. Media was spiked with GRN163L or MM every 3-4 days. At collection, supernatant was collected to be combined with pellet. Attached cells were washed in 1xPBS, trypsinized, pelleted, resuspended in 1xPBS, and incubated in staining buffer containing propidium iodide, RNAse A, and NP40 detergent for 30 minutes at 4°C. Flow cytometry analysis was performed with FACScan. 1 µM of doxorubicin treatment for 48 hours was used as a positive control for each cell line. Experiment was performed in triplicate for each treatment group. ** $p < 0.01$, 1-way ANOVA.

phosphorylated in an ATM/ATR dependent manner (γ-H2AX), leading to the recruitment of homology-directed repair complexes containing BRCA1 (Krum, la Rosa Dalugdugan et al. 2010). We used Western blot analysis to assess the effects of GRN163L treatment on phosphorylation of γ-H2AX or protein expression of p21, an inhibitor of G_1/S cell cycle progression. As telomere shortening is known to trigger a DNA damage response (DDR) and, ultimately, replicative senescence, we reasoned that long-term treatment with GRN163L might result in different DDR profiles in BRCA1 wild-type versus mutant cells. After 3 weeks of continuous treatment with GRN163L, BRCA1 mutant UWB1.289 cells showed an increase in the phosphorylated form of H2AX. This induction was not seen in UWB1.289+BRCA1 cells until the 6-week timepoint (Figure 2.17A), indicating either that damage caused by GRN163L is more efficiently repaired in the BRCA1 wild-type cells or damage does not occur as robustly at this timepoint in UWB1.289+BRCA1 cells. UV-irradiated K-562 cells were used as a positive control. We also used immunofluorescence to visualize γ-H2AX foci in UWB1.289 and UWB1.289+BRCA1 cells following 1-week treatment with GRN163L or MM (Figure 2.17B). Using this method, we saw an increase in γ-H2AX positive cells in UWB1.289 doxorubicin- and GRN163L-treated groups compared to the BRCA1 wild-type cell line. These results agree with those presented in Figure 2.17A and suggest that GRN163L treatment induces γ-H2AX foci as early as one week after treatment and that DNA damage persists at 3 and 6 weeks in UWB1.289 cells (Figure 2.17). After 1 and 3-week treatment with GRN163L, HCC1937 pBp cells showed an increase in γ-H2AX protein expression relative to the untreated sample from the same timepoint (Figure 2.18A). HCC1937+BRCA1 cells do not show this same induction via Western blot, though some

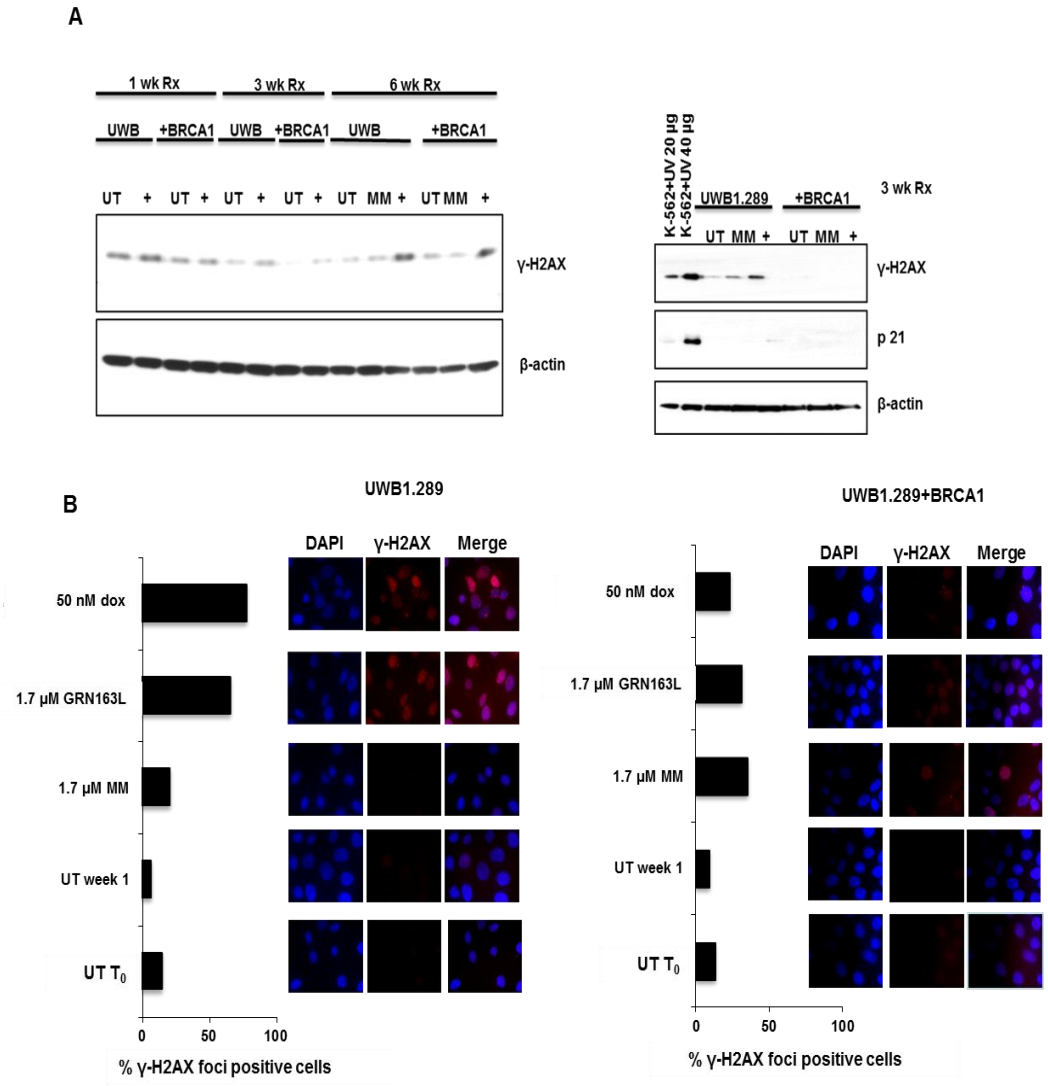


Figure 2.17. UWB1.289 cells show increased γ-H2AX expression compared to UWB1.289+BRCA1 cells following 1-week (immunofluorescence) or 3-week (Western blot) continuous treatment with GRN163L.
(A) Western blot analysis. Cells were continuously grown untreated (UT) or treated for 1, 3, or 6 weeks with GRN163L (+ denotes 1.7 µM GRN163L) or 1.7 µM Mismatch (MM) oligonucleotide. At collection, cells were washed in 1 x PBS, trypsinized, and pelleted. Supernatant was collected and combined with attached cells in the pellet for Western blot analysis as described in Materials and Methods. (B) Immunofluorescence. Cells were plated on 4-well chamber slides and grown in the presence of GRN163L or MM for 7 days. Media was removed every 3 days and replaced with fresh media or fresh media containing the appropriate oligonucleotide. On day 7, media was removed and slides were washed in ice cold 1 x PBS and fixed for 15 min. at room temperature in a 3:1 mixture of MeOH:acetic acid. Slides were blocked in 5% BSA in PBS for 1 hr at room temperature, and incubated with γ-H2AX antibody (Millipore) as described in Materials and Methods.

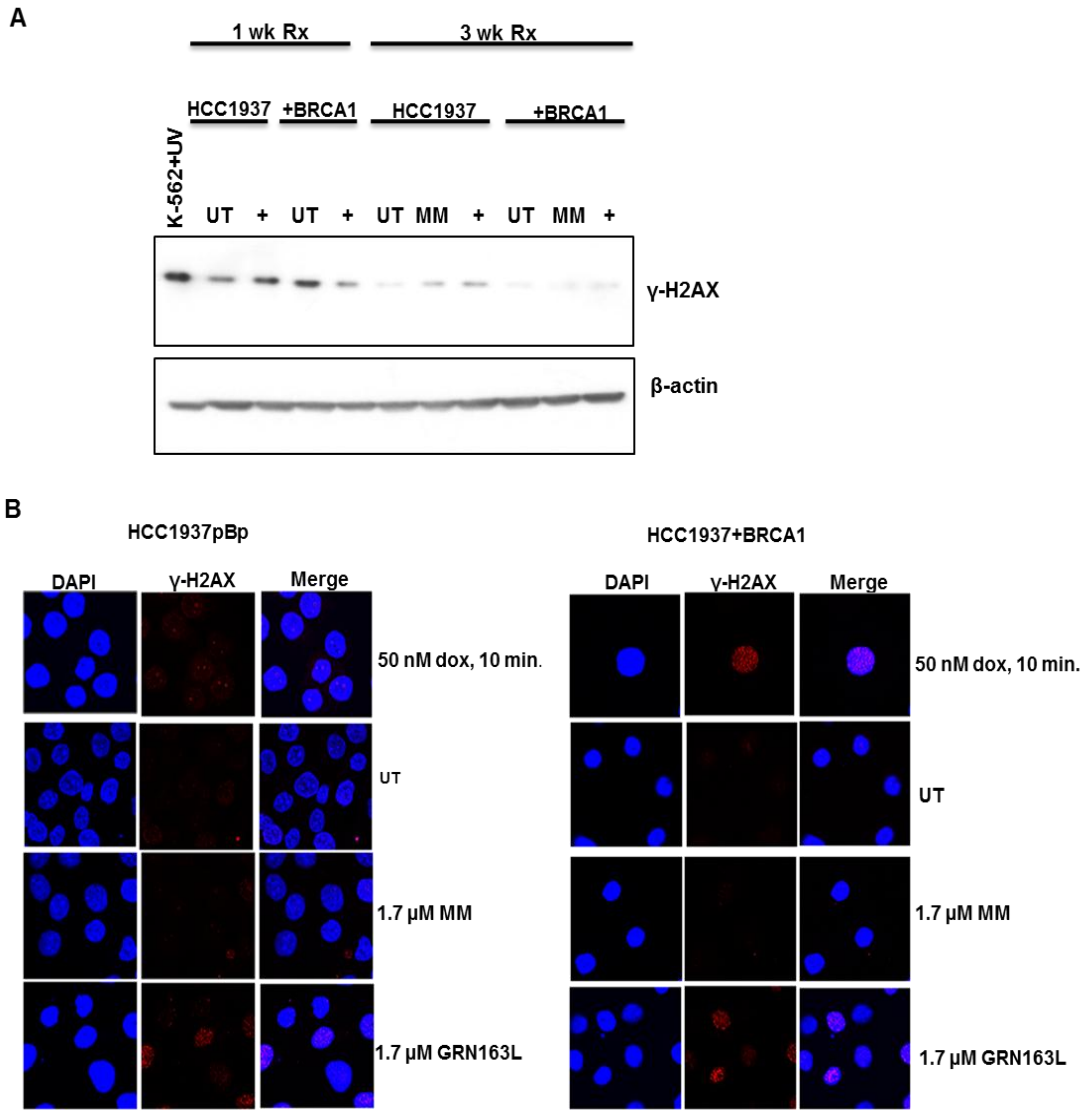


Figure 2.18. GRN163L induces γ-H2AX expression in HCC1937 pBp and HCC1937+BRCA1 cells after 1- or 3-week treatment.
(A) Western blot analysis. Cells were continuously grown untreated (UT) or treated for 1 or 3 weeks with GRN163L (+ denotes 1.7 μM GRN163L) or 1.7 μM Mismatch (MM) oligonucleotide. At collection, cells were washed in 1 x PBS, trypsinized, and pelleted. Supernatant was collected and combined with attached cells in the pellet for Western blot analysis as described in Materials and Methods. (B) Immunofluorescence. Cells were plated on 4-well chamber slides and grown in the presence of GRN163L or MM for 21 days. Media was removed every 3 days and replaced with fresh media or fresh media containing the appropriate oligonucleotide. On day 7, media was removed and slides were washed in ice cold 1 x PBS and fixed for 15 min at room temperature in a 3:1 mixture of MeOH:acetic acid. Slides were blocked in 5% BSA in PBS for 1 hr at room temperature, and stained for immunofluorescence as described in Materials and Methods.

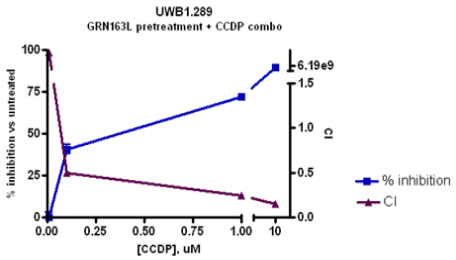
γ-H2AX foci are evident using immunofluoresnce after 3-week treatment with GRN163L (Figure 2.18A and B).

GRN163L pretreatment or concurrent treatment synergizes with DNA damaging agents

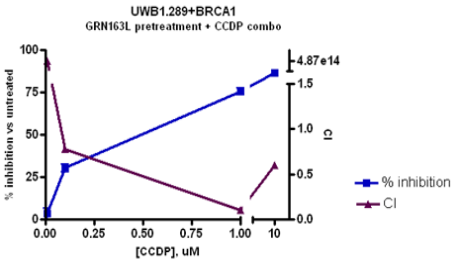
Reduced telomerase expression has been reported to compromise DNA repair in primary fibroblasts (Masutomi, Possemato et al. 2005). Given the ability of GRN163L to inhibit telomerase and induce DNA damage, we next investigated whether concurrent treatment with GRN163L might sensitize cells to DNA damaging agents commonly used in the treatment of breast and ovarian cancer. When UWB1.289 and UWB1.289+BRCA1 ovarian cancer cell lines were treated with GRN163L and cisplatin simultaneously, this combination was found to be antagonistic at all concentrations tested (data not shown). Next, we pretreated UWB1.289 and UWB1.289+BRCA1 cells for 6 weeks with 0.85 μM GRN163L to induce telomere shortening (Figure 2.13B**,** data shows shortening up to 3 weeks**)**. After a 6-week treatment, GRN163L was removed and cells were treated with various concentrations of cisplatin. The effects of GRN163L pretreatment augmented the action of cisplatin in both HCC1937 pBp and HCC1937+BRCA1 cell lines (Figure 2.19A). The results of 0.85 μM GRN163L treatment followed by cisplatin treatment were compared to untreated cells and to cells treated with 0.85 μM GRN163L for the duration of the experiment. Next, we examined the combination effects of GRN163L and topoisomerase II inhibitor doxorubicin in breast cancer cell lines HCC1937 pBp and HCC1937+BRCA1. The combination of GRN163L and doxorubicin added simultaneously was synergistic at the majority of concentration combinations tested in both cell lines (Figure 2.19B).

A

UWB1.289		
[GRN163L], uM	[CCDP], uM	CI
0.85	0.01	6.19E+09
0.85	0.1	0.501
0.85	1	0.243
0.85	10	0.155



UWB1.289+BRCA1		
[GRN163L], uM	[CCDP], uM	CI
0.85	0.01	4.87E+14
0.85	0.1	0.782
0.85	1	0.108
0.85	10	0.601



B

HCC1937 pBp		
[GRN163L], uM	[Doxorubicin], uM	CI
0.325	0.081	0.397
0.625	0.156	0.236
1.25	0.313	0.369
2.5	0.625	0.469
5	1.25	0.611
10	2.5	1.161
20	5	2.571

HCC1937+BRCA1		
[GRN163L], uM	[Doxorubicin], uM	CI
0.325	0.081	0.066
0.625	0.156	0.111
1.25	0.313	0.189
2.5	0.625	0.328
5	1.25	0.574
10	2.5	1.223
20	5	2.471

Figure 2.19. GRN163L pretreatment augments the action of cisplatin (A) and concurrent GRN163L treatment synergizes with doxorubicin (B).
Cells were grown in 96-well plates in the presence of fresh media, doxorubicin, GRN163L, a combination of doxorubicin and GRN163L, or cisplatin. After 72 hr, media was removed and cells were fixed in 100% methanol and stained with methylene blue. The methylene blue colorimetric assay measures proliferation based on optical density (Oliver, Harrison et al. 1989). Synergism was defined as a combination index (CI) value < 1 and was determined using Calcusyn software and the Chou-Talalay method (Chou and Talalay 1984).

Discussion

In summary, our work demonstrates differences in response to GRN163L between BRCA1 mutant and BRCA1 wild-type cell lines. Importantly, these differences only became apparent upon longer treatment timepoints at which the telomere-shortening effects of GRN163L were evident. At all treatment timepoints using the TRAP telomerase activity assay, regardless of whether treatment was simultaneous or next-day, we saw no significant differences in telomerase inhibition comparing BRCA1 mutant and BRCA1 wild-type cell lines as groups. This finding is not entirely surprising, considering we found no differences among these cells in terms of baseline telomerase activity (Figure 2.3A). Additionally, no timepoint tested in the TRAP assay was sufficient to see effects dependent upon baseline telomere length and the telomere-shortening effects of GRN163L. The differences in sensitivity were not due to differences in telomerase activity, as all cell lines were found to have the same activity levels at baseline. This finding contradicts a previous report detailing lower levels of telomerase activity following exogenous BRCA1 overexpression (Xiong, Fan et al. 2003). This discrepancy could be due to differences in methodology (Xiong et al. use of exogenous BRCA1 stable and transient transfection vs our use of established cell lines with endogenous BRCA1 or BRCA1 stably transduced).

The increased sensitivity of HCC1937 pBp and UWB1.289 cells to GRN163L relative to their BRCA1 wild-type counterparts could be due to differences in baseline telomere length, as demonstrated by the TeloTAGGG assay (Figure 2.4). The TeloTAGGG assay revealed a difference in baseline telomere length between HCC1937 pBp and HCC1937+BRCA1 cell lines and between UWB1.289 and UWB1.289+BRCA1

cell lines, with the BRCA1 mutant cell line of each isogenic pair having shorter telomeres. As predicted based on this result, HCC1937 pBp and UWB1.289 cells are more sensitive to GRN163L compared to their wild-type BRCA1 counterparts after 3-week treatment in clonogenic survival assays (Figure 2.10). This difference in sensitivity is evident in longer treatments with the HCC1937 pBp cell line (see population doubling experiment, Figure 2.12A and B), but not with the UWB1.289 cell line (Figure 2.12 C and D). Interestingly, we do, however, see that both UWB cell lines (which had among the longest telomeres at baseline) continue to grow and proliferate longer in the presence of drug relative to the HCC cell lines (which had among the shortest telomeres at baseline) (Figure 2.4 and Figure 2.12). This result could be a limitation of the assay as cells must be continually cultured and passaged in the presence of drug for several weeks and those cells less affected are most likely to be those collected after this time period.

Given the relatively short baseline telomere lengths of HCC1937 pBp and HCC1937+BRCA1 cell lines, we predicted they would be more sensitive to telomerase inhibition compared to cell lines with longer telomeres at baseline. In fact, we see these cell lines begin to die off much earlier than the UWB1.289/UWB1.289+BRCA1 cell line pair in population doubling studies (Figure 2.12). This finding correlates with both of the UWB cell lines having relatively longer average telomere lengths at baseline compared to most of the other cell lines in the panel (Figure 2.4).

In long-term population doubling studies, we observed HCC1937 pBp cells die off earlier than HCC1937+BRCA1 cells, and this result could be predicted based on the TeloTAGGG assay, as HCC1937 pBp cells had a slight, but statisitically significant, reduction in baseline telomere length compared to HCC1937+BRCA1 cells (Figure 2.4).

We cannot, however, rule out the possibility of a few very short telomeres in the HCC1937 pBp cell line, which cannot be detected using the resolution of the TeloTAGGG assay. As predicted by the shortest telomere hypothesis, the shortest one or two telomeres will predict sensitivity to telomerase inhibition, regardless of the average telomere length of a cell population (Harley 2008). Measuring only the shortest telomeres in a population has its challenges, and, even when detected, it is very difficult to establish a cause and effect relationship between the shortest telomeres and inhibitor response.

We demonstrate the effects we see following GRN163L treatment are due to the telomere shortening properties of the drug, with telomere shortening occurring over a 3- or 6- week period after treatment with GRN163L, but not MM oligonucleotide (Figure 2.13). The differences in sensitivity seen between BRCA1 mutant and BRCA1 wild-type cell lines was likely not due to changes in cell cycle dynamics, as we saw no significant differences in cell cycle distribution following 6-week continuous treatment with GRN163L (Figures 2.16 and 2.17). Though we anticipated a change in cell cycle distribution after long-term treatment with GRN163L, we may not have seen this due to a number of factors. For instance, lack of a normal DNA damage response in BRCA1 mutant cells suggests they will be less likely to halt the cell cycle due to DNA damage and will continue proliferating in the presence of damage (Deng 2006). Furthermore, UWB1.289 and UWB1.289+BRCA1 cells harbor an acquired somatic mutation in p53 (DelloRusso, Welcsh et al. 2007), and recent work suggests cell cycle arrest following prolonged mitotic arrest and telomere deprotection is p53-dependent (Hayashi, Cesare et al. 2012). In addition, based on the population doubling level results, a 6-week treatment

may not be sufficient to see the cell cycle effects of GRN163L, as HCC1937 pBp cells, the first to die off, do not begin to do so until approximately 7 weeks of treatment (Figure 2.12). Our result may be due to not being able to see cell cycle changes until the population ceases to grow after protracted GRN163L treatment.

Differences in sensitivity to GRN163L may be due to differences in the DNA damage response between UWB1.289 and UWB1.289+BRCA1 cell lines, with persistent γ-H2AX foci in UWB1.289 cells indicating an inability to repair DNA damage following treatment with GRN163L. These results led us to investigate whether GRN163L works synergistically with DNA damaging chemotherapeutics, such as cisplatin and doxorubicin. We found the combination of GRN163L and cisplatin used concurrently were antagonistic. Six-week pretreatment and removal of GRN163L, following by addition of cisplatin for 72 hours, was synergistic at the majority of concentration combinations tested for both UWB1.289 and UWB1.289+BRCA1 cell lines. This result suggests the telomere-shortening effects of GRN163L, rather than the chemistry of the oligonucleotide itself, impart enhanced sensitivity to cisplatin. The combination of GRN163L and doxorubicin added simultaneously was synergistic at the majority of concentration combinations tested in HCC1937 pBp and HCC1937+BRCA1 cell lines. This result is in agreement with a report that GRN163L potentiates the effects of G_2-specific topoisomerase II inhibitor etoposide. The combination effect with GRN163L is thought to be due to the cell-cycle specific action of etoposide (Tamakawa, Fleisig et al. 2010).

We also cannot rule out the possibility that some mutations in BRCA1 confer more sensitivity to GRN163L than others. From our results, it is difficult to discern whether this is the case, as such an assessment must also take into account both baseline

telomere length and population doubling time of cell lines. If loss of heterozygosity (LOH) in BRCA1 mutant carriers occurs before reactivation of telomerase in cancer development, these patients may benefit from preventative treatment with a telomerase inhibitor. It is also possible that telomerase activity levels are higher at baseline higher in BRCA1 mutation carriers because they lack one functional copy of BRCA1. As such, BRCA1 mutation carriers may benefit from telomerase inhibitor treatment to attenuate telomerase expression levels and stave off cancer development. In conclusion, this work highlights the multiple factors that must be taken into account in predicting cancer cell sensitivity to telomerase inhibition, including BRCA1 status, and lays a foundation for further investigation into the mechanism of action of GRN163L in BRCA1 mutant and wild-type cells.

Materials and Methods

1. Cell culture

a. HCC1937 pBp (pBabepuro, empty vector) and HCC1937+BRCA1

HCC1937 pBp and HCC1937+BRCA1 cells were kind gifts from Dr. Stephen Elledge. HCC1937 pBp and HCC1937+BRCA1 cells were maintained in DMEM 10% fetal bovine serum (FBS). HCC1937+BRCA1 cells were periodically treated with 1 µg/mL puromycin to select for maintenance of the vector containing wild-type BRCA1. Cells were grown at 37°C and 5% CO_2 and split every 4-7 days. Media was replenished 2-3 times per week and cells were routinely tested for mycoplasma contamination (Sigma).

b. UWB1.289 and UWB1.289+BRCA1

UWB1.289 and UWB1.289+BRCA1 cells were purchased from the American Type Culture Collection (ATCC, Rockville, Md, USA) and maintained in RPMI:MEGM at a ratio of 1:1 supplemented with 3% FBS and SingleQuot additives (Clonetics). UWB1.289+BRCA1 cells were grown under G-418 (200 µg/mL) selection for maintenance of the vector containing BRCA1. G-418 was removed from media when UWB1.289+BRCA1 cells were treated with GRN163L or mismatch oligonucleotide. During long-term treatments (greater than 3 weeks), UWB1.289+BRCA1 cells were periodically selected with G-418 at splitting. Cells were grown at 37°C and 5% CO_2 and split every 4-7 days. Media was replenished 2-3 times per week and cells were routinely tested for mycoplasma contamination (Sigma).

c. MCF7

MCF7 cells (ATCC) were maintained in DMEM 10% FBS. Cells were grown at 37°C and 5% CO_2 and split every 3-4 days. Cells were routinely checked for mycoplasma contamination (Sigma).

d. MDA.MB.436

MDA.MB.436 cells (ATCC) were maintained in MEM 10% FBS, 1 mM sodium pyruvate, 1 M Hepes, and 2 x MEM vitamins. Cells were grown at 37°C and 5% CO_2 and split every 3-4 days. Cells were routinely checked for mycoplasma contamination (Sigma).

e. MDA.MB.468

MDA.MB.468 cells (ATCC) were cultured in DMEM 10% FBS and maintained at 37°C and 5% CO_2. Cells were split every 3-4 days and routinely tested for mycoplasma contamination (Sigma).

f. SUM149PT

SUM149PT cells were a kind gift from Dr. George Sledge (Stanford University, formerly of Indiana University School of Medicine). SUM149PT cells were maintained in Ham's F-12, 5% cosmic calf serum (CCS), 5 µg/mL insulin, and 1 µg/mL hydrocortisone. Cells were grown at 37°C and 5% CO_2 and split every 3-4 days. Cells were routinely checked for mycoplasma contamination (Sigma).

2. Oligonucleotides

The lipid-modified N3′→P5′ thiophosphoramidate oligonucleotide GRN163L (5′-Palm-TAGGGTTAGACAA-NH_2-3′) has a sequence complementary to the hTR template

region (also known as imetelstat). Either a mismatch (30S GRN140833, 5'-Palm-TAGGTGTAAGCAA-3', mismatch sequences underlined) or sense oligonucleotide (GRN140832, 5'-Palm-ATCCCAATCTGTT-3') was used as a negative control for these studies (both are referred to as MM in the text) (Herbert, Gellert et al. 2005). All oligonucleotides used in this study were provided by Geron Corporation.

3. Treatment with GRN163L

Cells were allowed to attach overnight prior to treatment with GRN163L or MM. Where multiple treatment concentrations were used, media was removed the day after plating and replaced with a 1:2 serial dilution series of GRN163L or MM. After 24 or 48 hours, 1.0×10^5 cells were collected for the TRAP (Telomeric Repeat Amplification Protocol) assay. For clonogenic survival assays, cells were treated with GRN163L or MM the day after plating. Drug was refreshed by spiking the media or by replacing the media with fresh media containing GRN163L or MM every 3-4 days. Where multiple treatment concentrations were used, a 1:2 serial dilution series of GRN163L or MM control was prepared (Figure 2.20). Alternatively, cells were treated at plating (simultaneous treatment). After 12 or 24 hours simultaneous treatment, 1.0×10^5 cells were collected for the TRAP assay. For simultaneous treatment clonogenic survival assays, cells were treated with GRN163L or MM at plating and drug was refreshed by spiking the media every 3-4 days for a period of three weeks prior to plating at low density (Figure 2.21).

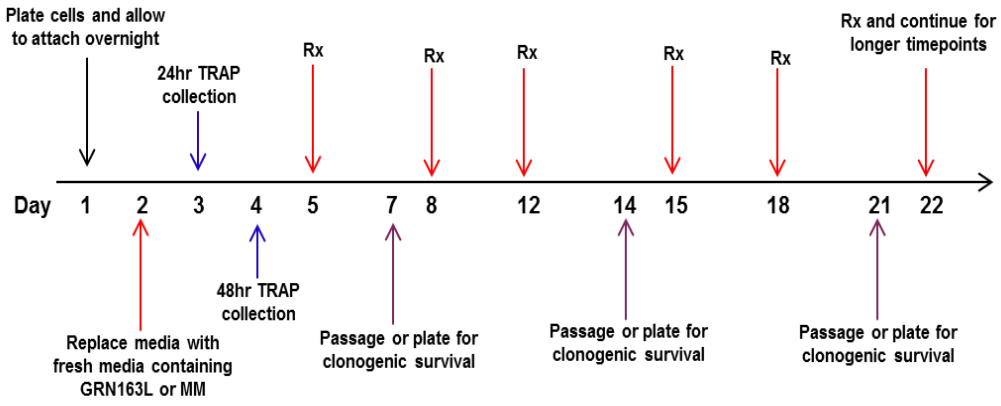


Figure 2.20. Next-day treatment set-up.
For next-day treatment, cells were plated and allowed to attach overnight. The next morning, media was removed and replaced with fresh media, or fresh media containing GRN163L or MM oligonucleotide. For long-term studies, cells were passaged once per week, and media or drug was replenished every 3-4 days.

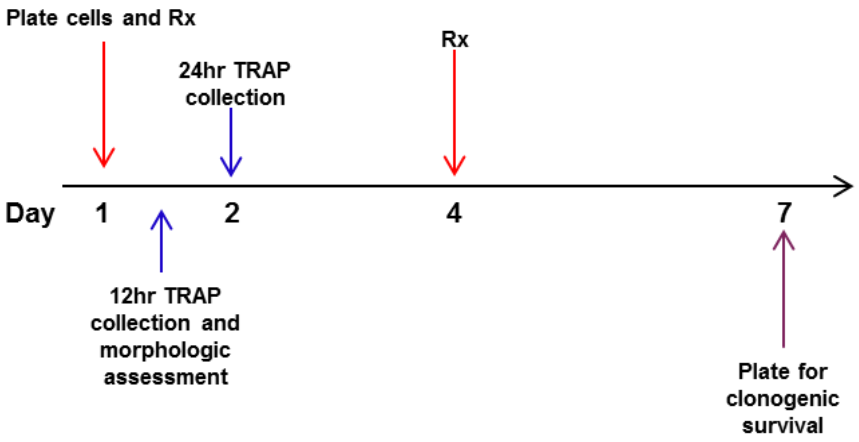


Figure 2.21. Simultaneous treatment set-up.
For simultaneous treatment, cells were untreated or treated with GRN163L or MM oligonucleotide at time of plating. Fresh media or drug was added on Day 4 when cells were used for clonogenic survival assays following 1 week simultaneous treatment.

4. Telomerase activity by the Telomeric Repeat Amplification Protocol (TRAP)

TRAP assays were performed as previously described (Hochreiter, Xiao et al. 2006). Briefly, cells were lysed in NP-40 lysis buffer (1000 cells/μL) and telomerase products were extended via 30-minute incubation with a Cy5 fluorescently labeled telomerase template primer. Extended products were amplified using PCR, and products were run on 10% nondenaturing acrylamide gels. Gels were visualized using a PhosphorImager and ImageQuant software. Products were quantified using ImageJ software and presented as the ratio of the telomerase product to the internal control band.

5. TeloTAGGG telomere measurement assays

Cells were grown untreated or in the presence of GRN163L or MM over a 3- or 6-week period. After each timepoint as indicated in the Results section, attached and floating cells were washed, pelleted, and used for DNA extraction per the manufacturer guidelines (Qiagen, Valencia, CA). DNA was digested using *Rsa*1/*Hin*f1 to leave telomeric DNA. Digested fragments were separated on a 0.8% agarose gel, blotted to a nylon membrane (Roche) using capillary transfer and 20 x SSC (Invitrogen) buffer. Following overnight transfer, DNA was UV-crosslinked to the membrane (Spectrolinker) and a non-radioactive DIG-labeled telomeric probe was hybridized to the membrane. The membrane was visualized using chemiluminescence. Mean TRF lengths were calculated using ImageJ software (Herbert, Hochreiter et al. 2006). Briefly, a grid of 30 boxes was positioned over each lane, and the signal intensity and size (kb) corresponding to each box was determined (Figure 2.22).

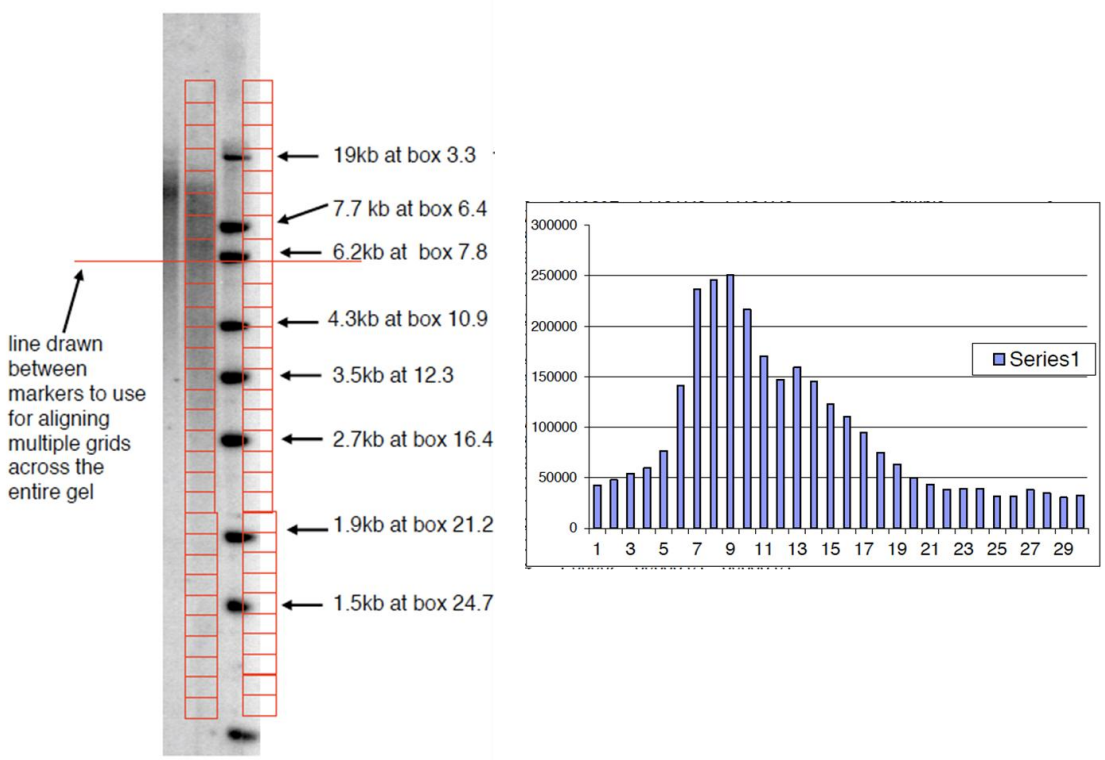


Figure 2.22. Measurement of telomere length using TeloRun.
Mean telomere lengths and telomere length distributions were calculated using the TeloRun program. Briefly, a grid of 30 boxes was drawn and superimposed on the molecular weight marker lane. The grid was then copied and laid over each sample lane of the gel (left). Relative to the where the molecular weight marker was in the grid, a density distribution plot correlating to kilobase lengths was constructed (right) for each sample when the grid was overlaid on sample lanes. The x-axis of the distribution plot denotes the number of the box on the grid (starting with box 1 at the top), and the y-axis denotes density. The peak of the density distribution plot correlates to the average telomere length for each sample. A line was drawn between markers from opposing sides of each gel to ensure the grid was properly aligned for each sample lane in relation to the alignment of the grid on the molecular weight marker (adapted from TeloRun, http://www4.utsouthwestern.edu/cellbio/shay-wright/research/sw_lab_methods.htm).

Average telomere length was visualized by drawing a line across the lanes as described previously (Hochreiter, Xiao et al. 2006).

6. Cell counting for long-term treatment population doubling study

Cells were plated at uniform density in 6-well dishes and passaged approximately once per week if needed. Cells were counted at each passage using a counter (Beckman Coulter Zm) and replated at uniform density and continually treated for the duration of the experiment. Drug was replenished every 3-4 days by spiking the media or replacing the media with fresh media containing GRN163L or MM. Population doublings were calculated as the log[(the number of cells collected)/(number of cells initially plated)]/log2 for each passage. Data is presented as cumulative population doubling level for each treatment group.

7. Clonogenic survival

Following a 1-, 2-, 3-, or 12-week treatment with GRN163L or MM oligonucleotide, 750-1000 cells were seeded on 10 cm^2 plates and grown undisturbed and untreated for 14-18 days. Media was then aspirated and plates were washed with 1 x HBSS before fixing with ice cold 100% methanol. Plates were stained with 0.5% crystal violet in 25% methanol for 10 minutes at room temperature, rinsed in double-distilled water, and allowed to dry overnight. Colonies consisting of at least 50 cells were scored and recorded as surviving fraction versus untreated.

8. Methylene blue proliferation assay for combination studies

Cells were grown in 96 well plates in replicates of eight in the presence of fresh media and varying concentrations of: cisplatin, GRN163L, doxorubicin, or a combination of doxorubicin and GRN163L (or cisplatin and 6-wk GRN163L-treated cells). After 72 hours, media was removed and cells were fixed in 100% methanol for 15 minutes at room temperature and stained with 0.05% methylene blue for 15 minutes with rocking at room temperature. Plates were rinsed with diH_20 and allowed to dry overnight. The next morning, plates were de-stained with 100 μL/well of 0.5 M HCl with shaking at room temperature. Optical density readings were collected at 610 nm using an absorbent plate reader and recorded as fraction affected relative to the untreated group. Synergism was defined as a combination index (CI) value <1 was determined using Calcusyn software and the Chou-Talalay method (Chou and Talalay 1984).

9. Western blot analysis

Cells were treated continuously for three weeks with GRN163L or MM. At collection, cells were washed in 1xPBS, trypsinized, pelleted, and snap frozen until lysis in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 37 mM ß-glycerol phosphate, 47 mM NaF, 1% NP40, 0.1% SDS, 0.5% Na deoxycholate, 10% glycerol, and protease inhibitor cocktail). Supernatant was collected and combined with attached cells in the pellet. 25 or 50 μg protein was loaded into 4-15% Bis-Tris SDS-PAGE (denaturing, reduced) gradient gels (NuPAGE) and run from 50 min-120 min (BRCA1) at 200 V. Protein was transferred to PVDF membrane at 10 V overnight at 4°C. Membranes were blocked overnight at 4°C in 5% BSA or 5% milk in PBS-T. BRCA1 MS110 antibody

(Calbiochem) was used at 1:200. γ-H2AX (Millipore) antibody was used at 1:1000 in 5% BSA in PBS-T. P-95 antibody (Signalway) was used at1:5000 in 5% BSA in PBS-T. β-actin (Sigma) was included as a loading control and was used at 1:5000. P21 antibody (Cell Signaling) was used at 1:2000 in 5% milk in PBS-T.

10. Flow cytometry

Cells were plated at uniform density to permit passage once per week for the duration of the experiment. Media was spiked with GRN163L or MM every 3-4 days. At the indicated timepoint, supernatant was collected and attached cells were collected as well by trypsinization. Cells were pelleted, resuspended in 1 x PBS and incubated in staining buffer (1 x PBS, 50 μg/mL propidium iodide, 200 ng/mL RNAse A, and 0.3% NP40) for 30 minutes at 4°C. Flow cytometry analysis was performed with FACScan (Becton Dickinson Biosciences). 1μM of doxorubicin treatment for 48 hours was used as a positive control for each cell line. The experiment was performed in triplicate for each treatment group.

11. Immunofluorescence

Cells were plated on 4-well chamber slides and allowed to grow for 2-3 days before subconfluent cells were fixed with methanol and acetic acid at a ratio of 3:1 for 15 min at room temperature. No additional permeabilization step was used. Slides were washed and 3 times in 1 x PBS and blocked in 5% BSA for 1 hour at room temperature. Primary antibody for γ-H2AX (Millipore) was used at 1 μg/mL in 5% BSA overnight at 4°C. Secondary antibody rhodamine red-X (Molecular Probes) was used at 1:1000 in 5% BSA

in 1 x PBS. Slides were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories) and fluorescent images were captured using a Leica DM5000B microscope and SPOT software (Diagnostic Instruments). Fifty cells were counted per treatment group, and the number of cells positive for at least 1 γ-H2AX focus was recorded as a percentage of the total cells counted.

12. Statistical analyses

Graph Pad Prism 4.0 software was used to conduct one-way ANOVA analysis, in which each treatment group was compared to all other treatment groups to determine statistical significance (where $p < 0.05$ was considered statistically significant). Calcusyn software (Biosoft) was used to determine combination effects via the Chou and Talalay method. A combination index (CI) value of < 1.0 indicates a synergistic interaction, a CI of 1.0 shows an additive effect, and a CI > 1.0 denotes an antagonistic effect (Chou and Talalay 1984).

CHAPTER THREE

ANALYSIS OF TELOMERE LENGTH DISTRIBUTION AND TELOMERE-ASSOCIATED GENE EXPRESSION PROFILES IN BRCA INDIVIDUALS

Abstract

Telomere dysfunction, telomere shortening, and deregulation of shelterin components have been linked to a variety of age-related ailments, including cancer. Recent evidence suggests the BRCA1 tumor suppressor has a role in telomere maintenance, and genetic anticipation coupled with telomere shortening has been reported in BRCA1 carriers. The direct effects of BRCA1 haploinsufficiency on telomere maintenance and predisposition to cancer development are largely unknown. To address these questions, we utilized RNA and DNA from peripheral blood leukocytes to assess telomere gene expression profiles and telomere length distribution in BRCA1 carriers, BRCA2 carriers, and sporadic and familial breast cancer individuals. We found overexpression of telomere-associated and telomere-proximal genes, including genes involved in negative regulation of telomere length, in BRCA1 carriers compared to all other cancer types. This study provides a potential mechanism by which telomere dysfunction may occur in BRCA carriers prior to loss of the remaining wild-type allele and links BRCA1 haploinsufficiency to changes in telomere length and gene expression. In addition, this work further distinguishes BRCA1 cancers from BRCA2 and other familial cancers and provides evidence that therapeutic targets aimed at telomerase or shelterin interactions may benefit this patient population.

Introduction

Multiple studies of cells from *BRCA* mutation carriers suggest "one-hit" effects occur even before loss of the remaining wild-type allele and precede development of disease. For instance, cultured epithelial cells from heterozygous carriers of *BRCA1* or *BRCA2* mutations exhibit genomic instability characterized by gene copy number loss and decreased homology-directed repair capacity in *in vitro* assays (Konishi, Mohseni et al. 2011). Other work suggests this instability may be due to deregulation of genes involved in the G_2/M cell cycle transition and DNA damage response in BRCA1 heterozygous mutant cells, while BRCA2 heterozygous mutant cells show deregulation of genes involved in the G_1/S cell cycle checkpoint. Interestingly, BRCA1, but not BRCA2, heterozygous epithelial cultured cells show upregulation of the secretoglobin family of genes and expression profiles similar to those seen in stem and progenitor cells (Bellacosa, Godwin et al. 2010). This finding corroborates with work suggesting a role for BRCA1 in regulating stemness and differentiation in breast progenitor cells (Liu, Ginestier et al. 2008). Expression profiles of heterozygous BRCA1 mutant cells also show similarities to hereditary breast cancers from BRCA1 individuals. A separate study demonstrated increased proliferation and clonogenic capacity, coupled with EGFR activation, in primary mammary epithelial cells from BRCA1 mutation carriers (Burga, Tung et al. 2009). Telomere dysfunction has been hypothesized to account for the unstable phenotype of cells derived from heterozygous BRCA mutation carriers, and may explain their predisposition to cancer development.

Telomere dysfunction and telomere shortening have been linked to a variety of human age-related ailments, including cardiovascular disease (Fyhrquist and Saijonmaa

2012), neurodegenerative disease (Panossian, Porter et al. 2003; Guan, Maeda et al. 2008), chronic inflammatory disease (Andrews, Fujii et al. 2010), and cancer (Armanios and Blackburn 2012). An analysis of case control studies revealed an association between short telomeres and elevated risk for cancers of the bladder, head and neck, lung and kidney (Wu, Amos et al. 2003). Genetic anticipation, or earlier age of onset and sometimes severity of disease in successive generations, has been attributed to telomere shortening in familial cancer syndromes such as Li-Fraumeni and dyskeratosis congenita (Tabori et al 2007, Vulliamy et al 2004). A recent report described an association between genetic anticipation and shortened telomeres in hereditary breast cancers (BRCA1, BRCA2, and BRCAX), but not in sporadic breast cancers (Delgado et al 2011). A similar study of ovarian cancer found significantly shorter telomeres in peripheral blood leukocytes in both sporadic and hereditary cases compared to healthy controls; thus, the roles of BRCA1 and BRCA2 in telomere shortening in ovarian cancer are unclear (Martinez-Delgado, Yanowsky et al. 2012). A separate report found no differences in telomere length of peripheral blood leukocytes between ovarian cancer cases and controls, but suggests single nucleotide polymorphisms (SNPs) in the TERT gene are associated with elevated ovarian cancer risk (Terry, Tworoger et al. 2012). In addition, lifestyle factors such as stress, diet, weight, and cigarette smoking are correlated with telomere length, but likely also have roles in development of disease though additional mechanisms (Epel, Blackburn et al. 2004; Valdes, Andrew et al. 2005; Paul 2011).

Such studies are largely association-based, and the factors driving telomere shortening and dysfunction and their contributions to disease development remain largely

unknown. Efforts to shed light on the mechanisms driving telomere dysfunction in cancer, though limited, have offered helpful clues. For instance, elevated levels of TRF2 have been reported in advanced breast cancers, and expression levels correlate with telomere shortening (Diehl, Idowu et al. 2011). Similarly, Hu et al. found elevated expression of TRF1, TRF2, TERT, and Ku70 in precancerous lesions, gastric cancer tissues, and gastric cancer with lymph node metastasis compared to normal gastric tissue. Importantly, levels of these proteins were significantly higher in gastric cancers with and without metastasis compared to precancerous lesions, and mean telomere length was inversely correlated with levels of TRF1, TRF2, TERT, and Ku70. Furthermore, BRCA1 was localized in the nucleus in normal gastric mucosa, but found primarily in the cytoplasm in precancerous lesions and gastric cancer, and cytoplastmic export of BRCA1 correlated with decreased telomere length (Hu, Zhang et al. 2010). In another recent study, Yanowsky and colleagues looked for sequence variation in telomere-associated genes in BRCA negative individuals with short telomeres and a family history of breast cancer (Yanowsky, Barroso et al. 2012). This study yielded noncoding variants, but no pathogenic role for the variants was found using functional predication analysis and control population studies. Evidence from Lou and colleagues suggests that telomere length can regulate gene expression (Lou, Wei et al. 2009). Namely, telomere shortening, but not replicative senescence or DNA damage signaling *per se*, led to upregulation of interferon-stimulated gene 15 (ISG15) prior to a DNA damage response triggered by telomere shortening. The increase in ISG15 expression was confirmed in human skin samples with increased age. This work provides insight into a possible mechanism for maintaining cellular homeostasis and reveals clues to a potential

mechanism by which accelerated telomere shortening could lead to an increase in inflammation-related gene expression and cancer development (Lou, Wei et al. 2009).

The objective of this study was to determine average telomere length and length distribution in peripheral blood leukocytes of BRCA mutation carriers compared to age-matched controls and sporadic and familial breast cancers. In addition, we aimed to identify factors that contribute to telomere dysfunction and genetic anticipation associated with cancer, particularly in BRCA individuals, and to correlate gene expression changes with telomere lengths. To this end, we assessed expression of telomere-associated genes and of telomere-proximal genes (genes within 1000 kb of the telomere) using the gene list from GEO Datasets GSE6799, previously published by Lou et al.(Lou, Wei et al. 2009). This work sheds light on the role of BRCA1 in telomere maintenance and on the mechanism of sensitivity of BRCA1 mutant cells to telomerase inhibition.

Results

Characteristics of patient samples and normal controls

All BRCA, sporadic, and familial blood samples were a generous gift from Dr. Lida Mina (IUSM). We obtained peripheral blood samples from ten BRCA1 carrier individuals, ten BRCA2 carrier individuals, nine individuals with familial breast cancer (individuals with no identified BRCA mutation but a family history of breast cancer), and eleven individuals with sporadic breast cancer. We also obtained peripheral blood from ten healthy control individuals to correct for age-related telomere shortening in telomere length assays. Individuals with sporadic breast cancer ranged in age from 36-70 years (mean age 52.09 +/- 12.84 years) (Table 3.1). The mean age at diagnosis of sporadic individuals was 38.45 +/- 10.61 years. 4/10 sporadic patients had lymph node involvement, and the majority (8/11) had ER+ disease (Table 3.2).

The mean age among BRCA1 carrier individuals was 46.6 +/- 11.64 years, and this did not differ significantly from the mean age of sporadic individuals (Tables 3.1 and 3.3, $p > 0.05$, one-way ANOVA). Of the ten BRCA1 carriers, five had developed breast cancer at the time of sampling, with a mean age at diagnosis of 39 +/- 4.2 years (Table 3.4). Though a trend was seen toward earlier development of breast cancer in BRCA1 carriers, the age of onset in BRCA1 individuals who developed cancer was not significantly different as compared to age of onset of sporadic breast cancer among our sample population ($p > 0.05$, one-way ANOVA). Of those BRCA1 carriers who developed cancer, none had lymph node involvement, and 4/5 were classified as triple negative, with one individual having ER+ disease (Table 3.4).

Table 3.1. Sporadic individual demographic information.

ID	Age	Sex	Height (cm)	Weight (kg)	Race
1	47	f	165	91.4	1R
4	65	f	170	78.6	1R, 13J
8	67	f	155	56.8	1R, 1J
9	45	f	177.5	87.2	1R
17	66	f	170	62.2	1R
19	70	f	172.5	86.3	1R
22	42	f	165	79.5	1R
24	55	f	170	76	1R
33	38	f	180	71.9	1R
34	36	f	167.5	79.5	3R
38	42	f	170	101.8	1R

ID corresponds to the assigned patient ID number given to each individual. For the race category, 1R denotes an answer of "White or Caucasian" on a questionnaire asking about race, and 3R denotes an answer of "Asian." 1J indicates that an individual is of Ashkenazi Jewish descent, and 13J indicates that an individual is unsure of whether she is of Ashkenazi Jewish descent.

Table 3.2. Sporadic individual breast cancer history.

ID	Age diag	BC	LyNo	# LyNo	ER	PR	HER2	Surg	Prevt	Rx	2DiagBC	2TypBC	MBC	RxMBC
1	27	2	0		+	-	?	1		R,C	0		0	
4	34	1	0		+	+	+	2			1	1	0	
8	55	1	1	4	-	-	+	1		R,C,B	0		1	C,H
9	33	1	1	2	+	+	+	1		R,C,H	0		0	
17	60	1	1		-	-	-			R,C,B	0		1	C
19	45	13	13		+	+	-		1	R,C,H	1	1	0	
22	38	1	0		+	+	+	1		C,B,H?	0		0	C
24	29	1	0		+	+	?	2		R,C,H	0		0	H
33	33	1	1	1	+	+	+	3	1	C,B,H	0		0	
34	32	1	1	0	-	-	-	1		R,C,B	0		0	
38	37	1	0		+	+	+	3	1	C,B,H	0		0	

ID corresponds to the patient ID number assigned to each individual. "Age diag" indicates the age at which a woman was first diagnosed with breast cancer. BC= Breast Cancer. A score of 1 under the BC category indicates the breast cancer was invasive, a score of 2 indicates pre-invasive breast cancer (Ductal Carcinoma In Situ, or DCIS), and 13 indicates type of breast cancer is unknown. LyNo stands for Lymph Nodes; in the LyNo column, a score of 0 indicates no lymph node involvement, a score of 1 indicates lymph node involvement, and a score of 13 indicates the patient is unsure of whether there was lymph node involvement. If a patient had lymph node involvement, the number of lymph nodes (#LyNo) is indicated in the next column, if known. ER, PR, and Her2 positivity are denoted by a "+" symbol. In the Surgery column (Surg), 1= lumpectomy, 2= mastectomy, and 3= bilateral mastectomy. If the patient had bilateral mastectomy, a score of 1 in the Preventative column (Prevt) indicates the procedure was done prophylactically for one breast. In the treatment category (Rx), R= radiation therapy, C= chemotherapy, B= biologic therapy, and H indicates hormonal therapy. In the 2DiagBC column, a score of 1= a patient who has had a second breast cancer, and 0= a patient who has not had a second breast cancer. In terms of second breast cancer type, a score of 1 means the second breast cancer was invasive. The presence (1) or absence (0) of metastatic breast cancer (MBC) and treatment for the metastatic breast cancer (RxMBC, C= chemotherapy, H= hormonal therapy) are also documented.

Table 3.3. BRCA1 individual demographic information.

ID	Age	Sex	Height (cm)	Weight (kg)	Race
3	63	f	165	60.5	1R,1J
11	51	f	162.5	61.4	1R
15	49	f	157.5	65	1H,11R,1J
20	52	f	172.5	75.5	1R
26	53	f	170	81.4	1R
28	45	f	170	106.8	1R
30	25	f	170	57.7	1R,1J
31	55	f	157.5	63.6	
32	29	f	162.5	59.1	1R
37	44	f	162.5	102.3	1R,1J

ID corresponds to the assigned patient ID number given to each individual. For the race category, 1R denotes an answer of "White or Caucasian" on a questionnaire asking about race. 11R? 1H means a woman answered "yes" to the question "Are you Hispanic or Latino?" 1J indicates that an individual is of Ashkenazi Jewish descent.

Table 3.4. BRCA1 individual breast cancer history.

ID	Age diag	BC	LyNo	ER	PR	HER2	Surg	Prevt	Rx	2DiagBC	2TypBC	MBC	RxMBC
3	46	1	0	-	-	?	3	1	C	0		0	
11	NA	0		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	NA	0		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
20	NA	0		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
26	39	1	0	-	-	-	1		R,C	1			
28	34	1	0	+	-	-	3	1	C,H	0		0	
30	NA	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
31	39	1	0	-	-	-	2		C	1	1	0	
32	NA	0		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
37	37	1	0	-	-	-	1,3	1	RC	0		0	

ID corresponds to the patient ID number assigned to each individual. Age diag indicates the age at which a woman was first diagnosed with breast cancer (NA indicates an individual who has not been diagnosed with cancer). BC stands for Breast Cancer. A score of 1 under the BC category indicates the breast cancer was invasive, a score of 0 indicates the individual has not been diagnosed with breast cancer. LyNo stands for Lymph Nodes; in the LyNo column, a score of 0 indicates no lymph node involvement. ER, PR, and Her2 positivity are denoted by a "+" symbol. In the Surgery column (Surg), 1= lumpectomy, 2= mastectomy, and 3= bilateral mastectomy. If the patient had bilateral mastectomy, a score of 1 in the Preventative column (Prevt) indicates the procedure was done prophylactically for one breast. In the treatment category (Rx), R= radiation therapy, C= chemotherapy, B= biologic therapy, and H indicates hormonal therapy. In the 2DiagBC column, a score of 1= a patient who has had a second breast cancer, and 0= a patient who has not had a second breast cancer. In terms of second breast cancer type, a score of 1 means the second breast cancer was invasive. The presence (1) or absence (0) of metastatic breast cancer (MBC) and treatment for the metastatic breast cancer (RxMBC, C= chemotherapy, H= hormonal therapy) are also documented.

Table 3.5. BRCA2 individual demographic information.

ID	Age	Sex	Height (cm)	Weight (kg)	Race
2	41	f	172.5	59.5	1R
10	44	f	162.5	63.6	1R
12	31	f	162.5	60	1R
13	47	f	162.5	49	1R
14	32	f	152.5	60	1R
16	43	f	170	57	1R
23	55	f	160	63.2	1R
29	36	f	172.5	63.6	1R
35	48	f	177.5	60.5	1R
36	45	f	160	80	1R

ID corresponds to the assigned patient ID number given to each individual. For the race category, 1R denotes an answer of "White or Caucasian" on a questionnaire asking about race.

The mean age among BRCA2 carrier individuals was 42.2 +/- 7.47 years, and this was not younger than the mean age of sporadic individuals or of BRCA1 individuals (Table 3.5, one-way ANOVA). Of the ten BRCA2 carriers, five had developed breast cancer at the time of sampling, with a mean age at diagnosis of 38.8 +/- 7.85 years (Table 3.6). The age of onset in BRCA2 individuals was not significantly different as compared to age of onset of sporadic breast or compared to age of onset of BRCA1 cancer among our sample population. Of those BRCA2 carriers who developed cancer, one had confirmed lymph node involvement, and 4 out of 5 carriers were classified as ER+, with one individual having triple negative breast cancer (Table 3.6).

The mean age among individuals who developed familial breast cancer was 53 +/- 5.63 years, and this was significantly older than the mean age of BRCA2 individuals, but was not different from the mean ages of sporadic or BRCA1 individuals (Table 3.7, one-way ANOVA). The mean age at diagnosis among those who developed familial breast cancer was 43.67 +/- 6.91, and this did not differ from the age of onset in sporadic, BRCA1, or BRCA2 individuals who developed cancer (one-way ANOVA). The majority of familial cancer patients (8 out of 9 patients) had ER+ disease, and one of these individuals had lymph node involvement. The remaining individual developed triple negative breast cancer (Table 3.8). For relative information and reproductive histories of sporadic, familial, and BRCA individuals used in this study, see Appendix.

Peripheral blood from ten healthy individuals encompassing the age range of all patient samples was obtained from the Komen Tissue Bank (age range of normal controls: 25-70). Importantly, because of potential environmental influences on telomere

Table 3.6. BRCA2 individual breast cancer history.

ID	Age diag	BC	LyNo	# LyNo	ER	PR	HER2	Surg	Prevt	Rx	2DiagBC	2TypBC	MBC	RxMBC
2	33	13	0		+	+	-	1		R,C	0		0	
10	39	1	1		+	+	-			C	0		0	
12	NA	0	NA		NA	NA	NA	NA	NA	NA				
13	42	2	0		-	-	-	3	1	C	0		0	
14	NA	2	0		NA	NA	NA	3	1		0		0	
16	30	1	1	6	+	+	-	2		R,C,H	0		0	
23	50	13	13		+	+	-			C	0		1	
29	NA	0	NA		NA	NA	NA	NA	NA	NA				
35	NA	0	NA		NA	NA	NA	NA	NA	NA				
36	NA	0	NA		NA	NA	NA	NA	NA	NA				

ID corresponds to the patient ID number assigned to each individual. Age diag indicates the age at which a woman was first diagnosed with breast cancer (NA indicates an individual has not been diagnosed with breast cancer). BC stands for Breast Cancer. A score of 0 under the BC category means no breast cancer, 1 indicates the breast cancer was invasive, a score of 2 indicates pre-invasive breast cancer (Ductal Carcinoma In Situ, or DCIS), and 13 indicates type of breast cancer is unknown. LyNo stands for Lymph Nodes; in the LyNo column, a score of 0 indicates no lymph node involvement, a score of 1 indicates lymph node involvement, and a score of 13 indicates the patient is unsure of whether there was lymph node involvement. If a patient had lymph node involvement, the number of lymph nodes (#LyNo) is indicated in the next column, if known. ER, PR, and Her2 positivity are denoted by a "+" symbol. In the Surgery column (Surg), 1= lumpectomy, 2= mastectomy, and 3= bilateral mastectomy. If the patient had bilateral mastectomy, a score of 1 in the Preventative column (Prevt) indicates the procedure was done prophylactically for one breast. In the treatment category (Rx), R= radiation therapy, C= chemotherapy, B= biologic therapy, and H indicates hormonal therapy. In the 2DiagBC column, a score of 1= a patient who has had a second breast cancer, and 0= a patient who has not had a second breast cancer. In terms of second breast cancer type, a score of 1 means the second breast cancer was invasive. The presence (1) or absence (0) of metastatic breast cancer (MBC) and treatment for the metastatic breast cancer (RxMBC, C= chemotherapy, H= hormonal therapy) are also documented.

Table 3.7. Familial individual demographic information.

ID	Age	Sex	Height (cm)	Weight (kg)	Race
5	46	f	183	66	1R
6	45	f	167.5	141	1R
7	56	f	152.5	70	1R
18	51	f	172.5	70.5	1R
21	52	f	165	61.4	1R
25	54	f	167.5	69.5	1R
27	64	f	160.5	56.4	1R
39	54	f	165	56.8	1R
40	55	f	162.5	55	1R

ID corresponds to the assigned patient ID number given to each individual. For the race category, 1R denotes an answer of "White or Caucasian" on a questionnaire asking about race.

Table 3.8. Familial individual breast cancer history.

ID	Age diag	BC	LyNo	# LyNo	ER	PR	HER2	Surg	Prevt	Rx	2DiagBC	2TypBC	MBC	RxMBC
5	38	13	0		+	+	?	1		R,C,H	0		0	
6	39	1	0		-	-	-	3		C,B	0		0	
7	48	1	0		+	+	?	1		R,C,H	0		0	
18	46	1	1		+	+	+	1		R,C,B,H	0		0	
21	43	1	0		+	+	-	1,3		H	0		0	
25	48	1	0		+	+	-			H	0		1	
27	52	1	0		+	+	-	3	1	H	0		0	
39	49	2	0		+	+	-	1		R,B,H	0		0	
40	30	1	0		+	+	?	2		C,	0		1	C,H

ID corresponds to the patient ID number assigned to each individual. Age diag indicates the age at which a woman was first diagnosed with breast cancer. BC stands for Breast Cancer. A score of 1 under the BC category indicates the breast cancer was invasive, a score of 2 indicates pre-invasive breast cancer (Ductal Carcinoma In Situ, or DCIS), and 13 indicates type of breast cancer is unknown. LyNo stands for Lymph Nodes; in the LyNo column, a score of 0 indicates no lymph node involvement, a score of 1 indicates lymph node involvement, and a score of 13 indicates the patient is unsure of whether there was lymph node involvement. If a patient had lymph node involvement, the number of lymph nodes (#LyNo) is indicated in the next column, if known. ER, PR, and Her2 positivity are denoted by a "+" symbol. In the Surgery column (Surg), 1= lumpectomy, 2= mastectomy, and 3= bilateral mastectomy. If the patient had bilateral mastectomy, a score of 1 in the Preventative column (Prevt) indicates the procedure was done prophylactically for one breast. In the treatment category (Rx), R= radiation therapy, C= chemotherapy, B= biologic therapy, and H indicates hormonal therapy. In the 2DiagBC column, a score of 1= a patient who has had a second breast cancer, and 0= a patient who has not had a second breast cancer. In terms of second breast cancer type, a score of 1 means the second breast cancer was invasive. The presence (1) or absence (0) of metastatic breast cancer (MBC) and treatment for the metastatic breast cancer (RxMBC, C= chemotherapy, H= hormonal therapy) are also documented.

Table 3.9. Healthy control demographic information.

Age at donation	# of first degree relatives with cancer history	Height (cm)	Weight (kg)	BMI	Ever used tobacco?
25	0	172.7	54.4	18.2	No
31	0	165.1	52.2	19.1	No
36	0	177.8	58.1	18.4	No
41	0	177.8	66.2	21	No
48	0	172.7	54.4	18.2	No
50	0	160	42.6	16.6	No
55	0	167.6	56.7	20.2	No
63	0	152.4	45.4	20	No
67	0	154.9	56.7	23.6	No
70	0	167.6	93	33.1	No

DNA from peripheral blood leukocytes of healthy individuals was obtained for age-matching. Healthy control individuals had no first degree relatives with cancer history, were within the normal BMI (body mass index) range of 18.5 to 24.9 (with the exception of the 70 year old individual, as no sample within normal BMI range was available for this age), and had never used tobacco (www.cancer.org).

length (Kim, Parks et al. 2009), none of the control individuals had ever used tobacco, and all except one (age 70, BMI 33.1) were within the BMI range considered normal (Table 3.9).

Patient samples show a trend toward shorter telomere length in BRCA1 carriers

Using TRF analysis and 3-4 independent replicates per patient sample, average telomere lengths were calculated for each patient sample and for normal controls as described in Materials and Methods (Figure 3.1, as an example). Telomeric DNA from the peripheral blood leukocytes used in this study yielded a relatively tight distribution of telomeric DNA for the majority of samples when run on a gel. Variability among technical replicates for each sample was relatively small. We did, however, see a wide range of telomere lengths within each group; though we saw a trend toward shorter telomere length in BRCA1 carriers compared to normal, sporadic, BRCA2, and familial individuals, this result did not reach statistical significance (Figure 3.2, including telomere length range in kb within each group).

BRCA1 and BRCA2 individuals show deregulation of telomere-associated genes

Unsupervised hierarchical clustering of telomere and telomerase-associated genes revealed three major groups of genes: those overexpressed in BRCA1 and BRCA2 individuals, those overexpressed only in BRCA1 individuals, and those not differentially expressed among BRCA and sporadic individuals (Figure 3.3). A comparison of gene expression in BRCA1 individuals versus all other individuals (BRCA2, familial, and sporadic) found differential expression of 46 genes ($p < 0.05$). Notably, 44 out of 46 genes in this dataset yielded with this analysis were overexpressed, with only two genes,

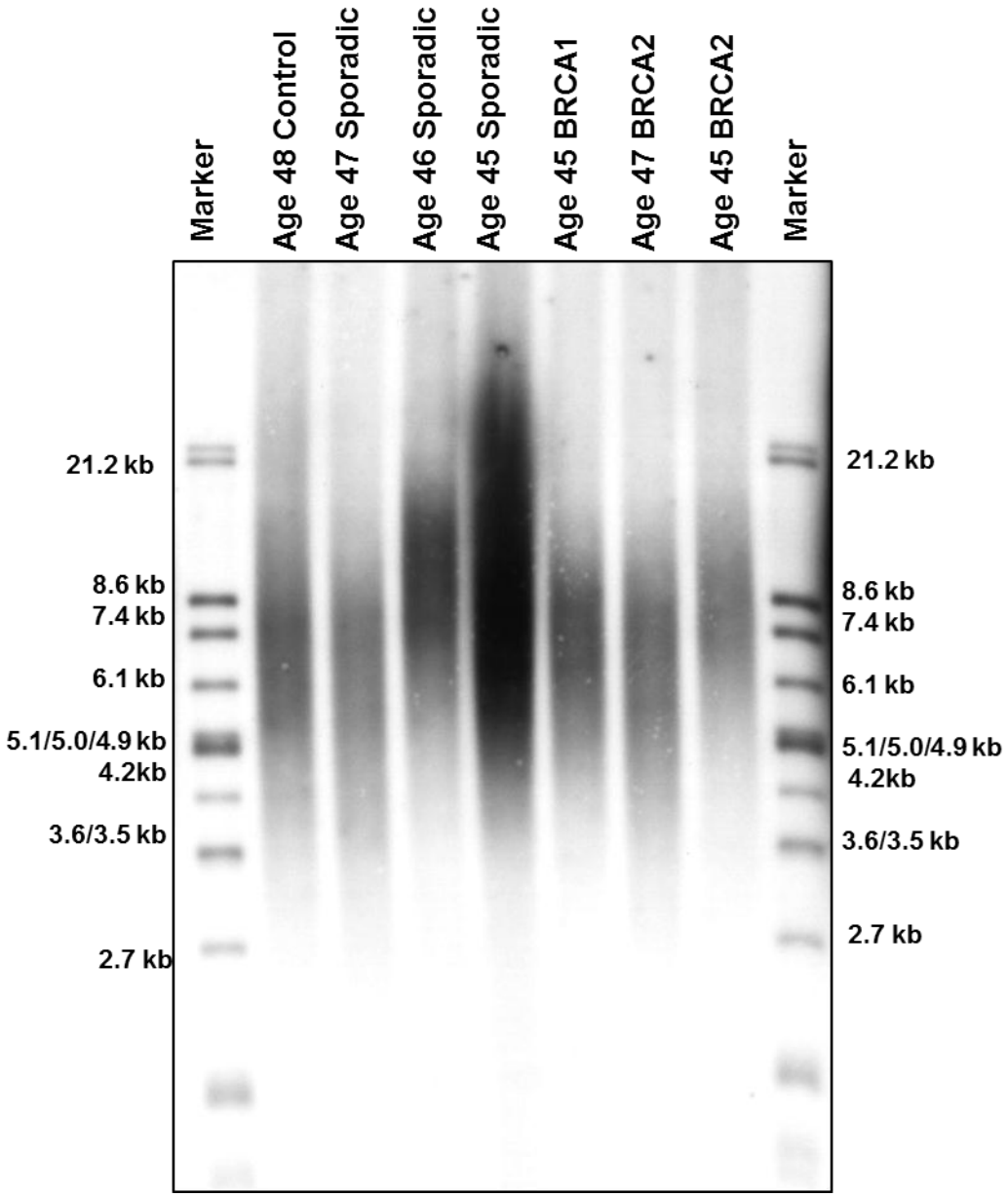


Figure 3.1. Telomeres of age-matched samples and normal control.
DNA was digested and used for TeloTAGGG analysis as described in Materials and Methods.

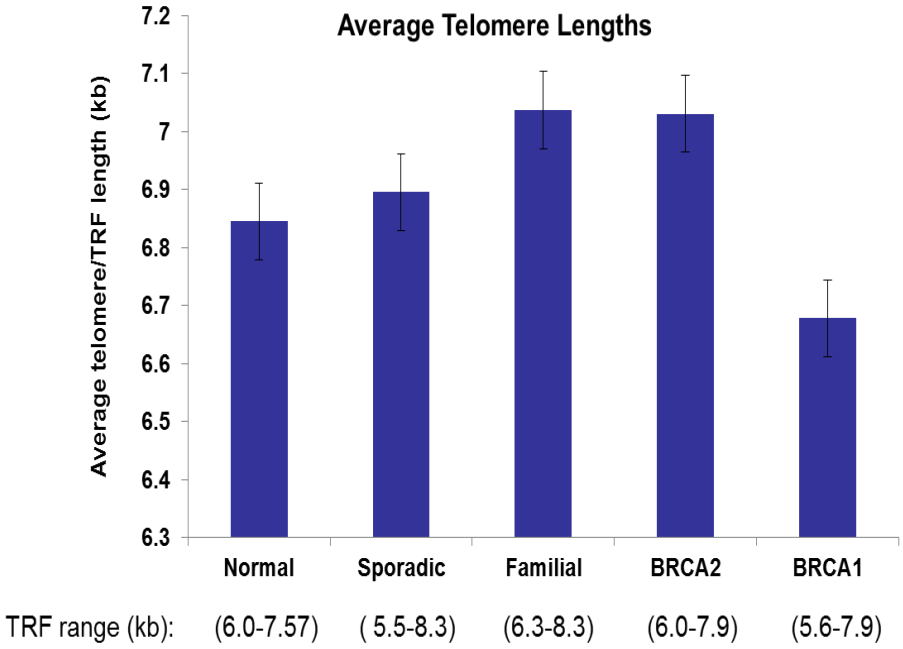


Figure 3.2. BRCA1 patient samples show a trend towards having the shortest telomeres.

Averages telomere lengths were calculated for each sample and normal control, and averages + SEM were calculated for each of the 5 groups (normal samples ranging in age from 25-70 were included as one group). The TRF range (in kb) for each group is listed below the chart. The sporadic breast cancer group showed the greatest range in telomere lengths, followed by the BRCA1 group.

IGFBP2 and *SERPINE1/PAI-1* gene, being underexpressed in BRCA1 individuals compared to all others. The top hit was the *PPP2R5C* gene, which encodes for PP2A, or protein phosphatase 2A, a serine/threonine phosphatase involved in negative control of cell growth and division (p= 0.000647, 1.43 fold increase vs all others). Fold change values ranged from a 1.54 fold increase in *TINF2 (TIN2)* expression to a 1.24 fold decrease in *IGFBP2* expression. For the complete list of differentially expressed genes in BRCA1 individuals compared to all other individuals, see Table 3.10.

We next separated BRCA1 individuals into those who had a cancer diagnosis at time of sampling, and those who did not, and compared each of these groups. A comparison of gene expression in BRCA2 individuals versus all other individuals (BRCA1, familial, and sporadic) showed underexpression of 8 genes, with the top hit being *UPF3A* (p value= 0.00785, 1.20 fold decrease vs all others). Fold change values ranged from a 1.18 fold decrease in *PPP2R3A* expression to a 1.34 fold decrease in *CDK5R2* expression. The complete list of telomere-associated genes underexpressed in BRCA2 individuals compared to all others is shown in Table 3.11. Interestingly, only 3 genes overlap between Tables 3.10 and 3.11, with *UPF3A* being significantly underexpressed in both BRCA1 and BRCA2 individuals, and CHFR and TTC21B being overexpressed in BRCA1 individuals but underexpressed in BRCA2 individuals (Tables 3.10 and 3.11).

When grouping all BRCA individuals (BRCA1 and BRCA2 with and without a cancer diagnosis) and comparing them with sporadic and familial cancer individuals using a p value cut-off of $p < 0.05$, we see differential expression of 83 out of 291 analyzed telomere-associated genes. All 83 genes identified in this analysis were

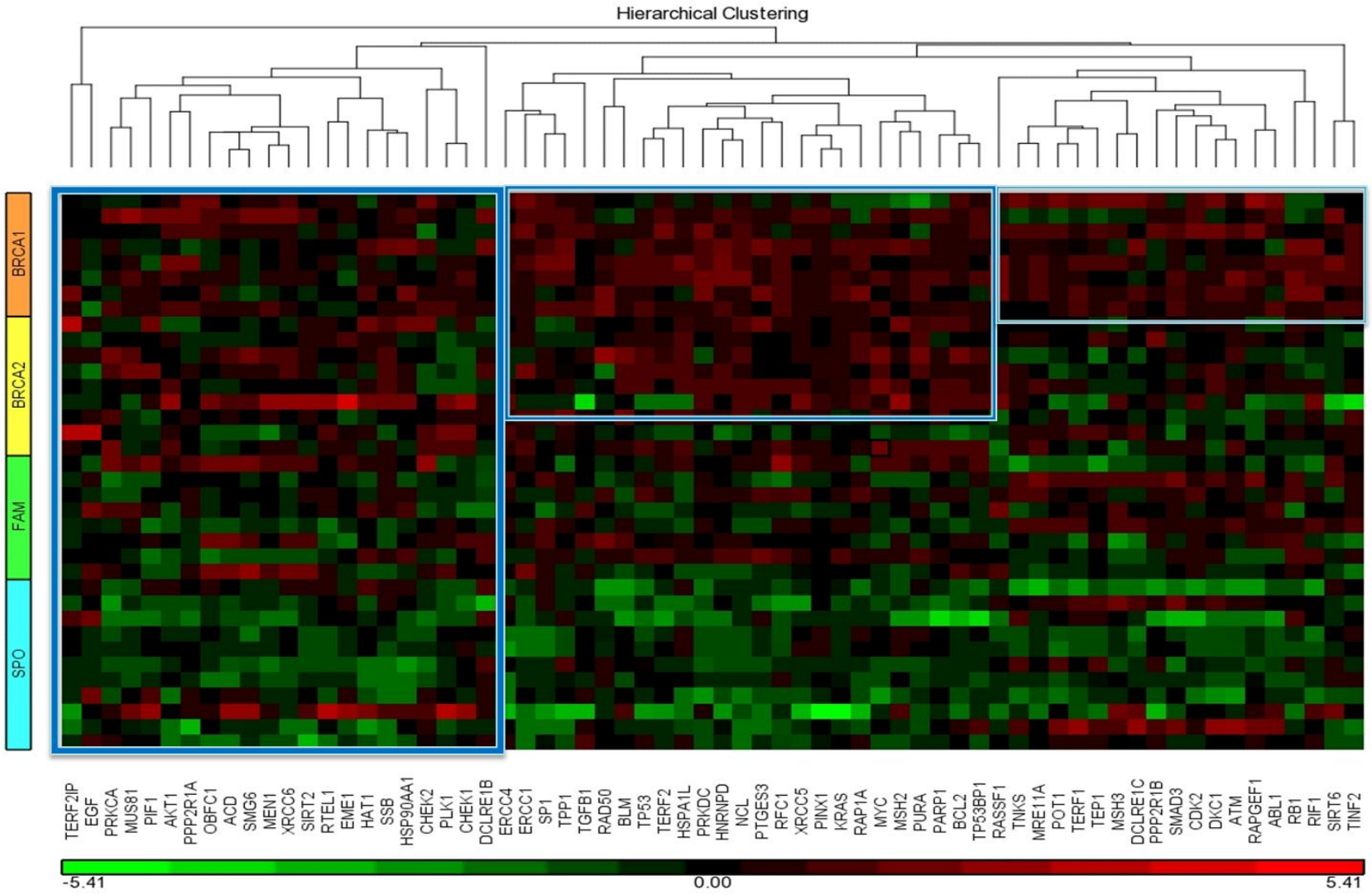


Figure 3.3. Unsupervised hierarchical clustering of expression of telomere and telomerase-associated genes reveals three distinct gene clusters. Distinct gene clusters are denoted by blue boxes. From left to right, the first cluster shows genes not differentially expressed among sporadic, familial, and BRCA individuals. The second cluster highlights genes overexpressed in BRCA1 and BRCA2 individuals, and the third cluster highlights genes overexpressed only in BRCA1 individuals.

Table 3.10. Significant expression changes in telomere and telomerase-associated genes in BRCA1 individuals vs sporadic, BRCA2, and familial individuals.

GENE	P VALUE	FOLD CHANGE
PPP2R5C	6.5E-04	1.43
MX1	3.0E-03	1.40
TINF2	4.0E-03	1.54
ISG15	5.2E-03	1.46
NFKB1	5.3E-03	1.32
UPF3A	7.5E-03	1.21
FN3KRP	7.8E-03	1.28
CHFR	8.0E-03	1.33
USP14	8.2E-03	1.25
CDK4	8.6E-03	1.28
PSMD11	9.3E-03	1.26
PPP2CB	9.5E-03	1.38
PRKDC	1.2E-02	1.21
CCND3	1.4E-02	1.27
PINX1	1.5E-02	1.29
RPL5	1.5E-02	1.25
ZNF324	1.5E-02	1.31
TERF2	1.6E-02	1.30
BARD1	1.8E-02	1.27
COMMD5	1.9E-02	1.35
JUNB	2.1E-02	1.38
SERPINE1	2.1E-02	-1.23
SRP68	2.2E-02	1.32
YWHAZ	2.3E-02	1.34
TTC21B	2.4E-02	1.35
HNRNPA1	2.4E-02	1.22
CCR7	2.4E-02	1.26
PDCD6	2.5E-02	1.19
HSP90AB1	2.5E-02	1.26
RPS11	2.6E-02	1.34
CCND2	2.6E-02	1.23
ZBTB45	2.8E-02	1.26
SIP1	2.8E-02	1.23
CTDP1	3.1E-02	1.27
ZNF34	3.1E-02	1.35
TRDMT1	3.2E-02	1.23
MFNG	3.4E-02	1.23
PSPH	3.5E-02	1.22
CECR5	3.7E-02	1.23
FYTTD1	3.8E-02	1.22
IGFBP2	3.8E-02	-1.24
PPP2R2A	3.8E-02	1.16
BIN1	4.1E-02	1.29
TERF1	4.2E-02	1.35
GNB2L1	4.3E-02	1.31
SDHA	4.4E-02	1.24

Significantly overexpressed genes from the third cluster of the heat map from Figure 3.1.

overexpressed in BRCA individuals compared to sporadic and familial individuals. 5 genes (*PECR, SOD1, HSP90AB1, RPL5,* and *SOAT1*) show highly significant overexpression, with p values of $p < 0.001$, and 26 additional genes have p values of $p < 0.01$ (Table 3.12).

We next compared gene expression in BRCA1 individuals to gene expression in all non-BRCA individuals (BRCA1 vs sporadic and familial). Using this analysis, and a p-value cut-off of $p < 0.05$, 85 genes were differentially expressed in BRCA1 vs non-BRCA individuals (Table 3.13). All expression changes, with the exception of *SERPINE1* (p= 0.0350, 1.21 fold decrease), were overexpression. As was the case with the analysis of BRCA1 individuals vs all other individuals, the *PPP2R5C* gene was the top hit (p= 0.000222, 1.5 fold increase). Next, we compared gene expression in BRCA2 individuals to gene expression in all non-BRCA individuals (BRCA2 vs sporadic and familial). Using this analysis, we found 31 genes that were differentially expressed (Table 3.14), with the vast majority (27 out of 31) of genes being overexpressed.

Analysis of Genes within 1000kb of the Telomere

Next, expression of genes within 1000 kb of the telomere was accessed in BRCA1, BRCA2, sporadic, and familial samples using previously published GEO dataset GSE6799. Unlike in the analysis of telomere and telomerase-associated genes, there is no clear pattern of clustering based on BRCA status, though clusters of overexpressed genes are more evident in some BRCA1 and BRCA2 individuals as compared to sporadic or familial individuals (Figure 3.4). Among genes in this dataset, a total of 46 genes were

Table 3.11. Significant gene expression changes in telomere and telomerase-associated genes in BRCA2 individuals vs BRCA1, sporadic, and familial.

GENE	P VALUE	FOLD CHANGE
UPF3A	7.8E-03	-1.20
MIER2	1.3E-02	-1.31
CDK5R2	1.7E-02	-1.34
PLEKHN1	2.5E-02	-1.26
PPP2R3A	2.7E-02	-1.18
CHFR	3.1E-02	-1.24
TTC21B	4.4E-02	-1.29
HES4	4.8E-02	-1.22

Table 3.12. Significant gene expression changes in telomere and telomerase-associated genes in BRCA individuals vs sporadic and familial individuals.

GENE	P VALUE	FOLD CHANGE	GENE	P VALUE	FOLD CHANGE
PECR	1.4E-04	1.25	GOLPH3L	1.6E-02	1.17
SOD1	3.0E-04	1.31	HNRNPC	1.6E-02	1.25
HSP90AB1	6.1E-04	1.35	ZNF132	1.6E-02	1.23
RPL5	8.4E-04	1.29	PDCD2	1.7E-02	1.20
SOAT1	9.5E-04	1.19	RIC8A	1.7E-02	1.26
ALDOA	1.4E-03	1.28	MINA	1.7E-02	1.17
CECR5	1.4E-03	1.31	SLC41A3	1.8E-02	1.23
MYC	1.7E-03	1.26	ATAD2	1.8E-02	1.18
SDHA	2.2E-03	1.32	YWHAZ	1.8E-02	1.29
PPP2R5C	2.5E-03	1.28	PGK1	1.9E-02	1.20
RPS11	3.3E-03	1.38	STAU1	1.9E-02	1.20
LDHA	3.3E-03	1.25	TRIM28	2.0E-02	1.30
SLC6A13	3.5E-03	1.32	DNMT3A	2.2E-02	1.20
CHMP2A	4.0E-03	1.32	DLL1	2.4E-02	1.24
VIM	4.8E-03	1.34	BLM	2.4E-02	1.14
CCND2	4.9E-03	1.25	DNAJB1	2.4E-02	1.27
BTF3	5.1E-03	1.30	SH3YL1	2.5E-02	1.25
RPS5	5.1E-03	1.31	CCR7	2.5E-02	1.20
GNB2L1	5.1E-03	1.36	ING1	2.5E-02	1.14
PSMD11	5.7E-03	1.22	XRCC5	2.6E-02	1.18
SSB	5.8E-03	1.26	TP53	2.7E-02	1.22
HNRNPA1	5.9E-03	1.23	SLC35B1	2.7E-02	1.18
IGFBP7	6.1E-03	1.22	TRIB3	2.8E-02	1.19
ADNP2	6.3E-03	1.23	FRG1	2.8E-02	1.16
PRKDC	6.6E-03	1.18	ISG15	2.8E-02	1.26
PINX1	6.9E-03	1.26	USP14	3.0E-02	1.16
PSPH	7.9E-03	1.24	NFKB1	3.2E-02	1.19
HDAC2	8.3E-03	1.19	PAK4	3.2E-02	1.17
SRP68	8.6E-03	1.30	COMMD5	3.4E-02	1.25
RPL22	8.7E-03	1.29	MORF4	3.4E-02	1.14
HSP90AA1	9.1E-03	1.20	PPP4R2	3.7E-02	1.16
CTCF	1.1E-02	1.29	MEN1	4.0E-02	1.24
PDCD6	1.2E-02	1.18	MRE11A	4.2E-02	1.15
CDK4	1.2E-02	1.21	ZNF141	4.3E-02	1.20
H2AFZ	1.2E-02	1.21	HTRA2	4.3E-02	1.26
MORF4L1	1.2E-02	1.17	PARP1	4.4E-02	1.14
ID2	1.4E-02	1.24	DNMT3B	4.5E-02	1.15
CCNG1	1.4E-02	1.19	HPRT1	4.6E-02	1.14
GAPDH	1.5E-02	1.26	XRCC6	4.7E-02	1.26
ITM2A	1.5E-02	1.20	ELN	4.8E-02	1.17
TERF2IP	1.6E-02	1.31	VIPR2	4.9E-02	1.18
RPL8	1.6E-02	1.29			

Table 3.13. Significant gene expression changes in telomere and telomerase-associated genes in BRCA1 individuals vs non BRCA individuals (sporadic and familial).

GENE	P VALUE	FOLD CHANGE	GENE	P VALUE	FOLD CHANGE
PPP2R5C	2.2E-04	1.50	RIC8A	1.6E-02	1.35
RPL5	1.1E-03	1.38	RPL22	1.6E-02	1.34
HSP90AB1	1.4E-03	1.42	SLC6A13	1.6E-02	1.33
PECR	1.9E-03	1.25	H2AFZ	1.6E-02	1.26
SOD1	2.0E-03	1.33	DNAJB1	1.8E-02	1.38
PSMD11	2.2E-03	1.34	HSP90AA1	1.9E-02	1.23
CECR5	2.8E-03	1.38	CTDP1	1.9E-02	1.31
PRKDC	2.8E-03	1.27	PDCD2	2.0E-02	1.25
CDK4	3.1E-03	1.34	MEN1	2.2E-02	1.37
RPS11	3.3E-03	1.51	ITM2A	2.2E-02	1.25
PINX1	3.3E-03	1.39	SLC35B1	2.2E-02	1.24
ISG15	3.6E-03	1.50	HNRNPC	2.2E-02	1.31
NFKB1	3.9E-03	1.35	SIP1	2.3E-02	1.25
SDHA	4.1E-03	1.39	TRDMT1	2.3E-02	1.26
CCND2	4.1E-03	1.33	BTF3	2.4E-02	1.30
HNRNPA1	4.3E-03	1.31	DNMT3A	2.4E-02	1.26
SRP68	4.9E-03	1.43	MFNG	2.6E-02	1.25
SOAT1	5.0E-03	1.20	LDHA	2.8E-02	1.23
USP14	5.0E-03	1.28	HDAC1	2.8E-02	1.31
MX1	5.7E-03	1.38	TRIB3	2.8E-02	1.25
TINF2	5.9E-03	1.53	ZNF274	2.8E-02	1.26
GNB2L1	6.1E-03	1.47	ZNF324	2.8E-02	1.28
PDCD6	6.4E-03	1.25	CHMP2A	3.0E-02	1.29
PSPH	6.6E-03	1.32	SH3YL1	3.1E-02	1.31
YWHAZ	7.8E-03	1.44	CHFR	3.1E-02	1.27
RPS5	7.9E-03	1.38	ZNF34	3.2E-02	1.37
VIM	8.1E-03	1.41	MRE11A	3.2E-02	1.21
CCR7	9.5E-03	1.32	IGFBP7	3.2E-02	1.21
PPP2CB	9.6E-03	1.40	ZNF16	3.3E-02	1.22
ALDOA	9.7E-03	1.29	TUBGCP2	3.4E-02	1.35
COMMD5	9.7E-03	1.42	ZNF141	3.5E-02	1.27
ADNP2	1.0E-02	1.28	SERPINE1	3.5E-02	-1.21
FN3KRP	1.1E-02	1.28	ECHS1	3.6E-02	1.27
MORF4L1	1.1E-02	1.23	SSB	3.7E-02	1.24
BARD1	1.2E-02	1.30	CDA	3.7E-02	1.26
MYC	1.2E-02	1.26	JUNB	4.3E-02	1.33
SLC41A3	1.3E-02	1.32	STAU1	4.3E-02	1.22
CTCF	1.3E-02	1.37	MAD1L1	4.6E-02	1.35
ID2	1.4E-02	1.32	RPL8	4.7E-02	1.30
CCND3	1.4E-02	1.28	GAPDH	4.8E-02	1.26
TERF2	1.4E-02	1.32	RGS11	4.8E-02	1.19
GOLPH3L	1.4E-02	1.23	MINA	4.8E-02	1.18
TRIM28	1.4E-02	1.42			

overexpressed in BRCA1 individuals compared to the other groups (sporadic, familial, BRCA2), with significance values of $p < 0.05$, with $p < 0.01$ for 8 genes, and $p < 0.001$ for one gene (Table 3.15). As was the case in the telomere and telomerase-associated gene dataset, the top hit was *PP2A*, and *PPP2CB*, the catalytic subunit of PP2A, was also overexpressed in BRCA1 individuals compared to all others. Next, expression profiles were compared for genes in the GSE6799 dataset among BRCA1 individuals who had developed breast cancer at time of sampling, and those who had not, and 20 genes were found to be deregulated in BRCA1 individuals with cancer compared to BRCA1 individuals without cancer (Table 3.16). Of the 20 genes, the majority (19 out of 20) were underexpressed, and one gene, *SERPINE1*, was overexpressed. As mentioned previously, SERPINE1 was one of only two genes underexpressed in an analysis of BRCA1 individuals vs sporadic, BRCA2, and familial individuals. The gene set in Table 3.16 is relatively unique in that the vast majority is underexpressed, and genes deregulated in Tables 3.15 and 3.16 only had one gene in common: *CERC5*. While *CECR5* was overexpressed in BRCA1 individuals compared to all other patient samples, the gene was significantly underexpressed in BRCA1 individuals who developed cancer compared to BRCA1 individuals who did not develop cancer.

In the BRCA2 group compared to all others, 14 genes were differentially expressed, with the majority (9 out of 14) being overexpressed (Table 3.17). The only overlap between the BRCA1 and BRCA2 groups when comparing each to all other patient samples was overexpression of *SOD1*, a superoxide dismutase. A comparison of BRCA2 individuals who developed breast cancer versus those who did not develop breast cancer yielded 14 differentially expressed genes, with 10 out of 14 being underexpressed

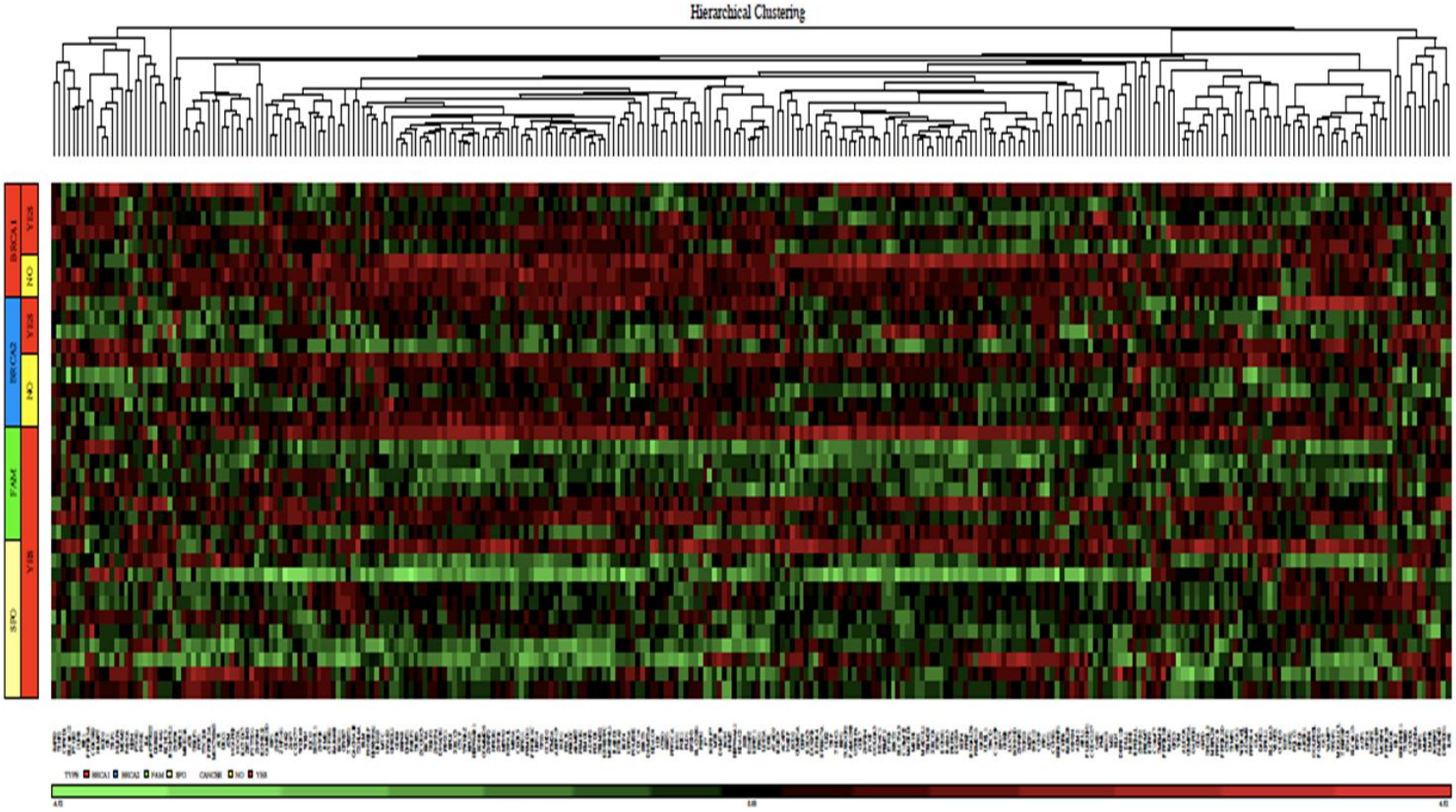


Figure 3.4. Heat map showing expression of genes from GSE6799 in all patient samples.

Table 3.14. Expression analysis from genes in GSE6799 dataset in BRCA1 individuals vs sporadic, BRCA2, and familial.

GENE	P VALUE	FOLD CHANGE
PPP2R5C	5.0E-04	1.42
ARMET	3.1E-03	1.31
ISG15	3.9E-03	1.46
MX1	4.1E-03	1.37
PSMB1	6.9E-03	1.26
PPP2CB	7.4E-03	1.37
UBE2J2	7.8E-03	1.34
TINF2	8.4E-03	1.50
USP14	9.0E-03	1.24
NFKB1	1.1E-02	1.29
PSMD11	1.1E-02	1.27
PRKDC	1.1E-02	1.21
RPS11	1.3E-02	1.36
YWHAZ	1.4E-02	1.34
PPP2R4	1.4E-02	1.30
CDK4	1.5E-02	1.25
HSP90AB1	1.5E-02	1.27
HNRNPA1	1.8E-02	1.24
FN3KRP	2.2E-02	1.24
BARD1	2.3E-02	1.26
SIP1	2.3E-02	1.24
SRP68	2.4E-02	1.30
SOD1	2.4E-02	1.21
PINX1	2.6E-02	1.27
EMG1	2.8E-02	1.21
MORF4L1	2.9E-02	1.18
CCND2	2.9E-02	1.23
TERF1	3.3E-02	1.35
SDHA	3.5E-02	1.25
CTDP1	3.5E-02	1.26
UPF3A	3.6E-02	1.16
CCND3	3.6E-02	1.22
COMMD5	3.8E-02	1.30
GNB2L1	3.9E-02	1.30
JUNB	4.0E-02	1.32
ZNF141	4.1E-02	1.24
PPP2R2A	4.3E-02	1.16
TTC21B	4.3E-02	1.29
TERF2	4.3E-02	1.25
CECR5	4.3E-02	1.23
TRIM28	4.4E-02	1.30
ZBTB45	4.5E-02	1.23
VIM	4.5E-02	1.27
RPS5	4.6E-02	1.25
DNAJB1	4.9E-02	1.27
TEP1	4.9E-02	1.24

Table 3.15. Expression analysis of genes from GSE6799 dataset comparing BRCA1 individuals who developed cancer vs BRCA1 individuals who did not develop cancer.

GENE	P VALUE	FOLD CHANGE
MFNG	4.2E-03	-1.65
NPAS2	7.0E-03	-1.51
CYP2E1	7.0E-03	-1.63
MEN1	8.9E-03	-1.84
CCR7	1.1E-02	-1.58
ZNF324	1.1E-02	-1.63
RGS19	1.1E-02	-1.66
ZNF10	1.4E-02	-1.50
ID2	1.5E-02	-1.59
ZNF34	1.8E-02	-1.79
DLL1	1.8E-02	-1.61
SH3YL1	2.3E-02	-1.62
CHFR	2.5E-02	-1.52
PSPH	2.8E-02	-1.45
SFRS17A	3.4E-02	-1.55
SERPINE1	4.1E-02	1.37
RPH3AL	4.3E-02	-1.57
FAM120B	4.7E-02	-1.61
CECR5	4.7E-02	-1.42
ZCCHC3	4.8E-02	-1.48

(Table 3.18). *CYP2E1* was underexpressed in both BRCA1 individuals with cancer and BRCA2 individuals with cancer, but no other genes were found to be commonly deregulated in comparing Tables 3.16 and 3.18. A comparison of BRCA1 individuals with cancer vs BRCA2 individuals with cancer found ten genes overexpressed in BRCA1 cancer individuals, including shelterin components *TRF2* and *POT1* (Table 3.19). Interestingly, no differences were seen in expression of the genes analyzed when comparing familial (individuals with a family history but no known BRCA1 or BRCA2 mutation) compared to all others (data not shown).

Discussion

Previous studies have correlated expression of telomere-associated genes with telomere length in cancer. One such recent study in breast cancer found an inverse relationship between expression of shelterin components TRF1, TRF2, TIN2, and POT1 and telomere length. The authors also provide evidence that shelterin components may be stimulated by mediators of stress and inflammation in cell culture models. This study used a quantitative PCR-based measurement of telomere content, focused primarily on ER+ cancers, and did not investigate the influence of BRCA status (Butler, Hines et al. 2012). Interestingly, this study found no correlation between TERT mRNA levels and telomere length in breast tumors.

Similarly, we found increased expression of several telomere and telomerase-associated genes, but not increased expression of TERT, in a comparison of BRCA1 individuals vs all other patient samples. In particular, we saw overexpression of shelterin components TIN2, TRF1, and TRF2 in BRCA1 individuals vs all other patient samples in

both a comparison using telomere-associated genes and a comparison using genes in the GSE6799 dataset. TIN2, TRF1, and TRF2 are all negative regulators of telomere length (Kim, Kaminker et al. 1999; Smogorzewska, van Steensel et al. 2000), and ingenuity pathway analysis confirmed the top pathway overexpressed in BRCA1 carriers was related to telomere length (data not shown). Among those genes related to control of telomere length, we see upregulation of PINX1, or Pin2/TRF1-interacting protein.

PINX1 is recruited to the telomere by TRF1 and is thought to act as an endogenous inhibitor of telomerase activity (Soohoo, Shi et al. 2011). Among BRCA1 carriers compared to all other patient samples, we see upregulation of a number of cyclins and members of the PI3K/Akt pathway, including NFKB1. In addition to telomere length and cell cycle regulators, we also saw upregulation of interferon stimulated gene 15 (ISG15). Lou and colleagues report ISG15 expression is regulated by telomere length, with an increase in ISG15 expression in human cell lines as telomeres shorten (Lou, Wei et al. 2009).

In contrast, BRCA2 carriers did not show deregulation of shelterin components in any comparison performed. In accessing BRCA1 carriers with cancer compared to BRCA2 carriers without cancer using genes in the GSE6799 dataset, we saw overexpression of POT1 and TIN2 in the BRCA1 carriers (Table 3.19). Generally, BRCA1 carriers showed overexpression of genes in this analysis. In a comparison of BRCA1 carriers who developed cancer compared to BRCA1 carriers who did not develop cancer, however, those who developed cancer showed almost exclusively underexpression of a particular subset of genes (Table 3.16).

Table 3.16. Significant expression changes among genes in GSE6799 for BRCA2 individuals vs all others (sporadic, BRCA1, and familial).

GENE	P VALUE	FOLD CHANGE
CDK5R2	1.0E-02	-1.37
PECR	1.7E-02	1.17
ZNF132	1.8E-02	1.27
UPF3A	2.9E-02	-1.16
PPP2R3A	3.0E-02	-1.16
MYB	3.5E-02	1.23
MYC	4.0E-02	1.21
SOD1	4.1E-02	1.18
CDKN2A	4.1E-02	-1.22
PLEKHN1	4.3E-02	-1.24
ALDOA	4.3E-02	1.20
HDAC2	4.6E-02	1.17
ATAD2	4.6E-02	1.18
CHMP2A	4.7E-02	1.23

Table 3.17. Significant gene expression changes in genes from GSE6799 among BRCA2 individuals with cancer vs BRCA2 individuals without cancer.

GENE	P VALUE	FOLD CHANGE
TUBB3	3.4E-04	1.73
DNMT3A	1.5E-03	-1.68
CCNE1	1.0E-02	1.49
UBE2J2	1.8E-02	-1.46
CDA	2.2E-02	-1.49
HBZ	2.2E-02	1.39
MYC	2.5E-02	-1.37
CDKN2A	2.5E-02	1.41
MGC70863	2.5E-02	-1.64
ING1	3.1E-02	-1.28
TXNL4A	3.3E-02	-1.55
CYP2E1	3.6E-02	-1.41
BLM	4.0E-02	-1.27
PSMD11	4.5E-02	-1.33

Table 3.18. Significant expression changes in genes in GSE6799 in a comparison of BRCA1 with cancer vs BRCA2 with cancer.

GENE	P VALUE	FOLD CHANGE
FYTTD1	1.2E-02	1.47
TEP1	1.5E-02	1.53
UBE2J2	2.2E-02	1.45
PPP2R5C	2.2E-02	1.44
POT1	2.4E-02	1.36
TINF2	2.4E-02	1.70
psiTPTE22	2.5E-02	1.34
PPP2R2A	3.6E-02	1.28
MGC70863	4.7E-02	1.55
PSMD11	4.9E-02	1.32

Our study employed a TeloTAGGG, Southern blot method of determining telomere length, which has some advantages over a quantitative PCR-based method. For instance, we are able to visualize and quantify the entire distribution of the telomere length and calculate an average length, whereas PCR-based methods are biased towards measurements of only the shortest telomeres in a sample population. This method does have its limitations, however, and gel to gel variation may be seen. In an attempt to mitigate this problem, the telomere length from each patient sample was measured 3-4 times and an average telomere length with standard deviation was calculated. Another drawback of this study is the small sample size for analysis, owing in part to the difficulty of obtaining samples from BRCA individuals. The small sample size contributed to the large degree of variability seen in telomere length within each sample grouping. In addition, the study is limited in that the normal controls used for comparison of telomere length were not from patient family members. Thus, despite efforts to control for smoking, BMI, and family history of breast cancer in the normal sample set, additional environmental influences may have played a role in determining normal telomere length. It would also have been ideal to use gene expression analysis from a true normal sample for comparison to our BRCA, sporadic, and familial patient samples. Nevertheless, this study provides important insights into how BRCA tumors differ molecularly from other breast cancer subtypes, and how those differences might contribute to telomere dysfunction and high levels of genomic instability. In addition, this work sheds light on previously uncharacterized differences between BRCA1 and BRCA2 tumors with relation to telomere and telomerase-associated genes and telomere-proximal genes.

Materials and Methods

1. Collection of patient blood samples

Following Institutional Review Board (IRB) approval (IRB # 1011003798; PI: Dr. Lida Mina) 10cc of peripheral blood was collected from 40 women (BRCA1, n=10; BRCA2, n=10; hereditary breast cancer without BRCA (FAM), n=9; and sporadic breast cancer (SPO), n=11). 5 BRCA1 and 5 BRCA2 samples were from women who had not developed cancer.

2. Acquisition of normal blood samples from Komen tissue bank

Following IRB approval (IRB # 1206009001), freeze-dried DNA extracted from peripheral blood of 10 healthy women was obtained from the Komen Tissue Bank at the IU Simon Cancer Center. DNA was resuspended in buffer AE (Qiagen, Valencia, CA) to yield a final concentration of 0.5 μg/μL and used in TeloTAGGG assays.

3. DNA extraction from peripheral blood lymphocytes

Genomic DNA was extracted from peripheral blood mononuclear cells using the DNeasy blood and tissue kit with some modifications to the manufacturer's protocol **(**Qiagen, Valencia, CA). Specifically. 400 μL of whole blood was incubated for 2 minutes at room temperature with 40 μL proteinase K and 8 μL 100 mg/mL RNase A. 400 μL of Buffer AL was added to each sample and samples were vortexed and incubated for 10 minutes at 56°C. Next, 400 μL 100% ethanol was added and each sample was mixed by vortexing. Approximately 1/4 of the mixture for each sample was applied to a DNeasy mini spin column placed in a 2 mL collection tube and spun at full speed for 1 minute. The flow

through and collection tube were discarded, and the remaining 3/4 of the mixture for each sample was applied to the same collection tube in three additional spins to obtain highly concentrated DNA samples. The remainder of the procedure was followed according to the manufacturer's protocol, and each sample was eluted in 40 μL Buffer AE. DNA concentration and quality was determined using a NanoDrop 2000 Spectrophotometer.

4. TeloTAGGG assays of telomere length

Genomic DNA was digested using *Rsa*1/*Hin*f1 to leave telomeric DNA (which do not have *Rsa*1/*Hin*f1restriction sites). Digested fragments were separated on a 0.8% agarose gel, blotted to a nylon membrane (Roche) using capillary transfer and 20 x SSC (Invitrogen) buffer. Following overnight transfer, DNA was UV-crosslinked to the membrane (Spectrolinker) and a non-radioactive DIG-labeled telomeric probe was hybridized to the membrane. The membrane was visualized using chemiluminescence.

5. Measurement of telomere length and age-adjustment

Mean TRF lengths were calculated using ImageJ software and TeloRun Excel spreadsheet program (Herbert, Hochreiter et al. 2006). Briefly, a grid of 30 boxes was positioned over each lane, and the signal intensity and size (kb) corresponding to each box was determined and averaged for each sample lane. Average telomere length was labeled on figures by drawing a line across the lanes as described previously (Hochreiter, Xiao et al. 2006).

6. Gene array analysis

Following RNA extraction and quality assessment, Illumina® Whole-Genome DASL™ microarray (Human Ref-8 BeadChips) analysis was performed. The raw data was

normalized and analyzed using Partek® Genomic Suite. Differentially expressed genes were identified using ANOVA analysis. The Indiana University Bioinformatics Core conducted gene array analysis and assisted with identification of differentially expressed genes.

7. Statistical analysis

Unsupervised hierarchical clustering was used to construct heat maps by the Indiana University Bioinformatics Core. P-values < 0.05 were considered significant.

CHAPTER FOUR

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

Personalized, targeted medicines are the preferred standard of care over harsh chemotherapies, though these options are not currently available to BRCA1 breast cancer patients. Telomerase inhibition offers a targeted treatment option that may prove less toxic than chemotherapy and radiation to most normal cells. The work presented in this thesis suggests that BRCA1 status, baseline telomere length, and expression profiles of telomere-associated and telomere-proximal genes are important factors to consider in implementing telomerase inhibitor treatment in breast cancer. We observed enhanced sensitivity of BRCA1 mutant breast and ovarian cancer cell lines to clinically relevant treatment concentrations of GRN163L, and this sensitivity correlated with shorter telomere lengths at baseline and increased expression of γ-H2AX for BRCA1 mutant versus BRCA1 wild-type cell lines.

This study is novel in that assessment of telomerase inhibitor sensitivity and telomere length as a function of BRCA1 status was conducted in established, isogenic cell line pairs. Notably, the HCC1937+BRCA1 and UWB1.289+BRCA1 cell lines were created by pooled, as opposed to clonal, selection to stably express BRCA1, thus decreasing the likelihood of selection for longer telomere length in the process of cell line establishment. In addition, using an established cell line model to study the relationship between BRCA1 and telomerase inhibition has advantages over utilizing transient overexpression or knockdown of BRCA1; transient overexpression or knockdown has the potential to alter growth kinetics and influence response to telomerase inhibitors due to

the cell cycle effects of BRCA1. Despite these challenges, it would be interesting to test the effects of GRN163L in a BRCA1-inducible, or BRCA1 targeted deletion model.

Given the differences in baseline telomere length between BRCA1 mutant and BRCA1 wild-type cell lines, it stands to reason that BRCA1 mutant cell lines require a shorter period of treatment with GRN163L to elicit telomere dysfunction. In an attempt to further investigate this possibility, rate of telomere shortening was accessed in BRCA1 mutant and BRCA1 wild-type cell lines over a three or six week period. In general, UWB1.289 and UWB1.289+BRCA1 cell lines, which had longer telomeres at baseline compared to the HCC1937 pBp and HCC1937+BRCA1 cell line pair, also showed a faster rate of telomere shortening, with rates in the UWB1.289 cell pair nearly double the rates in the HCC1937 cell line pair. Though each BRCA1 wild-type cell line showed a slightly higher rate of base pair loss per week as compared to the BRCA1 mutant cell line, this result is difficult to interpret as telomere shortening in the cell lines accessed was not a linear process. For instance, the UWB1.289 cell line initially showed telomere shortening at an accelerated rate compared to the UWB1.289+BRCA1 cell line, but showed a degree of recovery by week three that was not seen in the UWB1.289+BRCA1 cell line.

This study also investigated the molecular responses to GRN163L treatment in BRCA1 mutant and BRCA1 wild-type cell lines through accessing cell cycle changes and expression of DNA double-strand break marker γ-H2AX. Though no cell cycle changes were seen following GRN163L treatment, induction of γ-H2AX in BRCA1 mutant cells compared to BRCA1 wild-type cells was seen as early as one week following initiation of GRN163L treatment. Expression of γ-H2AX persisted in the BRCA1 mutant cell lines,

suggesting an impairment in DNA repair caused by telomerase inhibitor treatment. Though γ-H2AX is a marker of telomere dysfunction, this study did not discern whether γ-H2AX was present at the telomere, and this assessment would be important to future studies.

Despite using four different cell lines with mutations from various regions in the full-length BRCA1 gene, the contributions of specific types of mutations in determining sensitivity to GRN163L could not be delineated. One exception to this may be the observation that SUM149PT cells, which harbor an acquired somatic mutation in BRCA1, do not show sensitivity to GRN163L in clonogenic survival assay after three-week treatment (Figure A1). In contrast, the BRCA1 mutations in HCC1937 pBp and UWB1.289 cells, though from different regions of the BRCA1 gene, are pathogenic, germline mutations resulting in frameshifts and protein inactivation.

Based upon the findings presented in Chapter Two and from recent work suggesting BRCA1 carriers harbor shorter telomeres at baseline compared to other breast cancer subtypes, telomerase inhibition may be a viable treatment option for BRCA1 cancer patients, either alone or in combination with DNA damaging agents. This work demonstrates BRCA1 acts synergistically with doxorubicin, a commonly used drug in treatment of breast cancer. Thus, combination treatment with doxorubicin and GRN163L may lower the effective dose of doxorubicin required to elicit a patient response. In addition, GRN163L pretreatment for a six-week period, following by addition of cisplatin, showed a greater effect compared to cisplatin treatment alone. Thus, cells with shortened telomeres are more sensitive to cisplatin, and this result has been reported by others (Uziel, Beery et al. 2010). For this reason, treatment with telomerase inhibitors

prior to chemotherapy may minimize the treatment duration and, thus, the toxic side effects of chemotherapy. This finding has broad-ranging implications that may extend to all cancers that express telomerase.

Other homologous recombination factors that act in concert with BRCA1, including NBS1, Rad51, and BRCA1, genes with known roles in telomere maintenance, are mutated in a proportion of hereditary breast and ovarian cancers. It would be interesting to access baseline telomere lengths in these individuals and to determine sensitivity to telomerase inhibition in these genetic backgrounds. In addition, triple negative breast cancer cells have what has been described as a "BRCAness" phenotype, and may also be sensitive to telomerase inhibition. This study also raises questions about considering expression profiles of shelterin components in predicting baseline telomere length and response to chemotherapy and/or telomerase inhibition, General overexpression of telomere and telomerase-associated genes and of genes within 1000 kb of the telomere was observed in BRCA1 carriers and, to a lesser degree, in BRCA2 carriers. Notably, deregulated genes were almost exclusively overexpressed in BRCA1 individuals, and several of these genes have known roles in regulation of telomere length.

Based on the results presented in Chapters Two and Three and in studies from others, we propose a model by which shorter telomere lengths in BRCA1 mutant cancer cell lines at baseline, coupled with additional effects of BRCA1 loss or haploinsufficiency on telomere dysfunction and ability to repair DNA damage, lead to a shorter time required to cause telomere dysfunction in BRCA1 mutant cancer cell lines compared to BRCA1 wild-type cancer cell lines. Once telomere dysfunction is elicited in BRCA1 mutant cell lines, we propose that it is persistent and contributes to the high

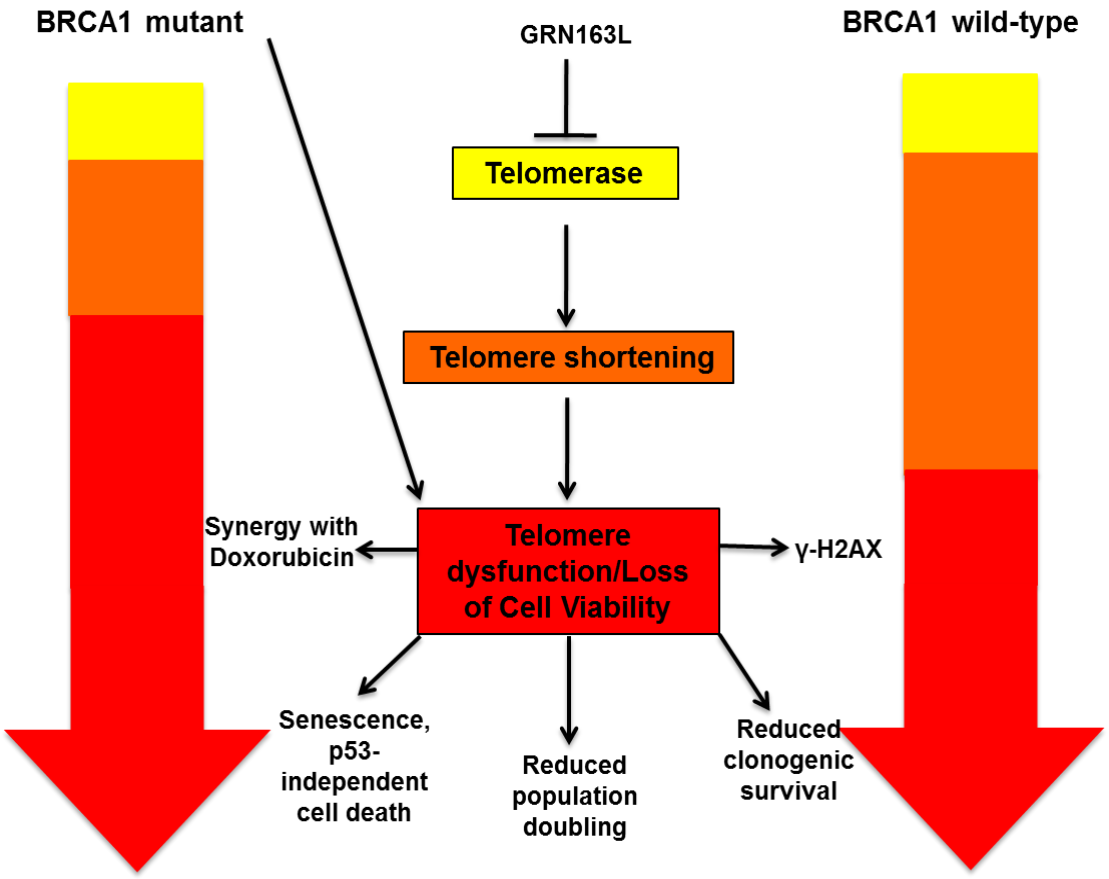


Figure 4.1. Proposed mechanism of action of GRN163L in BRCA1 mutant and wild-type cell lines.

Based on the results of this thesis, we propose a model in which BRCA1 mutant cells have shorter telomere lengths (orange) at baseline, due to overexpression of negative regulators of telomere length, and increased genomic and telomeric instability owing to the loss of the tumor suppressive function of wild-type BRCA1. This shorter telomere length (orange) makes BRCA1 mutant cells more susceptible to telomere dysfunction (red) following telomerase inhibitor treatment. In addition, since BRCA1 mutant cells are dysfunctional in the error-free repair of double strand breaks via homologous recombination, DNA damage elicited by telomerase inhibitors either persists, unrepaired, or is repaired by error-prone NHEJ, further contributing to genomic instability through facilitating formation of abnormal telomere structures.

degree of genomic instability that characterizes the BRCA1 cancer subtype (Figure 4.1). In addition, factors that may contribute to telomere dysfunction through accelerating telomeric loss are already present in BRCA1 mutant individuals (and BRCA1 carriers) as has been reported previously by others, and as the data presented in Chapter Three of this thesis suggests.

Future work should focus on further understanding the molecular responses to GRN163L in BRCA1 mutant cell lines through studying additional factors involved in telomere dysfunction, such as 53BP1. It would be interesting to determine whether the sensitivity to GRN163L seen in BRCA1 mutant cancer cell lines also occurs in BRCA1 carrier populations or in BRCA2 cancer or cancer populations. This research suggests loss of one copy of BRCA1 leads to overexpression of negative regulators in telomere length. It is tempting to hypothesize that this overexpression acts as a compensatory homeostasis mechanism to keep cell growth in check in BRCA1 haploinsufficient cells. We hypothesize wild-type BRCA1 acts as a tumor suppressor and component of telomere maintenance through two general mechanisms (Figure 4.2, *proposed mechanism*):

(1) Prior to telomere dysfunction, BRCA1 maintains a balance in expression levels of shelterin components that negatively regulate telomere length, in effect ensuring formation of the t-loop structure and protection of the telomere from DNA repair machinery; (2) BRCA1 mutation leads to overexpression of shelterin components; this imbalance tips the scales in favor of telomere shortening, resulting in telomere shortening and ultimately, telomere uncapping and activation of a DNA damage response at the telomere. Because BRCA1 is central to the error-free homologous repair of double-strand breaks, BRCA1 mutant cells attempt to repair telomere dysfunction via NHEJ,

resulting in fusion of nonhomologous chromosomes and additional chromosomal aberrations involving the telomere. Furthermore, loss of BRCA1 function leads to dysfunctional cell cycle checkpoints. In particular, BRCA1 mutant cells are unable to halt the cell cycle at the G2/M checkpoint, which is normally activated after telomere dysfunction. Additionally, BRCA1 is recruited to sites of double strand breaks by the MRN complex and is thought to be involved in generation of the single-stranded DNA required for homologous recombination (Chen, Nievera et al. 2008). It is possible that the MRN complex, which interacts with shelterin components at the telomere and is important for the formation of the single-stranded G overhang, also recruits BRCA1 to the telomere to facilitate this function (Deng, Guo et al. 2009), and this model has been proposed by others (Eliot Rosen, *in press*). In conclusion, this work supports consideration of BRCA1 status, baseline telomere length, and expression of telomere and telomerase-associated genes in individualized treatment plans in hereditary breast cancer, and also potentially in other cancer types.

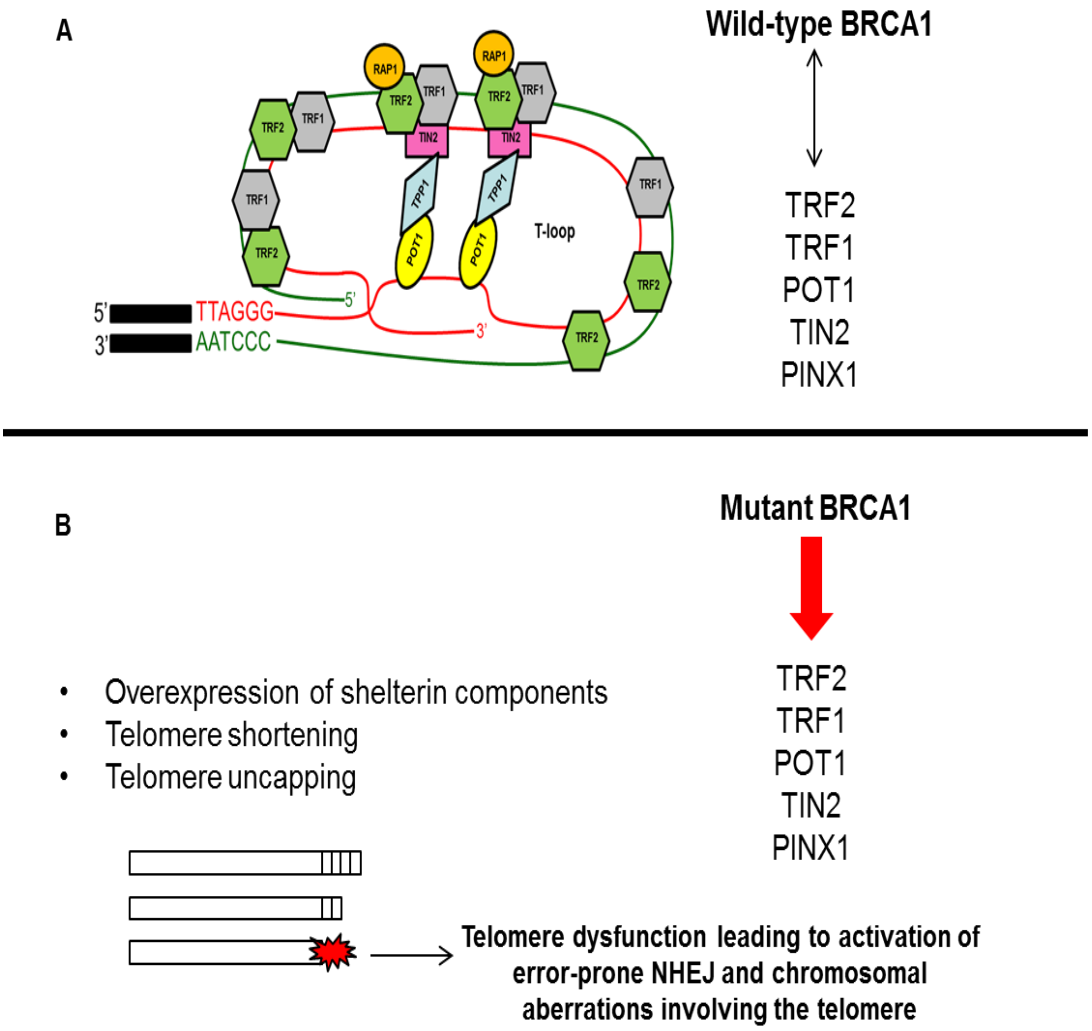


Figure 4.2. Proposed mechanism of action of BRCA1 at the telomere.
We hypothesize wild-type BRCA1 acts as a tumor suppressor and component of telomere maintenance through two general mechanisms: (A) Prior to telomere dysfunction, BRCA1 maintains a balance in expression levels of shelterin components that negatively regulate telomere length, in effect ensuring formation of the t-loop structure and protection of the telomere from DNA repair machinery. (B) BRCA1 mutation leads to overexpression of shelterin components; this imbalance tips the scales in favor of telomere shortening, resulting in telomere shortening and ultimately, telomere uncapping and activation of a DNA damage response at the telomere. Because BRCA1 is central to the error-free homologous repair of double-strand breaks, BRCA1 mutant cells attempt to repair telomere dysfunction via NHEJ, resulting in fusion of nonhomologous chromosomes and additional chromosomal aberrations involving the telomere.

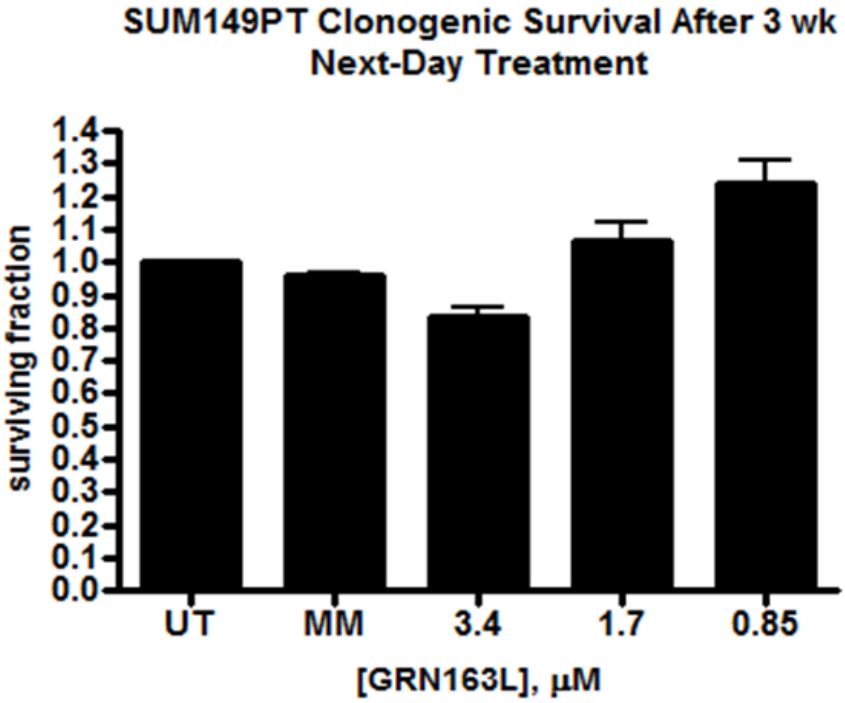


Figure A1. SUM149PT cells are not significantly affected by 3 week treatment with GRN163L.

Table A1. Sporadic individual relative information.

ID	Blood relative	Relative type	Cancer location	Age at diagnosis
1	0			
4	1	2	1	58
8	0			
9	1	3	1	82
17	0			
19	1	4		55
19		6	2	70
19		6	2	64
19		6		76
22	1	2	1	70
24	0			
33	0			
34	0			
38	0			

ID indicates the patient number assigned to each individual. A score of 0 in the Blood relative column indicates an individual with no family relative who has or has had breast or ovarian cancer, and a score of 1 indicates an individual with a blood relative who has or has had breast or ovarian cancer. In the Relative type column, 1= mother, 2= maternal grandmother, 3= paternal grandmother, 4= sister, 5= daughter, 6= maternal aunt, 7= paternal aunt, 8= maternal first cousin, 9= paternal first cousin, and 10= male blood relatives who have or have had breast cancer. In the cancer location column, 1= the relative had breast cancer in 1 breast, 2= the relative had breast cancer in both breasts, and 3= ovarian cancer. If applicable, the age of the relative's diagnosis is also given.

Table A2. Sporadic individual reproductive history.

ID	Age of menarche	# pregnancies	Age FP	HRT	Duration HRT	Currently on HRT	Type HRT
1	11	2	33	0			
4	14	2	25	0			
8	12	2	20	1	4	0	0
9	11	0		0			
17	11	3	20	1	1	0	13
19	13	1	32	0			
22	14	2	30	0			
24	12	5	31	0			
33	15	0		0			
34	13	1	32	0			
38	13	3	30	0			

ID indicates the patient number assigned to each individual. Age of menarche is the age of first menstrual period, Age FP= age of first pregnancy, a score of 0 in the HRT (hormone replacement therapy) column indicates an individual has never taken hormone replacement therapy, and 1 indicates an individual who has taken hormone replacement therapy. The Duration of HRT is listed in years, if applicable. In the Currently on HRT category, 0= not currently taking hormones, 1= currently taking hormones. In the Type of HRT column, 1= Estrogen, 2= Estrogen and Progesterone, and 13= patient is unsure of type.

Table A3. BRCA1 individual relative information.

ID	Blood relative	Relative type	Cancer location	Age at diagnosis
3	1	1	1	40
3		4	2	25
3		4	1	30
3		8	1	40
11	1	1	1	38
11		6	3	52
11		8	1	42
15	1	1	3	
15		2	1	
15		8	1	
20	1	3	1,3	53
26	1	1	1,2	38
26		3	3	56
26		6	1,2	42
26		8	1,2	34
28	1	2	1	41
28		6	1	34
28		6	3	49
28		8	2	34
30	1	1	3	55
30		2	1	59
30		3	1	76
30		6	2	32
30		6	1	54
30		7	1	41
31	1	1	2,3	51
31		4	1	49
32	1	1	2	39
32		2	2,3	
37	1	3	2	35
37		7	2	35

ID indicates the patient number assigned to each individual. A score of 0 in the Blood relative column indicates an individual with no family relative who has or has had breast or ovarian cancer, and a score of 1 indicates an individual with a blood relative who has or has had breast or ovarian cancer. In the Relative type column, 1= mother, 2= maternal grandmother, 3= paternal grandmother, 4= sister, 5= daughter, 6= maternal aunt, 7= paternal aunt, 8= maternal first cousin, 9= paternal first cousin, and 10= male blood relatives who have or have had breast cancer. In the cancer location column, 1= the relative had breast cancer in 1 breast, 2= the relative had breast cancer in both breasts, and 3= ovarian cancer. If applicable, the age of the relative's diagnosis is also given.

Table A4. BRCA1 individual reproductive history.

ID	Age of menarche	# pregnancies	Age FP	HRT	Duration HRT	Currently on HRT	Type HRT
3	13	2	24	0			
11	13	2	25	0			
15	11	2		1	4	1	1
20	13	2	21	0			
26	12	4	23	0			
28	13	2	30	0			
30	14	0	NA	0			
31	13	3	24	0			
32	12	0		0			
37	13	1	35	0			

ID indicates the patient number assigned to each individual. Age of menarche is the age of first menstrual period, Age FP= age of first pregnancy, a score of 0 in the HRT (hormone replacement therapy) column indicates an individual has never taken hormone replacement therapy, and 1 indicates an individual who has taken hormone replacement therapy. The Duration of HRT is listed in years, if applicable. In the Currently on HRT category, 0= not currently taking hormones, 1= currently taking hormones. In the Type of HRT column, 1= Estrogen, 2= Estrogen and Progesterone, and 13= patient is unsure of type.

Table A5. BRCA2 individual relative information.

ID	Blood relative	Relative type	Cancer location	Age at diagnosis
2	1	7	2	54
2		7	1	40
2		9	2	
10	1	1	1	56
10		4	1	36
12	1	1	1	54
12		2	1	26
13	13			
14	1	2	2	45
14		3	1	71
16	1	3	3	55
23	1	3	1	56
23		9	1	29
29	1	1	2	36
29		2	1	50
29		6	1	50
29		6	1	50
35	1	1	3	67
35		10	1	
36	1	3	3	
36		4	1	
36		7	1,3	
36		7	1,3	

ID indicates the patient number assigned to each individual. A score of 0 in the Blood relative column indicates an individual with no family relative who has or has had breast or ovarian cancer, and a score of 1 indicates an individual with a blood relative who has or has had breast or ovarian cancer. In the Relative type column, 1= mother, 2= maternal grandmother, 3= paternal grandmother, 4= sister, 5= daughter, 6= maternal aunt, 7= paternal aunt, 8= maternal first cousin, 9= paternal first cousin, and 10= male blood relatives who have or have had breast cancer. In the cancer location column, 1= the relative had breast cancer in 1 breast, 2= the relative had breast cancer in both breasts, and 3= ovarian cancer. If applicable, the age of the relative's diagnosis is also given.

Table A6. BRCA2 individual reproductive history.

ID	Age of menarche	# pregnancies	Age FP	HRT	Duration HRT	Currently on HRT	Type HRT
2	11	2	28	0			
10	13	0		0			
12	12	0		0			
13	12	3	27	0			
14	14	0		0			
16	13	3	23	0			
23	15	3	19	0			
29	12	4	23	1	3	1	1
35	12	4	31	0			
36	9	2	26	0			

ID indicates the patient number assigned to each individual. Age of menarche is the age of first menstrual period, Age FP= age of first pregnancy, a score of 0 in the HRT (hormone replacement therapy) column indicates an individual has never taken hormone replacement therapy, and 1 indicates an individual who has taken hormone replacement therapy. The Duration of HRT is listed in years, if applicable. In the Currently on HRT category, 0= not currently taking hormones, 1= currently taking hormones. In the Type of HRT column, 1= Estrogen, 2= Estrogen and Progesterone, and 13= patient is unsure of type.

Table A7. Familial individual relative information.

ID	Blood relative	Relative type	Cancer location	Age at diagnosis
5	1	1	2	38
5		2	3	
5		6	2	
5		6	2,3	
5		8	2	
6	1	1	2	52
7	1	4	1	35
18	1	4	1	
21	1	2	1	46
25	1	1	1,3	83,73
25		7	2	60
27	1	1	1	44
27		2	1	50
39	1	3	1	76
39		4	1	46
40	1	1	1	67
40		4	1	29

ID indicates the patient number assigned to each individual. A score of 0 in the Blood relative column indicates an individual with no family relative who has or has had breast or ovarian cancer, and a score of 1 indicates an individual with a blood relative who has or has had breast or ovarian cancer. In the Relative type column, 1= mother, 2= maternal grandmother, 3= paternal grandmother, 4= sister, 5= daughter, 6= maternal aunt, 7= paternal aunt, 8= maternal first cousin, 9= paternal first cousin, and 10= male blood relatives who have or have had breast cancer. In the cancer location column, 1= the relative had breast cancer in 1 breast, 2= the relative had breast cancer in both breasts, and 3= ovarian cancer. If applicable, the age of the relative's diagnosis is also given.

Table A8. Familial individual reproductive history.

ID	Age of menarche	# pregnancies	Age FP	HRT
5	14	6	26	0
6	12	0		0
7	13	4	24	0
18	12	2	31	0
21	13	2	28	0
25	15	3		0
27	15	2	31	0
39	13	4	34	0
40	12	2	30	0

ID indicates the patient number assigned to each individual. Age of menarche is the age of first menstrual period, Age FP= age of first pregnancy, a score of 0 in the HRT (hormone replacement therapy) column indicates an individual has never taken hormone replacement therapy, and 1 indicates an individual who has taken hormone replacement therapy. The Duration of HRT is listed in years, if applicable. In the Currently on HRT category, 0= not currently taking hormones, 1= currently taking hormones. In the Type of HRT column, 1= Estrogen, 2= Estrogen and Progesterone, and 13= patient is unsure of type.

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CURRICULUM VITAE

Elizabeth Ann Phipps

Education:

2008-2013, Ph.D. in Medical and Molecular Genetics (minor: Cancer Biology) Indiana University, Indianapolis, IN
Overall GPA: 3.95/4.00

Dissertation: *Consequences of Telomerase Inhibition and Telomere Dysfunction in BRCA1 Mutant Cancer Cells*

2002-2006, B.A. Biology, *Cum Laude* Hanover College, Hanover, IN
Overall GPA: 3.56/4.00

Related courses (Graduate level)

Biochemical Basis of Biological Processes, Molecular Biology and Genetics, Cellular Basis of Systems Biology, Experimental Design and Research Biostatistics, Biochemical and Molecular Genetics, Clinical Genetics, Human Cytogenetics, Human Population Genetics, Cytogenetics of Malignancies, Neoplastic Determinants, Cancer Signaling Gone Awry, Animal Models of Human Disease, Developmental Genetics, Gene Transfer Approaches, Molecular Cancer Genetics, Responsible Conduct of Research

Positions and Experience:

INDIANA UNIVERSITY SCHOOL of MEDICINE, DEPARTMENT of MEDICAL and MOLECULAR GENETICS, Indianapolis, Indiana
Ph.D. candidate, dissertation research in the laboratory of Dr. Brittney-Shea Herbert, April 2011-July 2013

INDIANA UNIVERSITY SCHOOL of MEDICINE, PHD GRADUATE PROGRAM Indianapolis, Indiana
Ph.D. student in the laboratory of Dr. Linda Malkas, August 2008-March 2011

PRITZKER LAB of MOLECULAR SYSTEMATICS, Chicago, Illinois
Research Assistant; Editorial Assistant June 2005-August 2005; February 2008-July 2008

ADVANCED TESTING LABORATORIES at Procter & Gamble, Mason, Ohio
Microbiologist, September 2006-January 2008

UPENN CLINICAL RESEARCH, DEPARTMENT of PSYCHOLOGY, Philadelphia, Pennsylvania
Research Intern for Dr. Stephen Kanes, January 2005-May 2005

Laboratory Skills:

- Cell culture (experience with culturing mouse embryonic fibroblasts, human mammary epithelial cells, human cancer cells) and related assays, including cell proliferation, drug screening, clonogenic survival, cell viability, drug combination experiments, and flow cytometry
- TRAP assay (measures telomerase activity levels)
- Familiar with mouse models of breast cancer
- Antibody development and peptide therapeutics
- Confocal and fluorescent microscopy
- DNA and RNA extraction (human blood and cell lines)
- Western blotting
- Southern blotting, including TeloTAGGG assays of telomere length
- Universal STELA
- PCR, Reverse transcriptase PCR, and real-time PCR (including focused gene arrays)
- DNA sequencing
- Immunofluorescence and immunostaining of cells and tissues
- Computer software- Microsoft Office, Graph Pad Prism, Calcusyn, Adobe Photoshop, ImageJ, Sequencher

Honors and Professional Memberships:

2012	Indiana University Simon Cancer Center AACR Travel Award
2011-2013	Graduate Student representative for IU Genetics Department
2010-2013	Department of Defense BCRP Predoctoral Fellowship
July 2011	Participant in telomere techniques workshop at UTSW (Dr. Shay)
2010-2013	American Association of Cancer Research, Associate Member
2002, 2004-2005	Hanover College Dean's List
2005-2006	Mortar Board Senior Honor Society
2004	Hanover College Rivers Institute Grant
2002	Alpha Lambda Delta Freshman Honor Society
2002	Hanover College Merit Scholarship
2002	Lilly Endowment Community Foundation Scholarship

Fellowships/Research Support:

BC093058 (Phipps) 05/01/2010-04/30/2014 $120,903.00
DOD CDMRP BCRP
Breast Cancer Research Program Predoctoral Fellowship
The goal is to provide support for my training in breast cancer biology and my research project on BRCA1.
Role: PI

Publications:

1. Phipps EA, Gryaznov SM, Herbert BS. BRCA1 Mutant Breast and Ovarian Cell Lines Exhibit Enhanced Sensitivity to GRN163L. (*submitted).*

2. Phipps EA, Goswami CP, Liu Y, Sledge GW, Mina LA, Herbert BS. Analysis of Telomere Length Distribution and Telomere-Associated Gene Expression Profiles of BRCA Individuals. *Manuscript in preparation.*
3. Smith SJ, Phipps EA, Dobrolecki LE, Mabrey K, Gulley P, Shen F, Dillehay KL, Dong Z, Fields G, Hickey RJ, Malkas LH. A Peptide Mimicking a Region in Proliferating Cell Nuclear Antigen (PCNA) Specific to Key Protein Interactions is Cytotoxic to Breast Cancer. (*submitted* to *Breast Cancer Research*).
4. McDaniel H, Phipps E, Goebel WS, Shen F, Yuan YC, Deng XT, Wang C, Liu Z, Warden C, Horne D, Hickey R, and Malkas L. Focused gene array analysis of DNA repair genes in human neuorblastoma cell lines. *Pediatric Blood & Cancer* 56: 964-965, 2011.
5. Summers KC, Shen F, Sierra Potchanant EA, Phipps EA, Hickey RJ, Malkas LH. Phosphorylation: the molecular switch of double-strand break repair. Int J Proteomics. 2011; 2011:373816; PMCID: PMC3200257.
6. Snider B M, Phipps EA, Smith SJ, Herbert B-S, Hickey RJ, Malkas LH. (December 2009) DNA Replication: Mammalian. In: ENCYCLOPEDIA OF LIFE SCIENCES. John Wiley & Sons, Ltd: Chichester http://www.els.net/ [DOI: 10.1002/9780470015902.a0001041.pub2].

Conferences Attended:

1. *AACR San Antonio Breast Cancer Symposium*, San Antonio, TX, 2012.
2. *Diseases of Aging Keynote Symposia*, Tokyo, Japan, 2012.
3. *American Association of Cancer Research Annual Conference,* Chicago, IL, 2012.
4. *DOD Era of Hope Conference*, Orlando, FL, 2011.
5. *Buffalo DNA Replication and Repair Meeting*, Buffalo, NY, 2010.
6. *American Association of Cancer Research Annual Conference,* Washington, DC, 2010.
7. *Amelia Project Annual Cancer Research Conference*, Indianapolis, IN, 2009, 2010, 2011.

Oral Presentations:

1. Consequences of Telomerase Inhibition and Telomere Dysfunction in BRCA1 Mutant Cancer Cells. St. Jude Children's Hospital Postdoctoral Candidate Seminar, Memphis, TN, May 2013.
2. Consequences of Telomerase Inhibition and Telomere Dysfunction in BRCA1 Mutant Cancer Cells, Rush University Postdoctoral Candidate Seminar, Chicago, IL, April 2013.
3. Consequences of Telomerase Inhibition and Telomere Dysfunction in BRCA1 Mutant Cancer Cells. IU Medical and Molecular Genetics Department Seminar, Indianapolis, IN, April 2013.
4. Investigating the Role of BRCA1 in Sensitivity to Telomerase Inhibition. IU Simon Cancer Center AACR Travel Award Recipient Seminar, Indianapolis, IN, June 2012.
5. TRAP (Telomeric Repeat Amplification Protocol). Department of Medical and Molecular Genetics Technical Skills Seminar, Indianapolis, IN, August 2011.

6. Loss of DNA Replication Fidelity in Cancer: The "Mutator Phenotype" Hypothesis Revisited. IU Medical and Molecular Genetics Department Seminar, Indianapolis, IN, November 2010.
7. Phylogeography of *Enhydris enhydris* and *Enhydris plumbea*. Butler Undergraduate Research Conference, Indianapolis, IN, April 2006.
8. Phylogeography of *Enhydris enhydris*. Hanover College Bio-Day, Hanover, IN, April 2006.

Poster Presentations:

1. Investigating the Role of BRCA1 in Sensitivity to Imetelstat, a Telomerase Template Antagonist. *AACR San Antonio Breast Cancer Symposium,* San Antonio, TX, December 2012.
2. Investigating the Role of BRCA1 in Sensitivity to Imetelstat, a Telomerase Template Antagonist. *Aging and Diseases of Aging Keynote Symposia,* Tokyo, Japan, October 2012.
3. Investigating the Role of BRCA1 in Sensitivity to Imetelstat, a Telomerase Template Antagonist. *American Association of Cancer Research Annual Conference,* Chicago, IL, April 2012.
4. Investigating the Role of BRCA1 in Sensitivity to Imetelstat, a Telomerase Template Antagonist. *Department of Medical and Molecular Genetics Annual Research Symposium*, Indianapolis, IN, September 2011.
5. Investigating the Role of BRCA1 in Sensitivity to Imetelstat, a Telomerase Template Antagonist. *DOD Era of Hope Conference*, Orlando, FL, August 2011.
6. Evaluating the Therapeutic Potential of a Peptide Segment of Cancer-associated PCNA in DNA Repair Deficient Breast Cancers. *Department of Medical and Molecular Genetics,* Indianapolis, IN, September 2010.
7. Evaluating the Therapeutic Potential of a Peptide Segment of Cancer-associated PCNA in DNA Repair Deficient Breast Cancers. *Buffalo DNA Replication and Repair Meeting,* Buffalo, NY, June 2010.
8. Evaluating the Therapeutic Potential of a Peptide Segment of Cancer-associated PCNA in DNA Repair Deficient Breast Cancers. *American Association of Cancer Research Annual Conference,* Washington DC, April 2010.
9. Evaluating the Therapeutic Potential of a Peptide Segment of Cancer-associated PCNA in DNA Repair Deficient Breast Cancers. *Department of Medical and Molecular Genetics Annual Research Symposium,* Indianapolis, IN, September 2009.
10. A Peptide Antibody Directed Against Cancer-associated PCNA Expressed in Breast Cancer Has Therapeutic Potential. *Amelia Project Cancer Research Conference*, Indianapolis, IN, February 2009.