Title
A role for the BLM RecQ helicase in efficient telomere replication

Permalink
https://escholarship.org/uc/item/3rb019hx

Author
Barefield, Colleen (Naeger)

Publication Date
2011

Peer reviewed|Thesis/dissertation
A role for the BLM RecQ helicase in efficient telomere replication

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biology

by

Colleen (Naeger) Barefield

Committee in Charge,
Professor Jan Karlseder, Chair
Professor Amy Kiger
Professor Richard Kolodner
Professor Vicki Lundblad
Professor Jean Wang
Professor Yang Xu

2011
Copyright
Colleen Barefield, 2011
All rights reserved.
The dissertation of Colleen Marie (Naeger) Barefield is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

________________________________________

________________________________________

________________________________________

________________________________________

________________________________________

Chair

University of California, San Diego
2011
First and foremost, I wish to dedicate this thesis to my family, who has been an un-ending source of love, support, and encouragement. I am constantly amazed by their acceptance of my seemingly endless academic endeavors, even though my aspirations have kept me away from them for so long. I can’t find the words to convey how much you have meant to me; you are each truly a part of this work.

And I dedicate this thesis to Adrian, who has provided love and support from the other side of the world.
# Table of Contents

Signature Page.............................................................................................................. iii

Dedication Page ........................................................................................................... iv

Table of Contents ........................................................................................................ v

List of Abbreviations .................................................................................................. vii

List of Tables ............................................................................................................... viii

List of Figures ............................................................................................................. ix

Acknowledgements ..................................................................................................... xi

Vita.................................................................................................................................... xiv

Abstract of the Dissertation ........................................................................................ xv

Chapter 1. Introduction ............................................................................................... 1

  - Chromosome end maintenance and genomic stability ......................................... 1
  - Telomere structure and length maintenance ......................................................... 4
  - Shelterin complex and chromosome end protection .............................................. 7
  - Telomeres suppress DNA damage recognition .................................................... 10
  - DNA damage and repair activation at dysfunctional telomeres ........................... 13
  - WRN RecQ helicase and telomere maintenance .................................................. 17
  - BLM RecQ helicase in genome maintenance ....................................................... 20
  - Telomeres as fragile sites ..................................................................................... 25
  - Summary ................................................................................................................ 26

Chapter 2. The BLM RecQ helicase contributes to telomere maintenance in mammalian fibroblasts ........................................................................................................ 27

  - Background ............................................................................................................ 27
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Results</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Acknowledgements</td>
<td>73</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>BLM RecQ helicase recruitment to dysfunctional telomeres</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Background</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>103</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Conclusions</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>111</td>
</tr>
</tbody>
</table>
List of Abbreviations

WRN – Werner RecQ helicase
WS – Werner's syndrome
BLM – BLM RecQ helicase
BS – Bloom's syndrome
IF-FISH – Immunofluorescence-Fluorescence in situ hybridization
TD – Telomere Defects
STL – Sister telomere loss
TFEs – Telomere free ends
UFBs – Ultra fine bridges
LRIs – Late replicating intermediates
HR – Homologous Recombination
NHEJ – Non homologous end-joining
HJ – Holliday junction
List of Tables

Table 1-1. RecQ helicases in mammalian cells ...............................................................18

Table 2-1. Features of 'normal' IMR90 and Bloom’s syndrome fibroblast cell lines obtained from Coriell Laboratories .................................................................38

Table 2-2. Telomere defects and anaphase bridge formation are elevated in Bloom's syndrome fibroblast cell lines .................................................................44

Table 2-3. Quantification of chromatids with telomere defects and DAPI positive anaphase bridges in IMR90-E6E7 shBLM and GM02548-E6E7 BS cells expressing full length BLM .................................................................50

Table 3-1. Telomere defects in BLM and TRF1-deficient fibroblast cells ...........95
List of Figures

Figure 1-1. Telomere dysfunction contributes to genome instability ..................3

Figure 1-2. Telomere structure ........................................................................9

Figure 1-3. Telomeres and DNA damage suppression .................................12

Figure 1-4. Possible mechanisms of formation of late-replicating intermediate structures (LRIs) and ultra-fine bridges (UFBs) ..................................................24

Figure 2-1. Predicted for model for WRN activity in efficient replication of the telomeric lagging strand ..........................................................30

Figure 2-2. Characterization of purified BLM antibody ................................34

Figure 2-3. BLM is detected at a subset of telomeres in a cell-cycle dependent manner ..........................................................................................35,36

Figure 2-4. Telomere defects and anaphase bridges in Bloom's syndrome and IMR90 fibroblast cell lines .............................................................41

Figure 2-5. Expression of the catalytic subunit of telomerase, hTERT, reduces chromosome fusions and telomere defects in BS cells .......................46

Figure 2-6. Expression of hTERT does not suppress genomic fusion events induced by irradiation ..........................................................47

Figure 2-7. Telomere defects and DNA anaphase bridge formation in IMR90-E6E7 shblm and GM02548-E6E7+BLM fibroblasts ........................................49

Figure 2-8. Loss of leading and lagging strand telomeres in BLM-deficient HeLa cells ..........................................................................................52

Figure 2-9. DNA damage foci and telomere damage-induced foci (TIFs) are elevated in BS fibroblasts ........................................................55, 56

Figure 3-1. Loss of both BLM and WRN RecQ helicases exacerbates telomere defects ..........................................................................................78

Figure 3-2. BLM shows distinct localization patterns between IMR90 and WS fibroblasts ..........................................................80
Figure 3-3. Ultra-fine bridges (UFBs) occur in normal fibroblast cell lines, but are more frequent and persist longer in AG05229 WS fibroblasts ..........................82, 83

Figure 3-4. A subset of Ultra fine bridges (UFBs) extend at one or both ends from telomeric regions ...........................................................................................................84

Figure 3-5. Expression of WRN in AG05229 cells reduces the frequency of UFB formation and suppresses UFB formation at telomeres .................................86

Figure 3-6. Treatment with aphidicolin induces changes in BLM localization patterns..........................................................................................................................88

Figure 3-7. Treatment with aphidicolin increases UFB formation..................90

Figure 3-8. TRF1 knockdown increases frequency of UFB formation.........93

Figure 3-9. TRF1 knockdown in IMR90-E6E7 and AG05229-E6E7 fibroblast cell lines increase telomere defects in metaphase chromosomes.........................94

Figure 4-1. Model proposing a role for BLM in processing late replication intermediates that form at telomeres.................................................................111
Acknowledgements

I am grateful for the financial support provided by the Cellular and Molecular Genetics Training Grant awarded to the University of California, San Diego. Funding from the CMG training grant supported the first three years of my graduate career. Also, I thank the Aileen S. Andrew Foundation for contributing to my research stipend in the final years of my doctoral education.

I am extremely grateful to the co-authors on the published article in PNAS, Laure Crabbe, Anna Jauch, and Heidi Holtgreve-Greve, for their significant contributions in the WRN helicase project. Figure 2-6 was re-printed directly from our publication:


Thank you to my outstanding thesis committee, for contributing their valuable time and experience to my graduate training and to this thesis project. They have always steered me in the most productive direction, and I greatly appreciate their support.

I would also like to thank all the former and current members of the Karlseder laboratory. It has been an honor and a pleasure working with each of these amazing scientists, and I can’t imagine a better group of people to work with. They have graciously shared their valuable experience and expertise.
whenever I have need of it, and provided constructive suggestions and critiques on the focus of my thesis work.

I particularly want to acknowledge our lab manager, Candy Haggblom. I appreciate her meticulous organization, attention to detail, cleverness, incredible technical experience, and unending patience. Because of her efforts, the Karlseder lab is a remarkably well-run unit; she is irreplaceable.

I would like to recognize two former post-doctoral researchers, Ramiro Verdun and Marcela Raices. Ramiro’s mentorship was invaluable, particularly during the initial stage of the BLM RecQ helicase project. He provided sound guidance on the day-to-day issues of experimental design, troubleshooting, and understanding basic science. But Ramiro also taught me some of the most important lessons about the scientific process, how to ask a ‘good question’, and how to keep it all in perspective. Thank you to Marcela Raices for her expert technical advice. She is an amazing, generous scientist and readily provided detailed suggestions for almost any protocol.

Laure Crabbe was in the process completing her graduate studies when I first joined the Karlseder lab. We only worked together a few months before she left and in that short time I acquired a significant amount of relevant knowledge, such as the techniques I would use constantly for the next six years. I took up where she left off on the RecQ helicase project. The protocols, cell lines, plasmids, and preliminary data were of exceptional quality and provided an excellent base on which to build my own thesis.
Perhaps the most important people I wish to thank are the current members of the Karlseder lab; I could not ask for a more skilled, engaging group of colleagues. These wonderful people have made it so much fun to come into work every day: Roddy O’Sullivan, Makoto Hayashi, Daniel Lackner, Tony Cesare, Teresa Rivera-Garcia, and Liana Oganesian. Thank you to Liana Oganesian… partly for providing technical assistance on in vitro activity assays, but mostly for her outstanding pep talks.

I offer my deepest appreciation to my thesis advisor, Professor Jan Karlseder. In five and half years in his lab, I have learned more than I thought possible. I am grateful to him for offering so many remarkable opportunities to meet amazing individuals and expand my life experiences. The knowledge I have gained under his mentorship will surely serve me for the rest of my scientific career and beyond.

And finally, this thesis is absolutely made possible by my amazing friends. There were long hours, stresses and frustrations, but their humor, support, and commiseration truly made my graduate career a wonderful experience.
Vita

2005-2011: Ph.D. in Biology, Division of Biological Sciences, University of California, San Diego. Supported by the Cell and Molecular Genetics Training Grant.

2007-2010: Teaching Assistant, University of California, San Diego

2003-2005: Research Technician at Washington University School of Medicine and St. Louis Children’s Hospital

2004-2005: Washington University- St. Louis (St. Louis, MO). No degree awarded.

2001: Research Assistant, Pacuare Nature Reserve, Limón province, Costa Rica

1999-2003: B.A. in Biology, Grinnell College (Grinnell, Iowa).

Publications


Abstract of the Dissertation

A role for the BLM RecQ helicase in efficient telomere replication

by

Colleen Marie (Naeger) Barefield

Doctor of Philosophy in Biology

University of California, San Diego, 2011

Professor Jan Karlseder, Chair

Two cancer predisposition disorders observed in humans, Werner syndrome (WS) and Bloom syndrome (BS), are caused by loss of function of the RecQ helicases WRN or BLM, respectively. BS and WS are characterized by replication defects, hyper-recombination events and chromosomal aberrations, which are hallmarks of cancer. Our lab has shown how inefficient replication of
the G-rich telomeric strand contributes to chromosome aberrations in WS cells, demonstrating a link between WRN, telomeres and genomic stability. Here I provide evidence that the BLM RecQ helicase also contributes to chromosome-end maintenance. Chromosome fusions and telomeric defects are elevated in fibroblasts lacking BLM; these aberrations are suppressed by hTERT-mediated telomere elongation, confirming a telomere-specific dysfunction in BS cells. Examination of metaphase chromosomes showed that telomere defects are significantly greater in BLM-deficient cells, similar to observations in cells lacking a functional WRN helicase. Furthermore, loss of both helicases exacerbates telomere defects and chromosome aberrations, indicating that BLM and WRN function independently in telomere maintenance. Fluorescence analysis revealed that BLM localization, particularly its recruitment to telomeres, changes in response to replication dysfunction. WRN deficiency or aphidicolin exposure led to an increase in late-replicating intermediates, observed as BLM-positive ultra-fine bridges (UFBs), particularly UFBs originating from telomeric DNA. I propose that the BLM RecQ helicase (likely in complex with the proteins TOPOIIIα, RMI1, RMI2) contributes to telomere maintenance, though most likely in a capacity distinct from WRN RecQ helicase.
Chapter 1. Introduction

Chromosome end maintenance and genomic stability

Maintenance of genome stability is an essential principle that is absolutely necessary for cell viability, efficient function and regulation of cellular pathways, and ultimately, for organismal health. A single DNA damage event can interrupt cell progress through the essential process of cell division, potentially compromise genome integrity, or lead to cell death. DNA breaks occur throughout the mammalian genome, but the linear ends of the 46 chromosomes in mammalian cells are highly susceptible to degradation and damage (McClintock 1941). Complex and specialized structures, the telomeres, ‘cap’ the end of each chromosome arm, preventing fusions and inappropriate recombination (Blackburn 2001).

In mammals, telomeres are composed of non-essential nucleotide repeats and associated proteins. The linear termini of chromosomes are protected by maintaining telomere length and by carefully regulating the diverse array of proteins that contribute to telomere replication and processing (reviewed in Palm and de Lange 2008). Chromosome ends with dysfunctional or critically short telomeres are recognized as DNA breaks and become substrates for fusion events (McClintock 1941). A single exposed DNA end is sufficient to rapidly trigger a finely tuned cellular response that ceases mitotic processes and repairs any damage in the genome (Hemann et al., 2001). The proteins that act in DNA damage and repair pathways are employed at multiple types of DNA lesions.
Indeed, damaged or shortened telomeres are processed by a subset of the same response factors that mediate repair of internal DNA double strand breaks (DSBs) (Karlseder et al., 2002; Smogorzewska et al., 2002; d'Adda di Fagagna et al., 2003).

The first responders to deprotected telomeres are DNA damage sensors that activate Homologous Recombination (HR) DNA repair pathway or – more commonly – the Non-homologous end-joining (NHEJ) pathway (Smogorzewska et al., 2002; Dimitrova et al., 2009). NHEJ is more frequently employed in chromosome end maintenance and this DNA damage response mends DNA lesions by fusing chromosome ends. Inappropriate fusions lead to inter-chromosomal breaks in subsequent mitotic cycles, regenerating the double strand breaks and repeating the fusion event. Random breaks in the genome may abolish oncogenic suppressors or, alternatively, activate tumorigenic factors. Therefore, maintaining functional telomeres is crucial for suppressing damage signaling and inappropriate chromosome- or chromatid-type fusions (Smogorzewska, et al., 2002) (Figure 1-1).
Figure 1-1. Telomere dysfunction contributes to genome instability. When one sister telomere or both telomeres are critically shortened, they are detected as DNA damage and processed by NHEJ or HR. Shown here are three types of chromosome fusion events: (Far left) Chromatid-type fusion between two de-protected sister chromatids. (Center image) chromosome-type fusions between two chromosomes with telomere free ends. (Far right) Sister fusion between two de-protected sister chromatids.
Telomere structure and length maintenance

Efficient chromosome end protection requires that the telomeric nucleotide repeat sequence end with a conserved single stranded overhang, and be kept at sufficient length. The G-rich telomeric lagging strand terminates with a single stranded 3’ protrusion (Makarov et al., 1997; Wright et al., 1997). This G-tail is a small, but important, telomeric feature that enhances chromosome end protection by invading the intra-telomeric duplex, and hiding the terminal DNA overhang (see Figure 1-2). The single stranded G-tail forms a displacement loop (D-loop) when it invades homologous sequence; the structural consequence is a larger telomeric loop (t-loop). Electron microscopy has enabled researchers to visualize t-loops at a subset of chromosome ends (Griffith et al., 1999). The precise function of the t-loop remains unknown, but it is predicted to be a protective mechanism that sequesters the telomere terminus from DNA damage recognition factors. The t-loop must be ‘opened’ over the course of each cell cycle so replication machinery can copy the full telomere length. Another critical function of the G-tail is in telomerase-mediated elongation. For enzymatic addition of telomere repeats, telomerase requires access to the single stranded 3’ overhang (Kelleher et al., 2005).

In addition to physical protection, the telomeres are a buffer at chromosome ends that prevent loss of genetic information after each mitotic cycle. Telomere length is maintained by telomerase, a specialized ribonucleoprotein complex, which employs a reverse transcriptase activity to
specifically elongate telomere ends by enzymatic addition of TTAGGG repeats. (Greider and Blackburn, 1985). The core components of telomerase are hTERT, the catalytic protein that enzymatically extends the telomere terminus, and mTERC, the highly conserved and complex RNA structure. mTERC contains the RNA telomere sequence, which serves as template for hTERT to extend telomere repeats at chromosome ends (Greider et al., 1989). mTERC also contains a number of regions/sequences that participate in protein interactions (Mitchell et al., 1999; Chen et al., 2000; Zhu et al., 2004). A growing number of associated proteins are required for assembly and localization of the telomerase complex, which is now known to form multiple, distinct protein complexes that likewise engage in distinct cellular pathways, such as transcription regulation (Cohen et al., 2007; Venteicher et al., 2008; Venteicher et al., 2009).

Telomerase expression is repressed in somatic cells, but when active, telomerase prevents genomic instability resulting from telomere loss; this bypass of damage-induced senescence can lead to immortalization. Active telomerase is a marked feature of cancer cells and is present in around 90% of human tumors (Bartuch and Lundblad 2006). However, some tumors and immortalized cells maintain telomere length through a telomerase-independent process known as alternative lengthening of telomeres (ALT). This complex mechanism elongates telomeres by homologous recombination and copy switching, which results in extreme variation in telomere length (Dunham et al., 2000).
Somatic cells experience gradual telomere erosion over accumulated mitotic divisions (Harley et al., 1990); this ultimately restricts cellular lifespan, since critically short telomeres trigger cell cycle arrest or cell death (Hayflick et al., 1965). An intrinsic consequence of replicating a linear genome is the ‘end protection problem,’ which describes the inability of conventional polymerases to fully maintain the length of a linear DNA template, leading to shortening of chromosome ends during each mitotic cycle (Olovnikov et al., 1973; Watson et al., 1972). The predicted nucleotide loss should be between six to nine bases, due to the removal of the RNA primer at the final Okazaki fragment (Olovnikov et al., 1973). Additional enzymatic processing to generate the 3’ overhang after each replicative cycle appears to cause an actual loss of approximately 100-200 basepairs of total telomere length (Makarov et al., 1997; Huffman et al., 2000).

Therefore, as population doublings accumulate telomeres become critically short and no longer efficiently ‘cap’ the chromosome termini; these damaged ends trigger cell cycle arrest or cell death (Harley et al., 1990; Hayflick et al., 1965; d’Adda di Fagagna et al., 2003; Lundblad and Szostak, 1989; O’Sullivan et al., 2010). In fact, one short telomere is sufficient to trigger cell cycle arrest (Hemann et al., 2001). Critically shortened telomeres become substrates for DNA damage and repair, ostensibly because the reduced binding sites lead to insufficient levels of Shelterin proteins for suppressing damage recognition factors (Loayza et al., 2003; Smogorzewska et al., 2000).
Shelterin complex and chromosome end protection

Mammalian telomeres are composed of approximately 9-15 kilobases of TTAGGG repeats, which are bound by a core group of telomere-specific proteins and a growing number of non-specific, telomere-associated proteins. Shelterin, the core protein complex constitutively associated with telomeric DNA, is composed of TRF1, TRF2, TIN2, RAP1, POT1 and TPP1 (reviewed in Palm and de Lange, 2008). These proteins are necessary for proper chromosome end protection and act inter-dependently to regulate telomere length and processing (de Lange et al., 2005) (Figure 1-2).

TRF1 (telomere repeat binding factor 1) and TRF2 (telomere repeat binding factor 2) are constitutively bound to the double-stranded telomeric repeat regions. TRF1 is thought to promote efficient telomere replication, particularly by recruiting necessary factors that assist with unwinding of the parent strand and facilitate replication fork progression (Sfeir et al., 2009). TRF2 is a crucial component in telomere stability and chromosome end protection (van Steensel et al., 1998), and TRF2 forms an essential complex with RAP1 (repressor/activator protein 1) (Bae and Baumann 2007; Sarthy et al., 2009). TRF1 and TRF2 are equipped to bind – or recruit by secondary interactions – an ever-growing list of proteins that contribute in some capacity to telomere maintenance.

TIN2 (TRF2- and TRF1-interacting nuclear protein) provides structural stability to the Shelterin complex. TIN2 binds both TRF1 and TRF2, then directly links to TPP1, another protein that maintains structure and stability in this
complex. POT1 (protection of telomeres 1) is the only Shelterin protein that occupies the single-stranded, telomeric, 3’ overhang (Baumann and Cech 2001). TPP1 connects POT1 and TIN2 (via TIN2-TPP1-POT1), thus tethering the single-stranded protein to the rest of the Shelterin complex (reviewed in Palm and de Lange, 2008) (Figure 1-2). Human POT1 localization at the chromosome terminus is required for telomere length and overhang length maintenance (Loayza et al., 2003); it exhibits binding affinity to the telomeric lagging strand 3’ overhang, minimally requiring the DNA sequence: 5’-TAGGGTTAG-3’ (Loayza et al., 2004).
Figure 1-2. Telomere structure. Top image is a single metaphase chromosome, where DNA is stained with DAPI (red) and telomeres are visualized with a fluorescent telomeric oligonucleotide (blue). The basic structure of the telomeric DNA is shown on top, with emphasis on the G-rich single stranded overhang. This G-tail has particular importance in telomeric structure, since it is required to invade homologous intra-telomeric sequence, forming a displacement loop (d-loop). The resulting structure, the telomeric loop (t-loop), is a secondary mechanism for protecting the chromosome end from DNA damage factors. Shown in the box is an individual ‘unit’ of the six proteins that make up the Shelterin complex.
**Telomeres suppress DNA damage recognition**

An essential feature of telomeres, which ensures chromosome end protection and thus genome integrity, is suppression of DNA damage responders. Telomeric tracts accommodate telomere-associated proteins, such as the Shelterin components, which are critical for preventing ATM or ATR mediated DNA damage responses (Karlseder et al., 1999; Lazzerini Denchi et al., 2007; Dimitrova et al., 2009). Telomere binding by the TRF2/RAP1 protein complex protects telomeric sequence from the DNA damage responder, ATM, and thus suppresses activation of NHEJ (Karlseder et al., 1999; Celli and de Lange, 2005; Lazzerini Denchi et al., 2007; Sarthy et al., 2009). TRF2 presence at chromosome ends is critical for end protection; in fact, even cells with well-maintained telomere length, but a dysfunctional TRF2 protein, exhibit a dramatic increase in fusion events between telomeres at chromosome ends (Karlseder et al., 1999, 2004; Konishi and de Lange 2008). POT1 particularly suppresses ATR activation, which most commonly triggers HR-mediated repair, but ATR has also been shown to activate non-homologous end-joining repair (Lazzerini Denchi et al., 2007).

De-protected chromosome ends are recognized by the DNA damage machinery, which signals through the p53 and RB1-dependent pathways to induce senescence, cell cycle arrest, or apoptosis (Karlseder et al., 2002). Although the downstream repair pathways (non-homologous end-joining and homologous recombination) employ distinct proteins, the DNA damage signals
are propagated by the same responders; both ATM and ATR activate phosphorylation of the histone, γH2AX, and recruit the mediator, 53BP1 (Takai et al., 2003; d’Adda di Fagagna et al., 2003).

Telomere damage is accurately assessed by localization of DNA damage responders to telomeres (Takai et al., 2003; d’Adda di Fagagna et al., 2003). In fact, these events are cytologically identified by immunofluorescence against proteins activated during a DNA damage response, such as the modified histone γH2AX, and downstream mediators 53BP1 and MDC1. Telomere damage induced foci (or TIFs) are visualized by accumulation of these proteins at telomeric signal (Takai et al., 2003; d’Adda di Fagagna et al., 2003).
Figure 1-3. Telomeres and DNA damage suppression. (A) A single unit of Shelterin, which is composed of six proteins constitutively found at chromosome ends. (B) TRF2 (and RAP1) are necessary to suppress ATM–mediated DNA damage response. If ATM recognizes unprotected telomeres, it recruits the responder complex, MRN (Mre11-RAD50-Nbs1) and triggers accumulation of the 53BP1, MDC1 and triggers accumulation of the 53BP1, MDC1 and γH2AX DNA damage markers. This ATM-mediated response activates the primary repair proteins that are necessary NHEJ, such as Ligase IV and Ku70/Ku80. (C) POT1 must bind the complete length of single stranded telomeric DNA to prevent an ATR-mediated DNA damage response. Though the proteins that facilitate DNA repair by homologous recombination are distinct from those in NHEJ, ATR also triggers accumulation of the DNA damage markers, 53BP1, MDC1, and γH2AX.
DNA damage and repair activation at dysfunctional telomeres

The DNA damage cascade activated at dysfunctional telomeres is remarkably similar to the response to ‘internal’ double stranded DNA breaks (DSB response) (Takai et al., 2003; d’Adda di Fagagna, et al., 2003). However, telomeres recognized as DSBs can induce two mechanisms of DNA repair: non-homologous end joining (NHEJ) and homologous recombination (HR). There are a number of similarities between these mechanisms, particularly in the factors that mediate DNA damage recognition and signaling (Takata et al., 1998). For example, both NHEJ and HR are first triggered by ATM or ATR-regulated signaling pathways, and thus activation of either pathway induces the same downstream targets, such as phosphorylated $\gamma$H2AX, MDC1, and 53BP1 (Takai et al., 2003; Dimitrova and de Lange, 2006; Lazzerini Denchi and de Lange 2007) (see Figure 1-3).

Although there is some overlap in protein activity, these two distinct strategies for DNA repair employ very different groups of proteins. Non-homologous end-joining employs the heterodimer, Ku70/Ku80, and DNA Ligase IV/XRCC4, which covalently joins two exposed double stranded DNA ends (for overview see (Critchlow et al., 1998; Correo et al., 2007; Lazzerini Denchi and de Lange 2007). Efficient homologous recombination in mammalian cells is more complex and requires a greater number of players. A few of the extensively studied HR factors that facilitate double strand break repair are the proteins involved in the initial recognition and processing, such as Mre11, Nbs1, Rad50.
(MRN complex) (reviewed in Jackson and Bartek, 2009), CtIP (Sartori et al., 2007; Chen et al., 2008), the helicase/nuclease complex BLM/Dna2, and the exonuclease, Exol (Nimonkar et al., 2008; Nimonkar et al., 2011). The enzymatically generated overhang is coated with the single stranded binding protein, RPA, and then RAD51 recruitment produces nucleoprotein filaments, which catalyze strand exchange ((reviewed in Yuan et al., 1999); San Filippo et al., 2008; Jackson and Bartek, 2009). Additional factors that are known to facilitate these initial steps include RAD52, BRCA1, and BRCA2; their precise contributions to this process have only recently emerged (Wong et al., 1997; Moynahan et al., 1999; Yuan et al., 1999; Yang et al., 2002; Carreira et al., 2009). There is some variation in proteins that participate in HR, depending on the genomic region and the type of HR. After these proteins have fully synthesized DNA at the lesion, dissolution of the intermediate structures produce the final repaired product. The cell's preferred method of dissolution is carried out by BLM helicase and associated topoisomerases and DNA-binding proteins, which generate non-crossover products ((reviewed in Chu and Hickson, 2009); Wechsler et al., 2011) (Figure 1-3).

While there are many complexities controlling which pathway is activated at any genomic lesion, employment of one or the other most likely depends on the cell cycle stage (Branzei et al., 2008). Non-homologous end-joining is a quicker, more efficient method; the ends of the double strand breaks are fused, regardless of sequence, and this process commonly occurs during G1. This mechanism is not intrinsically detrimental to genome stability, due to the high-
density of repeat sequences and non-coding DNA sequence (reviewed in Mao et al., 2008). However, non-homologous end-joining between two chromosome ends with critically shortened telomeres can generate chromosome-type fusions, which give rise to further intra-chromosomal breaks in subsequent cell cycles and ultimately compromise genome stability (Konishi et al., 2008; Ira et al., 2004).

Homologous recombination generally yields more accurate repair products, yet recombination mediated repair between inappropriately matched sequences can lead to severe chromosomal rearrangements (Aguilera and Gomez-Gonzalez, 2008). It requires a homologous sequence, using the sister chromatid as a repair template, and thus this pathway is restricted to S and G2 phases of the cell cycle (Rothman et al., 2003; Ira et al., 2004; Aylon et al., 2004).

Three types of homologous recombination (HR) have been shown to contribute to the ever-increasing complexity of telomere maintenance (reviewed in Palm and de Lange, 2008). The first is telomere-loop HR (t-loop HR), which is activated when the loop structure at chromosome ends is inappropriately resolved; this defect is predicted to occur in the absence of TRF2-mediated regulation. Holliday junctions are a structural consequence of the t-loop formation (see Figure 1-2), therefore this process requires proteins required for resolution of HJ structures, such as the WRN helicase (Wang et al., 2004; Li et al., 2008), XRCC3 resolvase (Liu et al., 2004), and Nbs1 (a component of the MRN DNA damage complex) (Wang et al., 2004). However, if these proteins
become deregulated the t-loop can be deleted entirely from chromosome ends, forming an extra-chromosomal telomeric circle (t-circle) independent of the chromosomal DNA, and leaving a truncated telomere (Wang et al., 2004).

The second HR process is telomeric sister chromatid exchange (t-SCE), which generates significant telomere length heterogeneity. T-SCEs result from hyper-recombination events and inappropriate crossovers, which are often caused by replication dysfunction or replication fork stalling (Laud et al., 2005). WRN helicase, as well as TRF2 and POT1 activity, suppress recombination intermediates at telomeres, and thus they suppress the telomere dysfunction induced by T-SCEs. T-SCEs are more frequent in cells with shortened telomeres and WRN deficiency (Laud et al., 2005). Loss of WRN function alone (or even in combination with moderate telomere dysfunction) induces T-SCEs; greater telomere dysfunction induces greater frequency of T-SCEs.

The final HR pathway at telomeres is recombination with telomeres and chromosome-internal telomeric sequence. This mechanism generates telomeric products similar to those observed after t-loop HR: an external deletion and extrachromosomal telomeres (Zhu et al., 2008). As in the first two HR pathways, WRN helicase activity is critical to suppress crossover events and unwinding inappropriate recombination intermediates, such as d-loops or Holliday junctions. The single-stranded telomere-binding protein, POT1, and XPF/ERCC1 (Zhu et al., 2003) are required to prevent this type of HR (He et al., 2006; Wu et al., 2006). This pathway is distinct from t-loop HR because the deleted circle
contains two regions of telomeric sequence and a non-telomeric, interstitial DNA sequence. These extrachromosomal DNA fragments are observed as telomere double minutes (TDMs).

**WRN RecQ helicase and telomere maintenance**

As described in the previous section, the Werner Syndrome RecQ helicase (WRN) is an enzyme with many roles in genome maintenance. The RecQ helicases are a highly conserved family of helicases, which were first discovered as a single homolog in *E. coli* (Umezu et al., 1990). These proteins have been well characterized in yeast, zebrafish, and flies (reviewed in Chu and Hickson, 2009). Five RecQ helicases have been identified in mammalian cells, and each have distinct activities in genome maintenance pathways (Table 1-1). Unlike its family members, WRN also contains exonuclease capabilities.

WRN activities in telomere maintenance, such as branch migration of Holliday junctions and degradation of mobile D-loops, are regulated by Shelterin components, TRF1, TRF2 and/or POT1 (Opresko et al., 2002, 2004, 2009). WRN helicase is highly efficient in alleviating secondary structures, such as G-quadruplexes, which are predicted to form in G-rich regions of the genome, such as telomeres (Opresko et al., 2003). These quadruplexes are likely to impede progress of the lagging-strand replication machinery and prevent synthesis of the daughter strand (Crabbe et al., 2004).
Table 1-1. RecQ helicases in mammalian cells. Shown are the documented diseases observed in individuals who lack a functional copy of the indicated RecQ helicase. The activity of each helicase is also indicated, as determined by *in vitro* activity assays.

<table>
<thead>
<tr>
<th>RecQ Helicase</th>
<th>Disease Affiliation?</th>
<th>DNA substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RECK1</td>
<td>None</td>
<td>• HJ dissolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Branch migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Forked DNA unwinding</td>
</tr>
<tr>
<td>RECK4</td>
<td>• Rothmund-Thomson</td>
<td>• Transcription factor binding</td>
</tr>
<tr>
<td></td>
<td>• RAPADILINO</td>
<td>• Strand annealing</td>
</tr>
<tr>
<td></td>
<td>• Baller-Gerold</td>
<td></td>
</tr>
<tr>
<td>RECK5</td>
<td>None</td>
<td>• d-loop dissolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Forked DNA unwinding</td>
</tr>
<tr>
<td>BLM</td>
<td>• Bloom’s syndrome</td>
<td>• d-loop dissolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• HJ dissolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• G-quadruplex resolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Branch migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• ssDNA annealing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Forked DNA unwinding</td>
</tr>
<tr>
<td>WRN</td>
<td>• Werner’s syndrome</td>
<td>• d-loop dissolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• HJ dissolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• G-quadruplex resolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Branch migration</td>
</tr>
</tbody>
</table>
Research from the Karlseder lab confirmed that WRN helicase activity is required for efficient lagging strand replication (Crabbe et al., 2004; Arnoult et al., 2009). Defective replication in cells lacking a functional WRN RecQ helicase leads to damage signaling at chromosome ends, which are then targeted and processed by DNA damage and repair proteins. WS cells in culture exhibit reduced proliferative lifespan and frequent chromosome translocations and deletions (Salk et al., 1981; Schulz et al., 1996). We were able to definitively link telomere loss with these chromosomal aberrations, thus showing that telomere dysfunction in WS cells contributes to the genome instability and cancer predisposition observed in WS patients (Chang et al., 2004; Crabbe et al., 2004; Crabbe et al., 2007).

Additional research has confirmed that chromosome instability is a consequence of telomere dysfunction induced by WRN-deficiency. Individuals lacking both the exonuclease and helicase activity of the WRN proteins are diagnosed with Werner’s syndrome. This condition leads to premature ageing, death around 50 years of age and a cancer predisposition (Brosh et al., 2007; Opresko et al., 2008). Patients most commonly succumb to cancers of mesenchymal origin (i.e. bone sarcomas) (Goto et al., 1996). The mouse model for the WRN disease phenotype is WRN depletion in a background of a deletion of the RNA subunit of telomerase, mTERC, which results in critically shortened telomere length. This indicates that the WRN deficiency induces pathophysiological defects in WS patients, due to telomere erosion and subsequent chromosome end defects (Chang et al., 2004).
**BLM RecQ helicase in genome maintenance**

A second RecQ helicase, BLM, has been extensively studied; therefore its contributions to genomic stability have been very well characterized. This ‘caretaker’ protein processes a range of DNA substrates and contributes to genome maintenance in several critical DNA repair pathways (reviewed in (Chu and Hickson, 2009). Like WRN proteins, there are several means by which BLM is predicted to contribute to efficient replication: stabilizing the replication fork, facilitating branch migration of homologous recombination intermediates, resolving inappropriate crossover events, and DNA end resection (Karow et al., 2000; Davies et al., 2007; Kikuchi et al., 2009; Nimonkar et al., 2011).

As described in the previous section, BLM participates in the initial steps of homologous recombination, by localization with the single stranded binding protein, RPA, and assisting RAD51 with invasion of the homologous sequence of the sister chromatid (Bischof et al., 2001; Wu et al., 2001). BLM also participates in later steps of HR, with another protein complex, composed of TOPO IIIα, RMI1, and RMI2 (BTR complex). In this capacity, BLM is particularly important in faithfully resolving HR structures, such as Holliday junctions, to generate non-crossover products (Wu and Hickson, 2003). In *S. cerevisiae*, this same mechanism has also been described, which incorporates the dissolution of HJs by the RecQ homolog, Sgs1, and its interacting partners, Top3 and Rmi1 (Ira et al., 2003; Cejka, et al., 2010).
Because it is responsible for a wide range of DNA maintenance activities, the absence of BLM RecQ helicase leads to an increase in genome aberrations, such as chromosome fusions and chromosome breaks. These events result from replication defects, such as stalled replication forks, and lead to crossover (Rassool et al., 2003). In fact, a common molecular marker for BS is an elevated frequency of Sister Chromatid Exchanges (SCEs) (German et al., 1965; Chaganti et al., 1974). BLM deficiency leads to Bloom’s syndrome (BS), a rare cancer-predisposition disease commonly observed in individuals of Ashkenazi Jewish descent. BS patients experience growth retardation, immunodeficiency, skin sensitivity to sunlight, and clinical reports document a remarkably broad range of cancer types that present as early as 25 years of age (German et al., 2007; (reviewed in Hananda and Hickson, 2007)).

BLM has been implicated, but not directly described, in telomere maintenance in unchallenged mammalian cells (Yankiwski et al., 2000; Stavropoulos et al., 2002; Opresko et al., 2005; Sfeir et al., 2010). It is particularly important in telomere maintenance in cells employing the alternative lengthening of telomeres (ALT) pathway (Stavropoulos et al., 2002). This mechanism is active in telomerase-negative cell types (Bryan et al., 1995) and utilizes recombination to maintain telomere length, which renders significantly heterogeneous telomere length (Bryan et al., 1997; Dunham et al., 2000).

A number of recent publications describe a new role for BLM in faithful chromosome segregation during mitosis. BLM forms a complex with its
interacting partners topoisomerase IIIα, RMI1, and RMI2 to process late-replicating intermediate (LRI) DNA structures that persist into anaphase. BLM and interacting proteins (BTR complex) process newly replicated but incompletely unwound late-replicating intermediates (LRIs) that persist into anaphase. These structures, termed ultra-fine bridges (UFBs), were not observed since they are undetectable by any known DNA counterstains. They were first observed with an antibody against PICH, which is a centromere-associated SNF2 family ATPase (Chan et al., 2007) (Figure 1-4). Surprisingly, these UFBs are observed even in ‘normal’ mitotic cells, but they are only detectable by antibody staining for BLM, TOPO IIIα, RMI1 and PICH.

While the exact DNA structures that compose these LRIs are still under speculation, a number of well designed studies has deduced that they are likely to arise from incompletely dissolved homologous recombination structures. The final step in HR is unwinding Holliday junctions to catenanes and hemicatenanes, which is performed by BTR dissolution (Chan et al., 2009; Naim et al., 2009) (Figure 1-4). While it is remarkable that incompletely resolved structures are allowed to persist into mitosis, previous research in yeast has shown that incomplete replication does not necessarily trigger cell cycle checkpoints (Torres-Rosell et al., 2007).

Ultra fine DNA bridges in anaphase are shown to frequently originate from centromeres, though more recent studies have found that late replicating intermediates targeted by the BTR complex can also form at fragile sites (Chan
et al., 2007; Chan et al., 2009). Fragile sites are difficult to replicate regions of the genome that are highly sensitive to treatment with aphidicolin or other replication inhibitors (Glover et al., 1984), which induce breaks, deletions, and persistent unresolved DNA structures (Chan et al., 2009). BLM and interacting proteins, TOPO IIIα, RMI1, RMI2, are known to localize to fragile sites, particularly following replication challenge by aphidicolin or MMC (Dutertre et al., 2000).
Figure 1-4. Possible mechanisms of formation of late-replicating intermediate structures (LRIs) and ultra-fine bridges (UFBs). (A) Replication fork progression may be delayed by external or internal influences: such as DNA lesions, replication challenge, or replication fork stalling and/or collapse. LRIs may be composed of a single Holliday junction or two converging Holliday junctions (shown here). The BTR complex (BLM helicase, topoisomerase III$\alpha$, RMI1 and RMI2) efficiently dissolves catenane or hemi-catenane late replicating intermediates, even LRIs that persist into mitosis. (B) Catenanes are composed of fully replicated, double stranded DNA that is unwound or tangled; the presence of catenanes as cells enter mitosis indicates a minor replication defect. As cells progress into the anaphase stage of mitosis, these structures are observed as ultra-fine bridges when spindle fibers pull the centromeres to opposite poles. The BTR complex quickly dissolves catenanes and UFBs are resolved early in anaphase. (C) If the delay is severe, vulnerable regions of the genome may only be partially replicated when the cells enter mitosis. These hemi-catenanes take longer to process and must be completely replicated before dissolution by the BTR complex; UFBs persist even in late-stage anaphases.
Telomeres as fragile sites

The term ‘fragile site’ is applied to regions in the genome that are difficult to replicate. While a number of fragile sites are composed of certain types of sequences, such as long repeat regions, or T-rich regions, there are no specific features that conclusively show why they are ‘difficult to replicate’. Replication of fragile sites is highly sensitive to partial replication inhibition induced by aphidicolin treatment, which leads to an increase in breaks, deletions, and recombination events (Glover et al., 1984).

Recent findings suggest that telomeres may also be fragile sites, following the observation that aphidicolin treatment induces telomere aberrations and replication fork stalling along telomeric tracks (Sfeir et al., 2009). The loss of TRF1 led to a greater frequency of ‘fragile telomere’ phenotype, which was exacerbated when TRF1 null MEFs were treated with aphidicolin. It was suggested that TRF1 contributes to efficient chromosome end replication by recruiting associated proteins that are specialized for facilitating replication of challenging regions, perhaps by preventing stalling or enabling restart (Sfeir et al., 2009). The helicases BLM and RTEL were suggested as possible factors recruited by TRF1 to telomeres, since loss of either led to an increase of ‘fragile telomeres’. This effect was epistatic with TRF1 loss, indicating that the presence of TRF1 is required for BLM and RTEL activity during telomere replication.
Summary

Here we show that BLM contributes to telomere maintenance under non-challenging conditions. Cells lacking BLM helicase activity display a greater frequency of telomere defects, such as sister telomere loss (STL) and telomere-free ends (TFEs). The role of BLM in telomere maintenance is evidently more significant in a genetic background of telomere dysfunction, such as in WS cells, where BLM depletion induces a greater frequency of telomere aberrations. Further examination of BLM helicase behavior in WRN-deficient fibroblast cells revealed a significant increase in BLM foci per cell and in the subset of BLM foci co-localizing with telomeres. In addition, not only do UFBs occur more frequently in WS cells, but these late-replicating intermediates also persist later in anaphase, which may be indicative of more extreme processing defects. BLM is known to be specifically employed at areas of the genome that undergo post-replication processing and I propose that BLM contributes to efficient telomere maintenance in this capacity, due to the difficult-to-replicate nature of telomeres.
Chapter 2. The BLM RecQ helicase contributes to telomere maintenance in mammalian fibroblasts

Background

Genomic instability commonly results from inefficient protection of chromosome ends by telomeres. Telomere stabilization can be compromised by ‘normal’ replication-dependent shortening and subsequent processing errors, and/or by deficiencies in Shelterin components or other telomere-associated proteins (Palm et al., 2008). Telomere replication is a highly complex process; progression of the replication fork through telomeric tracts is facilitated by the precise and dynamic recruitment of a surprising array of proteins. Our lab was one of many to describe the importance of DNA damage and repair proteins in normal telomere maintenance (Zhu et al., 2000; Verdun et al., 2005). Continued examination of novel telomere association proteins demonstrated that WRN RecQ helicase, a DNA repair protein involved in re-start of stalled replication forks, alleviation of Holliday junctions (such as the HJs formed at the t-loop) is also required for proper telomere maintenance (Chang et al., 2004; Crabbe et al., 2004; Opresko et al., 2004; Crabbe et al., 2007; Arnoult et al., 2009). Replication of the G-rich lagging strand poses a potential problem, since secondary structures are predicted to form at these regions and prevent replication machinery from accessing telomeres, thus inhibiting synthesis of the daughter strand (Chang et al., 2004; (reviewed in Williamson 2004)).

WRN helicase activity readily alleviates secondary structures, such as G-quadruplexes, which are predicted to form in G-rich regions of the genome, such
as telomeres (Opresko et al., 2003). Cells lacking a functional WRN helicase activity exhibit telomere dysfunction, which the Karlseder lab termed Sister telomere loss (STL) (Crabbe et al., 2004).

Telomere phenotypes on metaphase chromosomes were assessed by fluorescence in situ hybridization (FISH). When telomere length was reduced to below FISH detection levels (approximately 1 kilobase), chromatids appear to lack a detectable telomere signal. Critically shortened telomeres occurred primarily at the lagging strand, which was established by CO-FISH (chromosome-orientation FISH) of metaphase chromosomes (Crabbe et al., 2004). Based on analysis of telomere phenotypes in metaphase spreads, our lab proposed that the WRN RecQ helicase activity resolves G-quartets and facilitates lagging strand synthesis (Figure 2-1). Unwound secondary structures at the lagging strand would continually challenge progression of the replication fork; a severe blockage could prevent replication completion. This inefficient replication of the G-rich telomeric strand and the dramatic erosion of telomere length expose chromosome ends to fusions, which contribute to the genomic instability in WS patients (Chang et al., 2004; Crabbe et al., 2004; Crabbe et al., 2007). However, the complete loss of a sister telomere observed in WS cells was relatively rare (occurring at about 2% of chromatids), suggesting a complementary mechanism for facilitating lagging strand synthesis.

BLM is a likely candidate for some overlap with the WRN helicase activity in telomere maintenance. WRN and BLM are both necessary for proper genome
stability (Chu et al., 2009); they contribute to DNA repair by processing homologous recombination structures, through strand exchange, branch migration of Holliday Junctions (Constantinou et al., 2000; Opresko et al., 2005) and dissolution of HJs or D-loops (Bachrati et al., 2006). Because they are responsible for a wide range of DNA maintenance activities, the absence of either RecQ helicase leads to an increase in genome aberrations, such as chromosome fusions and chromosome breaks.
Figure 2-1. Predicted for model for WRN activity in efficient replication of the telomeric lagging strand. Shown here is the extreme chromosome terminus. In mammalian cells, the telomeric lagging strand is composed of 9-15 kilobases of TTAGGG repeats. The G-rich sequence is shown to readily, and stably, form G-quadruplex structures, which could potentially inhibit progression of the lagging strand replication machinery. In the absence of WRN helicase activity, G-quadruplexes remain un-resolved and the lagging strand is therefore inaccessible to the replisome.
Previous findings indicated a role for BLM at ‘normal’ mammalian telomeres, yet the exact purpose or mechanism was unknown. *In vitro* binding assays show that BLM can directly interact with the telomere binding proteins, TRF2 and WRN (Stavropoulos et al., 2002), as well as DNA damage proteins that participate in telomere processing, such as RAD51 and ATM (reviewed in Hickson 2003). Immunofluorescence studies showed frequent BLM and WRN co-localization events in a number of cell types (von Kobbe et al., 2002), and extensive *in vitro* binding assays described a direct protein-protein interaction between these RecQ helicases (Opresko et al., 2002). These studies also confirmed that BLM is detectable at a subset of telomeric foci in normal and SV40-transformed fibroblasts (Yankiwski, 2000), but it more frequently overlaps with telomeric signals in cell lines undergoing alternative lengthening of telomeres (ALT) (Stavropoulos et al., 2002). The diverse activity of BLM in DNA replication, DNA damage and repair pathways, and *in vitro* resolution of D-loops and G-quadruplexes, makes this helicase a likely contributor to telomere maintenance.
Results

BLM is present at telomeres

BLM is difficult to detect at targeted genomic regions, due to a number of features intrinsic to a helicase recruited to DNA in response to replication defects or DNA damage. BLM is present in very low abundance in the nucleus of IMR90 fibroblast cells, and endogenous levels are difficult to detect by standard biochemical techniques (Dutertre et al., 2000). Because BLM is recruited to DNA structures rather than a particular region or sequence, its specific genomic localization during a ‘normal’ cell cycle is relatively unpredictable. Furthermore, in vitro analyses demonstrating its rapid enzymatic activity might suggest that BLM would transiently localize to Holliday junctions or G-quadruplexes, where it rapidly processes non-standard DNA substrates (Karow et al., 2000; Sun et al., 1998).

Despite these challenges, we sought a possible interaction between BLM and telomeres by two methods: Immunofluorescence – fluorescence in situ hybridization (IF-FISH) and chromatin immunoprecipitation (ChIP). These methods are highly sensitive, so to reduce non-specific signal and enhance protein detection I designed a BLM antibody. I purified a fragment of the BLM N-terminus, which was dialyzed in PBS and injected into a rabbit. After approximately three months, the rabbit serum was collected and BLM antibody was purified over an immuno-affinity column. Extensive analyses were performed to confirm specificity and efficiency of the antibody for Western
blotting, immunoprecipitation, and immunofluorescence (Figure 2-2). The antibody was first described in a publication generated through collaboration with the Weitzman lab, which exhibited that BLM is targeted for degradation following HPV infection (Orazio et al., 2010). When performed in asynchronous IMR90 cells, both techniques detected BLM at telomeres, though in low abundance (Figure 2-3B,C).
Figure 2-2. Characterization of purified BLM antibody. To confirm the specificity and detection limits of the purified BLM antibody, we tested a number of cell lines by western blotting and immunofluorescence. BLM knockdown was achieved by stably transfecting IMR90-E6E7 fibroblasts with short hairpin RNA against BLM. To reconstitute BLM expression in BS cells, full length BLM cDNA was stably expressed by retroviral delivery. Positive cell lines were selected by puromycin treatment. For WB, cells were counted and lysed with Laemmeli buffer before SDS-PAGE. (A) BLM antibody distinguishes BLM knockdown in IMR90-E6E7 fibroblasts stable transfection with. Western blot of IMR90-E6E7 (B) IF-FISH images of synchronized IMR90 cells. BLM was detected by IF with a BLM antibody (red) and telomeres were visualized by FISH with a fluorescent labeled telomeric probe, FITC-[CCCTAA]$_4$ (green).
Figure 2-3. BLM is detected at telomeres in a cell-cycle dependent manner. IMR90 fibroblast cells were synchronized at the G1 boundary by double thymidine block, and synchronization was confirmed by FACS analysis. At pre-determined time-points, cells were collected and processed for FACS, IF-FISH, and ChIP. For IF-FISH, IMR90 cells growing on glass coverslips were washed and fixed with 4% paraformaldehyde. For ChIP analysis, IMR90 cells growing in 15cm dishes were harvested, washed, and cross-linked with 1% paraformaldehyde. IMR90 cells were synchronized in four independent experiments. (A) IF-FISH images of synchronized IMR90 cells. BLM was detected by IF with a BLM antibody (red) and telomeres were visualized by FISH with a fluorescent labeled telomeric probe, FITC-[CCCTAA]_4 (green). Arrows indicate colocalization events between BLM and telomeric foci. (B) Quantification of IF-FISH data, showing the percent of cells with over two colocalization events. The values shown here were calculated from five synchronization experiments and at least 50 cells were counted for each time-point. Figure 2-3 continued on next page.
Figure 2-3 (continued). BLM is detected at telomeres in a cell-cycle dependent manner. IMR90 fibroblast cells were synchronized at the G1 boundary by double thymidine block, and synchronization was confirmed by FACS analysis. At predetermined time-points, cells were collected and processed for FACS, IF-FISH, and ChIP. For IF-FISH, IMR90 cells growing on glass coverslips were washed and fixed with 4% paraformaldehyde. For ChIP analysis, IMR90 cells growing in 15cm dishes were harvested, washed, and cross-linked with 1% paraformaldehyde. IMR90 cells were synchronized in four independent experiments. (C) Immunoprecipitations were performed with the indicated antibody and dot blots were hybridized with radio-labeled TTAGGG and ALU probes. (D) Quantification of TEL DNA (top panel) or ALU DNA (bottom panel) in ChIPs with BLM or IgG.
A number of studies have shown that BLM protein expression and nuclear localization patterns vary in a cell-cycle dependent manner and protein accumulates in late S/G2 (Dutertre et al., 2000; Yankiwski, 2000). To accurately characterize a potential telomere-specific activity we synchronized IMR90 fibroblasts at entry into G1 by a thymidine-thymidine double block. Synchronization and cell cycle progression was assessed by FACS analysis with propidium iodide staining. At each stage in the cell cycle, cells were collected and/or prepared for IF-FISH or ChIP.

Following IF-FISH, we imaged synchronized IMR90 fibroblasts on a fluorescence microscope Figure 2-3A). Co-localization events were confirmed by measuring overlap between BLM and telomeric signal intensities with the Profile function in AxioVision software. We calculated the average colocalization frequencies from five independent synchronization experiments and IF-FISH analyses. Co-localization frequencies were also obtained with a Metasystems Metafer automated slide scanning and analysis microscope, which analyzed images captured with an automated, multi-slide Zeiss Imager fluorescence microscope and external Hamamatsu ORCA-ER digital camera (Waitt Biophotonics Core Facility at the Salk Institute). Metasystems technicians designed a program particularly for the Karlseder lab, which calculated the number of BLM foci, telomeric foci, and colocalization events per cell. These values corresponded with results obtained manually. Though BLM was present at a low subset of telomeres, cells in late G2 displayed a significant increase in multiple colocalization events (Figure 2-3B).
Table 2-1. Features of ‘normal’ IMR90 and Bloom’s syndrome fibroblast cell lines obtained from Coriell Laboratories. Relevant clinical data and genetic background of each cell line was available on the company’s website (http://ccr.coriell.org).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Genetic Features</th>
<th>Clinical Symptoms</th>
<th>Patient Info</th>
</tr>
</thead>
</table>
| IMR90     | None             | None              | 16 week fetus | 16 week fetus  
            |                  |                   | Caucasian     |
|           |                  |                   | Female       |
| GM02548   | Increase SCE     | Yes (data not available) | 6 YR Black |
|           | Chromosome breakage |               | Male         |
|           | Increase post UV irradiation |       |              |
|           | unscheduled DNA synthesis |     |              |
| GM03402B  | Increase SCE     | - Microencephaly  | 30 YR        |
|           | Chromosome breakage | - Congenital dwarfism | Caucasian   |
|           | Reduced DNA ligase I | - Facial telangiectasias | Male;       |
|           | activity         | - Photosensitivity | Ashkenazi   |
|           |                  | - Rectal carcinoma | descent     |
|           |                  | - Basal cell carcinoma |              |
We also collected cell lysates for immunoprecipitations with indicated antibodies (Figure 2-3D). DNA fragments bound by each antibody were purified, transferred to a membrane, and hybridized with a probe composed of telomeric repeats (Figure 2-3D, top panel) or the control Alu sequence (Figure 2-3D, bottom panel). Chromatin bound by BLM antibody or the negative control antibody (IgG) was quantified from signal intensities obtained with the ImageQuant software. BLM occupied a very low portion of telomeric chromatin and was slightly enriched in G2/late G2 (Figure 2-3C).

ChIP and IF-FISH data both consistently demonstrate that BLM can be detected at very low levels in normal mammalian telomeres, though these methods do not show consistent temporal recruitment of BLM to telomeres. One possible explanation for this discrepancy is BLM’s preference for non-standard DNA structures, which may not be retained through the multi-step ChIP. Further analysis of BLM recruitment to telomeres provides a more satisfactory explanation for the results of IF-FISH and ChIP assays and will be discussed in greater detail in Chapter 3 of this Thesis.
**Chromosome aberrations and telomere defects in BS cells**

The BS fibroblast cell lines used in experiments are derived from lung fibroblasts of patients with the disease, indicated by catalog numbers: GM02548 and GM3402B. IMR90 ‘normal’ untreated fibroblasts (reference #I90-15) are included as a control population (Coriell Laboratories). Individuals who develop BS inherit two mutated alleles, since one wild type copy of *blm* is sufficient to support genome maintenance (Table 2-1). BS is most prevalent among individuals of Ashkenazi Jewish decent.

BLM-deficient cells are characterized by replication dysfunction and DNA damage events that lead to chromosomal aberrations, such as breaks or fusions (Chaganti et al., 1974; German et al., 1993). A common experimental method to assess these aberrations is quantification of anaphase bridges (see Figure 2-4A). Fibroblasts from Bloom Syndrome patients had a higher rate of anaphase bridge formation compared to IMR90 fibroblasts (Table 2-2). Expression of human papilloma virus 16 (HPV16) E6 and E7 oncoproteins efficiently suppresses the p53 and pRb DNA damage checkpoints, causing fibroblast cells to progress through mitosis even in the presence of DNA damage (Smogorzewska et al., 2002). Oncoprotein expression increases the number of mitotic cells, including cells with DNA damage or chromosome aberrations, but does trigger these events. GM02548-E6E7 and GM03402B-E6E7 cell lines display a significant increase in anaphase bridges compared with IMR90-E6E7 (Table 2-2).
Figure 2-4. Telomere defects and anaphase bridges in Bloom's syndrome and IMR90 fibroblast cell lines. (A) Telomere defects (TDs), such as sister telomere loss (STL) and telomere free ends (TFEs) were counted by analysis of metaphase chromosomes. Chromosome DNA was stained with DAPI (in red) and telomeres were visualized with FISH with a fluorescently labeled telomeric PNA oligonucleotide (in green). Numbers displayed here are the percent of chromatids with telomere defects. At least 1500 chromatids were counted for each cell line; average values were obtained from three independent experiments. (B) These cells were grown on glass coverslips, methanol fixed, stained with DAPI and mounted on glass slides. Coverslips were scanned for cells in the anaphase stage of mitosis, which was performed on a Zeiss Axio Imager.Z1 fluorescence microscope.
Chromosome or chromatid fusion events are also a frequent consequence of dysfunctional or critically shortened telomeres, which are readily recognized by DNA damage proteins and rapidly processed by the fusion activity of Non-Homologous End Joining (NHEJ) machinery (Smogorzewska et al., 2002). We explored the possibility that frequent fusion events in BS cells could partly result from telomere dysfunction. As an initial screen, we examined the average length of telomeres in primary BLS fibroblasts by following the telomere shortening rates over accumulated population doublings. This analysis began with young cells and continued to onset of cellular senescence, which was determined by cell morphology and population doubling rates. The baseline for growth rate and telomere length was normal primary IMR90 fibroblasts.

At specific increments, cell lysates were collected from both BS cells and IMR90 cells over the course of their lifespan in culture. I performed a modified Southern blot, termed a Telomere-Repeat-Fragment (TRF) analysis, which measures the length of the G-rich overhang (native condition) and total telomere length (denatured condition). Analysis of accumulated population doublings showed that BS cells grew more slowly and reached senescence earlier compared to the IMR90 human fibroblast line. However, the TRF analysis showed no significant difference in telomere shortening rates in BLS cell lines compared to normal IMR90 cells. Telomeric fusions, which persisted throughout accumulated cell divisions, were detected in the BLS cell line with shorter telomeres.
Because there is no observable change in the bulk telomere population, individual telomere phenotypes were examined by performing FISH on metaphase chromosomes from BS fibroblasts. Chromosomes were stained with DAPI (shown in red) and telomeres are visualized by hybridization with a fluorescently labeled telomeric oligonucleotide (in blue) (Figure 2-4B). The frequency of telomere defects (TDs), such as sister telomere loss (STL) or telomere free ends (TFE) (for examples of TDs see Figure 2-4B), were significantly elevated in BS cell lines compared to normal IMR90 fibroblasts (Table 2-2). While changes in the FISH signal intensities were not quantified, it is noteworthy that telomeric signal intensities were much lower in BS metaphases compared to identically treated IMR90 metaphase chromosomes.

To confirm that telomere defects and chromosome fusions in BS cells were a direct consequence of the loss of BLM, BLM expression was reduced in IMR90-E6E7 fibroblasts by retroviral delivery of a short hairpin (pSuper.retro, Oligoengine) and confirmed by western blotting and immunofluorescence (Figure 2-7). BLM deficient IMR90-E6E7 cells displayed a frequency of anaphase bridge formation and telomere defects similar to the levels observed in BS fibroblasts (Figure 2-7C; Table 2-3).
Table 2-2. Telomere defects and anaphase bridge formation are elevated in Bloom’s syndrome fibroblast cell lines. Quantification of telomere defects at metaphase chromosomes and frequency of DAPI-positive anaphase bridges. Telomere defects are displayed here are the percent of chromatids with telomere defects. At least 1500 chromatids were counted for each cell line; average values were obtained from three independent experiments. The percent of anaphase cells with DAPI bridges was calculated from four independent experiments, where at least 50 anaphases were counted per cell line. Statistical analyses for telomere defects and anaphase bridges were performed with a two-tailed student’s t-test (*p < 0.05 and **p < 0.005).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Chromatids with Telomere Defects (%)</th>
<th>Anaphase Bridges (% Anaphases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMR90</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>IMR90-E6E7</td>
<td>1.2</td>
<td>4.6</td>
</tr>
<tr>
<td>GM02548</td>
<td>2.5**</td>
<td>23**</td>
</tr>
<tr>
<td>GM02548-E6E7</td>
<td>3.4**</td>
<td>29*</td>
</tr>
<tr>
<td>GM03402B</td>
<td>3.1**</td>
<td>21**</td>
</tr>
<tr>
<td>GM03402B-E6E7</td>
<td>3.7**</td>
<td>33**</td>
</tr>
</tbody>
</table>

* p < 0.05  
**p < 0.005
We also tried to suppress telomere defects by stably restoring BLM helicase activity in Bloom’s syndrome fibroblasts. Western blotting confirmed BLM expression (Figure 2-7A). Examination of anaphase bridges and metaphase chromosomes indicates that reconstituted BLM reduces chromosomal aberrations and telomere defects (Figure 2-7C; Table 2-3), though not as efficiently as hTERT expression (Figure 2-5).

*Telomerase-mediated telomere elongation in Bloom’s syndrome cells*

Deprotected chromosome ends are recognized by DNA damage proteins and become highly vulnerable substrates for processing that leads to formation of end-to-end chromosome fusions (Palm et al., 2008). The frequency of anaphase bridges and a reduction in telomeric foci strongly indicate that telomere defects contribute to the genomic instability in BS cells. To investigate the possibility that chromosomal aberrations observed in BS cells are partly a result of critically shortened telomeres, the catalytic subunit of telomerase, hTERT, was over-expressed in Bloom’s syndrome fibroblast cell lines. Telomerase is a ribonucleoprotein, which employs a reverse transcriptase activity to extend telomeric tracts by adding TTAGGG repeats specifically to chromosome ends. Though it has little or no activity in fibroblasts, exogenous expression of hTERT is sufficient to recruit necessary proteins and accessory factors that effect telomere lengthening. Elongation is not observed in cells expressing a dominant negative form (DN_hTERT), which is unable to catalytically add telomere repeats.
Figure 2-5. Expression of the catalytic subunit of telomerase, hTERT, reduces chromosome fusions and telomere defects in BS cells. BS cells with E6 and E7 oncoproteins were stably transfected with an empty vector Control, hTERT, or DN_hTERT. Cells were methanol-fixed for anaphase bridge analysis, or treated with colcemide and harvested for FISH of metaphase spreads. Where indicated, arrow bars represent standard deviation. p values were calculated by two-tailed student's t-test (*p < 0.05 and **p < 0.005). (A) Percent of anaphases with DAPI bridges in IMR90 and BS fibroblast cells. At least 55 anaphases per cell line, per experiment were counted from three independent experiments. The percentage of anaphase bridges is indicated. (B) The frequency of telomere defects (TDs) in IMR90 or BS fibroblasts, shown as a percent of chromatids. TDs were quantified by analysis of telomeres phenotypes in metaphase spreads. At least 1500 chromatids per cell line, per experiment were counted from three independent experiments. (C) Images showing FISH of metaphase chromosomes; telomeres were visualized with FITC-[CCCTAA]_4 probe, and DNA was stained with DAPI. Shown are chromosomes from IMR90-E6E7 cells with the control vector, and BS cell lines expressing the indicated vector. Arrows indicate chromosomes shown in magnification panels below each metaphase spread.
Figure 2-6. Expression of hTERT does not suppress genomic fusion events induced by irradiation. Quantification of DNA bridges in IMR90-control-E6E7 and IMR90-hTERT-E6E7 cells. Cells were irradiated with 3 Gy, cultivated for 24 hours, fixed, and stained with DAPI for analysis. At least 53 anaphases per condition per experiment were counted in three independent experiments. The percentage of cells with anaphase bridges is indicated, and arrow bars represent the standard deviation (previously published in Crabbe et al., 2007).
Chromosome fusions, measured by anaphase bridge formation, were suppressed in BS cells expressing hTERT, but not in cells with the empty retroviral vector or the vector containing DN_hTERT (Figure 2-4A). This is specifically a result of elongating critically shortened telomeres, as we have previously established that hTERT enzymatic activity is not sufficient to alleviate non-telomeric lesions (Figure 2-6).

We also assessed telomere phenotypes and chromosomal integrity by telomeric FISH of metaphase chromosomes. By enzymatically restoring the length of critically shortened telomeres we are also able to distinguish metaphase chromosomes with genuine telomere defects from chromosomes that lack a telomeric signal due to internal chromosome breaks. Telomeric signals were restored and uniformly intensified following hTERT expression (Figure 2-4C), which significantly reduced telomere defects in BS cells (Figure 2-4B). These data suggest that exposed chromosome ends resulting from critically shortened telomeres may account for a portion of chromosomal aberrations observed in Bloom’s syndrome cells.
Figure 2-7. Telomere defects and DNA anaphase bridge formation in IMR90-E6E7 shblm and GM02548-E6E7+BLM fibroblasts. IMR90-E6E7 fibroblasts were transduced with a non-specific shRNA (NS) or shRNA targeting BLM. Full length BLM was retrovirally delivered in GM02548-E6E7 BS fibroblasts. IMR90 and BS fibroblasts were treated with puromycin to generate stable cell lines. (A) Western Blotting and (B) Immunofluorescence confirmed reduction of BLM expression in IMR90-E6E7 cells expressing a short hairpin against BLM (shBLM), but not in cells with a non-specific sequence. Over-expression of BLM in GM02548-E6E7 was successful, but did not reach levels observed in normal fibroblasts (WB: bottom panel).
Table 2-3. Quantification of chromatids with telomere defects and DAPI positive anaphase bridges in IMR90-E6E7 shBLM and GM02548-E6E7 BS cells expressing full length BLM. At least 1500 chromatids and 75 anaphases were counted for each cell line and average values were obtained from three independent experiments. The $p$ values were calculated using a two-tailed student’s t-test (*$p < 0.05$ and **$p < 0.005$).

<table>
<thead>
<tr>
<th></th>
<th>Chromatids with Telomere Defects (%)</th>
<th>DAPI Bridges (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMR90-E6E7 scramble</td>
<td>1.5</td>
<td>7.3</td>
</tr>
<tr>
<td>IMR90-E6E7 shBLM</td>
<td>3.1*</td>
<td>25*</td>
</tr>
<tr>
<td>GM02548-E6E7 Control</td>
<td>3.9</td>
<td>36</td>
</tr>
<tr>
<td>GM02548-E6E7 BLM</td>
<td>2.9</td>
<td>21</td>
</tr>
</tbody>
</table>

** $p < 0.005$
* $p < 0.05$
No strand-specific telomere loss in BLM-deficient cells.

Telomere defects in BS cells occur at remarkably similar frequency to the telomere phenotypes in WS cells, which may indicate an overlapping role for BLM and WRN in chromosome end maintenance. WRN assists in lagging strand replication, most likely through unwinding of secondary structures that form at the G-rich lagging strand. BLM helicase is also highly efficient in resolving G-quadruplexes, which prompted examination of strand specific telomere phenotypes in cells lacking BLM. To distinguish between the leading and lagging strands on metaphase chromosomes, we performed chromosome-orientation FISH (CO-FISH). This method yields the most accurate results when performed in cell lines with longer telomeres so BLM was stably knocked down in HeLa LT cells (LT = Long Telomeres), via retroviral delivery of a short hairpin RNA (as described previously). Cells were treated with a telomerase inhibitor (BIBR) for five days before collection and CO-FISH analysis to prevent enzymatic lengthening of shortened telomeres caused by BLM deficiency.

Analysis of metaphase chromosomes from BLM-deficient HeLa cells revealed an overall increase in total telomere defects (TDs). We did observe an increase in STL at the leading and lagging strand telomeres, compared to the control HeLa cells. However, there was not a difference between leading strand STL and lagging strand STL (Figure 2-8). Therefore, BLM does not appear to preferentially facilitate leading or lagging telomere maintenance.
Figure 2-8. Loss of leading and lagging strand telomeres in BLM-deficient HeLa cells. Leading and lagging strand synthesis incorporates bromodeoxyuridine (BrdU) and bromodeoxycytidine (BrdC) into freshly synthesized DNA strands. Bromo-nucleotide–substituted DNA is degraded, and telomeres are hybridized with FITC-[CCCATT]₄ and TAMRA-[TTAGGG]₄ probes. (A) CO-FISH of control HeLa LT cells and HeLa LT cells with reduced BLM expression. Leading strand telomeres are shown in red and lagging strand telomeres in green. Cells were treated with the telomerase inhibitor BIBR1532 (Boehringer) 4 days before analysis. The arrows indicate missing sister telomeres or telomere free ends. (B) Percent of chromatids with the indicated telomere defects. Total STL combines leading and lagging strand STL events. Total telomere defects (Total TDs) combine all STL events and telomere free ends. Values were calculated from three independent experiments. At least 1000 chromatids per cell line, per experiment were counted from three independent experiments; arrow bars represent standard deviation. Statistical significance was determined with a student’s t-test (*p < 0.05 and **p < 0.005).
**DNA damage and TIF formation in BS cells**

Telomeres that become critically short or sustain any dysfunction that generates unprotected chromosome ends are rapidly recognized and bound by proteins in the DNA damage pathway. A DNA damage response triggered at chromosome ends and the subsequent accumulation of DNA repair factors are termed telomere-damage induced foci (TIFs). TIFs are cytologically visible as 53BP1 or γH2AX DNA damage markers accumulated at telomeric signal(s) (d'Adda di Fagagna et al., 2003; Takai et al., 2003) (Figure 2-9A,C). When performing analyses, I chose the frequencies of damage events and TIFs according to standard values employed by the telomere field (Sfeir et al., 2009; Sfeir et al., 2010). Typically, IMR90 cells display a low, background level of damage foci γH2AX TIFs.

IF-FISH in Bloom’s syndrome cells demonstrated an increase in cells with over five DNA damage foci, which was observed with both 53BP1 (Figure 2-9A and 2-8B, upper panel) and γH2AX (Figure 2-9C and 2-9D, upper panel). Furthermore, compared to untreated IMR90 cells, BLM deficient fibroblasts experienced a higher frequency of DNA damage signaling events at telomeres. We established TIF frequency as the percent of interphase nuclei with over five 53BP1 TIFs (Figure 2-9B, bottom panel) or over three γH2AX TIFs (Figure 2-9D, bottom panel). Five 53BP1 TIFs and three γH2AX TIFs were selected for analysis in accordance with the standard counts usually published in the telomere field. Elongation of truncated telomeric sequence by hTERT
suppressed the frequency of damage-induced foci and TIF formation (Figure 2-9B,D) thus confirming that a subset of DNA damage events in BS cells occurs at telomeres.
Figure 2-9. DNA damage foci and telomere damage-induced foci (TIFs) are elevated in BS fibroblasts. Production of IMR90-E6E7 and GM02548-E6E7 fibroblast cell lines expressing hTERT was described previously. Cells grown on glass coverslips were fixed with 2% PFA before IF-FISH. DNA damage events were identified as 53BP1 or γH2AX foci. TIFs were distinguished by localization of 53BP1 or γH2AX foci with telomeric signal. (A) Examples of IF-FISH in indicated fibroblast cell lines, showing DNA damage events and/or TIFs, visualized with 53BP1. TIFs are indicated with white arrows. (B) Percent of IMR90 and GM02548 BS cells with over five 53BP1 foci (top panel) and over five TIFs (bottom panel). Figure 2-9 continued on the next page.
Figure 2-9 (continued). DNA damage foci and telomere damage-induced foci (TIFs) are elevated in BS fibroblasts. Production of IMR90-E6E7 and GM02548-E6E7 fibroblast cell lines expressing hTERT was described previously. Cells grown on glass coverslips were fixed with 2% PFA before IF-FISH. DNA damage events were identified as 53BP1 or $\gamma$H2AX foci. TIFs were distinguished by localization of 53BP1 or $\gamma$H2AX foci with telomeric signal. (C) Examples of IF-FISH in indicated fibroblast cells, showing DNA damage events and/or TIFs, visualized with $\gamma$H2AX. TIFs are indicated with white arrows. (D) Percent of cells with over five $\gamma$H2AX foci (top panel) or over three TIFs (bottom panel). These values were obtained from at least two independent experiments, in which at least 100 cells from each cell line were counted. Statistical significance was calculated with a student’s t-test and arrow bars indicated standard deviation.
Discussion

The BLM RecQ helicase contributes to genome maintenance as a DNA repair protein that facilitates homologous recombination, particularly by efficient resolution of intermediate structures such as D-loops and Holliday junctions (reviewed in (Chu and Hickson 2009)). Due to low abundance in the nucleus and the rapid and transient processing of non-standard DNA substrates, BLM is difficult to detect at targeted genomic regions. IF-FISH and ChIP analyses in asynchronous cells indicate a low level of BLM at chromosome ends, which confirms previous reports of BLM localization with a subset of telomeres (Yankiwski et al., 2000). These same assays, performed in synchronized fibroblast cells, revealed that BLM presence at telomeres varies over cell cycle progression, though the results were inconsistent. ChIP analysis indicates BLM occupation of telomeres is highest during G2 (Figure 2-1C and D), but IF-FISH data consistently show BLM enrichment during late G2 or at the G2/M boundary (Figure 2-1A and B). This discrepancy may be due to experimental methods and efficiency of DNA recovery during the ChIP procedure, particularly in consideration of BLM binding to secondary DNA substrates.

We conclude that BLM localizes to chromosome ends at very low levels in unchallenged mammalian fibroblasts, and at greater frequency in cells undergoing the ALT (alternative lengthening of telomeres) pathway of telomere maintenance. Our observations in synchronized cells corroborate previous publications, which describe the cell cycle regulated dynamics of BLM
localization patterns, such as BLM foci coinciding with a portion of newly replicated DNA and with stalled replication forks during S phase (Wu et al., 2000; Constantinou et al., 2002). We found that BLM is recruited to telomeres during G2 phase of the cell cycle. How BLM activity contributes to chromosome end maintenance during G2 will be discussed in greater detail in Chapter three.

Bloom’s syndrome cells are defined by genomic instability and are known to display a higher rate of anaphase bridge formation compared to IMR90 fibroblasts; our data correspond to previous reports of DAPI bridge frequency in BS cells (Chan et al., 2007; German et al., 1993). Interestingly, anaphase bridge formation was significantly suppressed in BS fibroblasts expressing hTERT, but not in cells with the dominant negative form, DN_hTERT. These data strongly indicate defective chromosome end protection in BS cells, because enzymatic repair of critically shortened telomeres can reduce a portion of the genomic aberrations in BS cells. Because hTERT specifically elongates telomeric sequence at chromosome ends, its expression is unable to repair non-telomeric breaks throughout the BS genome and fusions are not entirely prevented.

Examination of individual telomeres on metaphase chromosomes revealed that the frequency of telomere defects (TDs), such as sister telomere loss (STL) and telomere free ends (TFE), were significantly elevated in BS cell lines compared to normal IMR90 fibroblasts. Before confirming that Bloom’s syndrome cells exhibited telomeric dysfunction, we needed to exclude the non-telomeric breaks at the distal regions of chromosome arms. By enzymatically
restoring the length of critically shortened telomeres, we were able to distinguish between metaphase chromosomes with genuine telomere defects, and chromosomes that lack a telomeric signal due to internal chromosome breaks. Telomeric signals were restored and uniformly intensified following hTERT expression, which led to a significant reduction in telomere defects in BS cells.

The FISH signals were not quantified, but it is noteworthy that telomeric signal intensities were markedly lower in BS metaphases compared to identically treated IMR90 metaphase chromosomes. Telomere defects, such as STL and TFEs, describe the extreme telomere phenotype, which are telomeres that have been shortened to below approximately one kilobase in length. This experimental limitation potentially excludes a subset of the population, which is telomeres that lose a considerable length of distal sequence due to faulty replication, but maintain sufficient length for detection by FISH.

Since this project began, Q-FISH (Quantitative FISH) fluorescent techniques have been advanced to accurately measure even moderate changes in signal intensity, which is a reliable indicator of telomere length. The greater sensitivity allows researchers to observe subtler phenotypes that may appear after only one or two population doublings. This has been exemplified by analysis of the WRN RecQ helicase, which was identified in efficient lagging strand synthesis by manually counting chromatids with complete loss of a sister telomere (Crabbe et al., 2004). Recent methods have employed Q-FISH on cells lacking WRN helicase activity and demonstrated that WRN participates in lagging
strand replication at every replicative cycle (Arnoult et al, 2009). This technique would be extremely valuable in characterizing BLM contribution to telomere maintenance, possibly by quantifying severity and rate of telomere loss after one or two replication cycles.

To exclude the possibility that non-specific variables in BS cells are responsible for telomere defects, BLM expression was suppressed in IMR90-E6E7 fibroblasts by retroviral delivery of a short hairpin (pSuper.retro, Oligoengine). BLM deficient IMR90-E6E7 cells displayed a frequency of anaphase bridge formation and telomere defects similar to the levels observed in BS fibroblasts. These data provide further support for a BLM-specific activity in telomere length maintenance and suppression of chromosome fusion events.

BLM helicase activity have been extensively characterized from analyses of isogenic BS fibroblast cells lines, PNSG13 (BLM⁻) and PSNF5 (BLM⁺) (Chan et al., 2007; Davies et al., 2007; Gaymes et al., 2002; Rassool et al., 2003). These matched cell lines differ only in BLM expression and provide an ideal system for BLM-specific analyses. Apparently BLM expression phenotypically ‘corrects’ SCE frequency, a hallmark of BS cells and a product of replication dysfunction (Gaymes et al., 2002). To independently generate a similar pair of cell lines, multiple attempts were made to generate a stable BS cell line by retroviral delivery of a reconstituted, full length BLM (GM02548-E6E7+BLM).

Western blotting detected BLM protein, but even the most efficient retroviral transduction of BLM cDNA produced cells expressing low levels of BLM
helicase (Figure 2-7A). This was remarkable considering that BLM cDNA was regulated by a strong CMV promoter and cloned into the same retroviral vector used for hTERT expression. However, BLM helicase activity is highly efficient even in very low abundance, therefore it is possible that a strict regulatory pathway is in place to prevent un-necessary helicase production. A significant discovery revealed that BLM expression in PSNF5 is reportedly 50% of ‘normal’ protein levels (data not shown), and this is sufficient to restore most aspects of BS cell functions to ‘normal’ levels (Rassool et al., 2003). BLM protein expression in GM02548-E6E7 was presumably similar to well-established isogenic BS cell lines, so we pursued analysis of chromosome fusion rates and telomere defects.

We observed that DAPI anaphase bridges were reduced from 41% in control cells to 20.5% in BLM+ fibroblasts. The suppression of anaphase bridges closely correlated with the previously published study from Ian Hickson’s group, which showed that BLM expression in BS cells reduced anaphase bridges from 48% in PNSG13 control cells to 29% in PSNF5 BLM+ cells (Chan et al., 2007). Telomere defects were also lowered from 3.9% chromatids in GM02548-E6E7-control cells, to 2.9% of chromatids in metaphases from GM02548-E6E7+BLM (Table 2-3). The moderate effects on DAPI anaphase bridge frequency and telomere phenotypes may be explained by insufficient quantities of BLM helicase, however, it is likely that ectopic BLM expression will suppress replication defects, rather than recover dramatically reduced telomeric length or reverse recombination events. In fact, not all features of replication defects are
suppressed, since PSNF5 cells demonstrate significant – but incomplete – reduction of DNA damage signaling events (Rassool et al., 2003) and DAPI anaphase bridge formation (Chan et al., 2007).

As described in the Introduction and in the background section of this Chapter, these helicases possess a remarkable degree of similarity. Our prediction for the BLM helicase contribution to telomere maintenance was through a mechanism similar to the WRN helicase role in lagging strand replication. This prediction was bolstered by observations that WS and BS cells demonstrate comparable rates of telomeric defects, which are dramatically reduced by hTERT expression in both cell lines. However, CO-FISH analyses indicate that BLM activity is not significantly strand specific, due to similar STL events at leading and lagging telomeric strands (Figure 2-8). Our data indicate that the BLM helicase possesses a distinct function in chromosome end maintenance, but these findings do not exclude the possibility that BLM may also alleviate secondary structures formed in the G-rich telomeric lagging strand.

We have proposed that critically shortened telomeres on metaphase chromosomes in BS cells will be processed into fusion products and contribute to genomic instability. Telomeres that become critically short, or otherwise experience replication dysfunction, are rapidly recognized and bound by proteins in the DNA damage pathway. A DNA damage response triggered at chromosome ends and the subsequent accumulation of DNA repair factors are termed telomere-damage induced foci (TIFs). This signaling event activates the
DNA repair pathways and commonly generates fusion products (d’Adda di Fagagna et al., 2002; Takai et al., 2003). IF-FISH of Bloom’s syndrome cells showed an increase in the number of cells with 53BP1 foci and γH2AX foci (Figure 2-9B, top panel and Figure 2-9D, top panel). We also observed an increase in TIF frequency compared to IMR90 fibroblast cells. Elongation of truncated telomeric sequence by hTERT suppressed the frequency of damage-induced foci and TIF formation (Figure 2-9A to D), thus confirming that a subset of DNA damage events in BS cells occur at telomeres.

A number of publications have noted BLM localization with telomeres, or predicted a telomere-specific function for this RecQ helicase, particularly in cells with an active ALT (alternative lengthening of telomeres) pathway (Yankiwski et al., 2000). However, there has been very little evidence connecting BLM activity with telomere maintenance. The data collected in this chapter reflect an extensive examination of BLM localization to chromosome ends, and a thorough characterization of telomere phenotypes in fibroblasts lacking a functional BLM RecQ helicase. However, we have shifted our prediction that BLM and WRN perform similar or overlapping functions. WRN localizes to telomeres during late S phase, while BLM binding is observed in late G2. Furthermore, CO-FISH data reveal that BLM deficiency impacts maintenance of both the leading and lagging telomeric strands. These findings support the conclusion that BLM does participate in telomere maintenance through a novel mechanism.
Anaphase and metaphase data show that cells deficient in BLM demonstrate critically shortened telomeres and chromosome fusions; elongation of shortened telomeres reduces the appearance of these genome instability markers. We further confirmed a BLM-specific role in telomere maintenance by analysis of BLM knockdown in IMR90 cells and BLM expression in BS cells. Therefore, we conclude that the BLM RecQ helicase participates in normal telomere maintenance pathways in unchallenged mammalian fibroblasts.
Materials and Methods

Cell Culture and production of cell lines

IMR90 primary lung fibroblasts, GM02548 and GM03402B primary BS fibroblasts, and AG05229 primary WS fibroblasts (Coriell Cell Repositories, Camden, NJ) were grown in Glutamax-DMEM (Gibco/Invitrogen, Carlsbad, CA) supplemented with 15% FBS, 0.1 mM nonessential amino acids and 100 units/ml penicillin/streptomycin. Cells were grown at 7.5% CO₂ and 3% O₂.

Retroviral infection to generate stable cell lines was performed as previously described (Smogorzewska et al., 2002). Phoenix amphotrophic packaging cells (Orbigen) were transfected with plasmids or shRNAs using calcium phosphate method. To generate stable cell lines, the culture media containing retrovirus was removed from phoenix amphotrophic cells at 36 hours, 48 hours, and 72 hours post transfection. The media was passed through a 0.45 µm filter, supplemented with 4 µg/ul polybrene, and directly applied to fibroblasts or HeLa LT cells. Following puromycin selection, cells were collected for downstream analyses.

Inhibition of the p53 and pRb pathways in fibroblast cells was achieved by expression of the E6 and E7 HPV oncoproteins, which were produced by LSXN packaging cells (Smogorzewska et al., 2002). Plasmids carrying cDNA of hTERT, DN_hTERT, and WRN were available in the Karlseder lab; hTERT vectors were graciously provided by Bill Hahn (Crabbe et al., 2004; Hahn et al., 1999). BLM knockdown was achieved by introducing shBLM
(TGCCAATGACCAGGCGATC) into the pSuper.retro puromycin retroviral vector (Oligoengine) (Crabbe et al., 2004). To reconstitute BLM in BS cells, BLM cDNA (Open Biosystems) was cloned into the pBabe-puro retroviral vector (Addgene).

**Fibroblast synchronizations and Chromatin Immunoprecipitation**

IMR90 fibroblasts cells grown in 15 cm dishes were synchronized in G1/S boundary phase by double- thymidine block. At two-hour intervals, cells were collected and processed for ChIP, FACs and IF-FISH. IMR90 cells in 15cm dishes were washed with 1x PBS and crosslinked by incubation with 1% formaldehyde in PBS. After 30 minutes, the crosslinking reaction was quenched in 0.1 M Glycine for five minutes and then cells were washed three times with 1xPBS. Cells were harvested in PBS from 15 cm plates and washed twice in 1x PBS. Pelleted cells were carefully resuspended in freshly made lysis solution with protease inhibitors (1% SDS, 0.5 M NaCl, 0.01 M Tris-HCl), and kept on ice for 15 minutes before storage at -80°C, when necessary. The DNA was sheared with a BioRuptor; cell lysates were given a total of 60 minutes sonication, 30 seconds on and 30 seconds off. At the mid-point, lysates were kept at 4°C for a 15-minute rest period. ChIPs were performed as described previously (Verdun et al., 2005).

**Telomere Southern Blots**

At increments of approximately five population doublings, cell lysates were collected from both BS cells and IMR90 cells over the course of their lifespan in culture. Genomic DNA was extracted and purified, then treated with frequent
cutter enzymes, Alu/MboI, digesting genomic sequence. The resulting undigested telomeric sequence was run overnight on an agarose gel. The gel was dried, washed with SSC and pre-hybridized in Church’s mix (0.5 M Sodium Phosphate buffer pH 7.2, 0.5 M EDTA, 1% BSA, 7% SDS) for approximately two hours. A radio-labeled telomeric probe was generated by incubating a telomeric primer, $^{32}$P-ATP and Klenow polymerase at 37°C for 1.5-2 hours, then column-purified. The pre-hybridized gel was incubated overnight at 37°C with radio-labeled telomeric probe in Church’s mix. The gels were thoroughly washed and exposed in a phosphoscreen cassette (Amersham Biosciences) before scanning on a Typhoon 8600 (Molecular Dynamics).

**Image Analysis**

Slides and coverslips were examined with a Zeiss Axio Imager.Z1 fluorescence microscope with apotome. Z-stack projections were captured with a Hamamatsu ORCA-ER digital camera and analyzed in AxioVision software (Released 4.6.3). Colocalization events in interphase cells were obtained by a second, automated method to bolster sample size and statistical confidence. Slides were imaged with the fluorescence MetaSystems slide scanner, which is composed of a multi-slide Zeiss fluorescence microscope managed through the MetaSystems Metafer software. Images were collected, processed, and analyzed by a specifically developed program that quantified the number of red foci, green foci, and co-localizations for each cell. The cumulative descriptive data were
combined to automatically generate population statistics for each cell line (program provided by Metasytems).

**Immunofluorescence, Immunofluorescence – Fluorescence in situ Hybridization (IF-FISH)**

Fibroblasts were grown on glass coverslips to approximately 75% confluence, washed in PBS and fixed in 4% PFA at room temperature for 10 minutes. Coverslips were washed with three PBS changes in 20 minutes, then incubated in blocking solution (0.3% Triton X-100, 5% Normal Goat Serum, 1mg/ml BSA in 1x PBS) for one hour prior to antibody staining. For BLM detection, coverslips were incubated with 7099 at 4°C for 16 hours. Secondary antibody staining was performed with Donkey anti-Rabbit Alexa 594 fluorescent conjugate, according to manufacturer recommended protocol (Invitrogen). For TIF detection with γH2AX cells were fixed in 2% PFA at room temperature for 10 minutes and immuno-stained according to the manufacturer’s recommendation (Cell Signaling).

When performing IF-FISH the secondary antibody fluorescent signal was fixed by a second incubation with 4% PFA at room temperature for 10 minutes. Coverslips were washed with three PBS changes in 20 minutes, ethanol dried, and stored overnight at room temperature. FISH was performed as previously described, using a telomeric PNA probe. DNA was DAPI stained and slides were mounted with Pro-Long Antifade Reagent (Invitrogen). Interphase cells and anaphases were imaged as described above, with a Zeiss Axio Imager.Z1.
Production of BLM antibody

To generate a BLM antibody, I generated and purified a BLM protein fragment using the Invitrogen Gateway protein expression system. 1.3 kilobases of the N-terminus of the BLM cDNA were amplified with manufacturer-specified 5' and 3' entry sequences. Purified amplification product was cloned into the pENTRY His96W Gateway expression vector (Invitrogen) and then transformed into cultured BL21 bacterial cells (Invitrogen). The expression product was composed of amino acids 1-438 of the BLM RecQ helicase with an N-terminus of nine histidine residues. Successful expression of the 9x His-tagged BLM fragment was confirmed by western blot with His antibody (Sigma). The protein was purified over a column of Talon Resin Affinity beads (Clontech).

For production of the anti-BLM antibody, the protein was dialyzed in PBS. A rabbit (#7099) was immunized with four 200 µg injections of the purified BLM fragment. The serum was applied to an affinity column composed of the purified 1-438 amino acid residues coupled to CNBr-activated sepharose beads (GE Healthcare). The column was washed with PBS and PBS/0.5 M NaCl before eluting the purified antibodies with 0.1 M Glycine, pH 2.5. BLM antibodies were collected in 500 µl fractions, neutralized with 35 µl of 1 M Tris-HCl, pH 9.5, and brought to a final concentration of 0.157 M NaCl and 10% glycerol. Purified BLM polyclonal antibody was characterized and tested for specificity by western blotting and immunofluorescence.
Antibodies and Western Blotting

Anti-BLM (Rabbit, #7099) (Orazio et al., 2010), Anti-TRF1 (Rabbit, #6839), and Anti-TRF2 (Rabbit, #6841) were produced at the Salk Institute. Commercial antibodies used: anti-γH2AX (Cell Signaling), anti-53BP1 (A300-273A; Bethyl Laboratories), and anti-WRN (ab200, Abcam). For Western Blotting, cells were harvested, washed in 1x PBS, counted, and lysed in Laemmeli buffer before loading whole cell lysates onto Tris-Acetate gels. Antibody dilutions were experimentally determined or based on manufacturers’ specifications.

Metaphase preparation and FISH

BS fibroblasts or untreated IMR90 fibroblasts were grown to 75% confluence and incubated with 0.1 µg/mL colcemide for two and a half hours before harvesting by trypsinization. Pelleted cells were carefully resuspended in a 0.075 M KCl hypotonic solution and incubated at 37ºC for seven minutes to induce swelling. Cells were fixed in ice cold Methanol: Acetic Acid (3:1) and stored at 4ºC overnight. Metaphase chromosomes were prepared by dropping cell suspensions on glass microscope slides, which were placed on a humidified heat block at 72ºC for one minute. Slides were dried overnight at room temperature before proceeding to hybridization.

Metaphase spreads were re-hydrated in PBS, fixed with 4% formaldehyde and pepsin treated for 10 minutes at 37ºC. Following a short PBS wash, cells were again fixed with 4% formaldehyde, washed and ethanol dried. FISH was carried out with a fluorescent PNA telomeric probe, FITC-OO-[CCCTAA]₄
(Applied Biosystems) as previously described (Smogorzewska et al., 2002). Hybridization solution was applied to each slide and denatured at 80°C for three minutes before incubation in a humidified chamber for three hours. DNA was DAPI stained and slides were mounted with Pro-Long Antifade Reagent (Invitrogen). Metaphases were imaged with a Zeiss Axio Imager.Z1, as described above.

**Metaphase preparation and CO-FISH**

HeLa LT cells were incubated with 10 µM BrdU and 3.3 µM BrdC for approximately 12-14 hours (time for one complete cell cycle). Cells were arrested in mitosis by adding 0.1 µg/mL colcemide to the culture media and incubating for two hours. Preparation of glass slides with metaphases spreads was performed as described above and dried overnight at room temperature before proceeding to hybridization.

Metaphase spreads were re-hydrated in PBS, treated with 0.5 mg/ml RNase for 10 minutes at 37°C and fixed with 4% formaldehyde. Cells were stained with 0.5 µg/ml Hoechst 33528 (Sigma) in 2x SSC for 15 minutes at room temperature, in the dark. Slides were submersed in 2x SSC and exposed to 365 nm UV light (Stratalinker 1800 UV irradiator) for 30 minutes; this exposed cells to the equivalent of 5.4 x10^3 J/m^2. To remove excess salt, slides were briefly rinsed with diH₂O before digestion with 1 U/µl Exonuclease III (New England Biolabs) for 15 minutes at room temperature. Slides were briefly rinsed in diH₂O, and then incubated with 70% formamide/ 2x SSC at 80°C for one minute. After PBS
washes, slides were dehydrated in an ethanol series, 70%, 95%, 100% ethanol, and air dried.

To perform CO-FISH, metaphase chromosomes were first hybridized with a fluorescently-labeled PNA probe binding the telomeric leading strand, TAMRA-OO-[GGGATT]₃ (Applied Biosystems). Slides were heat denatured at 80°C and incubated in a humidified chamber for two hours at room temperature. Following a 15 minutes in PNA wash A (70% Formamide, 0.01 M Tris-HCl pH 7.5), we then hybridized metaphases with a second PNA probe binding the lagging telomere probe, FITC-OO-[CCCTAA]₃. Hybridization was followed by two washes in PNA Wash A, and three washes in PNA Wash B (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.08% Tween-20). DAPI was added to the second wash in PNA Wash B, to a final concentration of 0.5 µg/mL. Slides were washed in an ethanol series: 70%, 95% and 100%, and completely air-dried before mounting with ProLong Antifade (Invitrogen).
Acknowledgements

I am extremely grateful to the co-authors on the published article in PNAS, Laure Crabbe, Anna Jauch, and Heidi Holtgreve-Greve, for their significant contributions in the WRN helicase project. Figure 2-6 was re-printed directly from our publication:

Chapter 3. BLM RecQ helicase recruitment to dysfunctional telomeres

Background

As ‘caretakers’ of genome stability, there are multiple mechanisms by which BLM and WRN are predicted to contribute to efficient replication and proper DNA damage and repair action. WRN and BLM RecQ helicases demonstrate similar in vitro activity at non-standard DNA structures, they colocalize in a number of cell types, and both are necessary for proper genome maintenance (Chu et al., 2009). It is also possible that BLM and WRN perform similar or overlapping roles in telomere maintenance, particularly because telomere defects characterized in BS cells occur at approximately the same frequency as WS cells, and hTERT-mediated telomere elongation in WS and BS fibroblasts virtually restores telomere dysfunction. However, the data presented in Chapter 2 indicate a subtle difference between BLM and WRN helicase activity in telomeric processing pathways (see Table 3-2). Therefore, we will investigate the potentially distinct activity of these two helicases.

A number of recent publications describe a new role for BLM in faithful chromosome segregation during mitosis. BLM forms a complex with TOPOIIIα, RMI1, and RMI2 (the BTR complex), which processes late replicating intermediates (LRI). These are composed of unresolved DNA structures that persist into mitosis and are detectable as ultra-fine bridges in anaphase. These fragile structures are known to contain newly replicated DNA (visualized by BrdU incorporation) and are predicted to form at intermediate DNA structures that
result from incompletely replicated DNA or fully replicated, but partially unresolved, double Holliday junctions. Though undetectable by DNA counterstains, these UFBs can be visualized with antibodies against associated proteins. UFBs are shown to most frequently originate from centromeres and fragile sites, though one report observed a number of bridges extending from telomeres (Chan et al., 2007; Chan et al., 2009).

Telomere replication defects are relatively common and a number of telomere-associated proteins are known to facilitate replication fork progression through this TTAGGG-repeat region of the genome. Interestingly, recent studies have shown that telomeres may behave similarly to genomic fragile sites. While most fragile sites experience a delay in replication timing, they do not commonly affect overall genome stability. However, these regions are hypersensitive to treatment with replication inhibitors, such as aphidicolin (Glover et al., 1984). Exposure to this compound induces fragile site expression – classically manifested as chromosome breaks and/or chromosome deletions – and causes an increase in the frequency of ultra-fine bridges extending from the common fragile site, FANCD2 (Chan et al., 2009; Naim et al., 2009). Aphidicolin treatment has also been shown to reduce telomere replication efficiency, particularly in the absence of a key telomeric protein, TRF1. A heightened sensitivity to mild replication stress led to the prediction that telomeres act as fragile sites (Sfeir et al., 2009).
The BLM-TOPOIIIα-RMI1-RMI2 complex is found at areas of post-replication processing, where it facilitates dissolution of persistent late-replicating intermediates. Though our data do not exclude the possibility that BLM also assists WRN with lagging strand synthesis, we propose a novel mechanism by which BLM contributes to efficient telomere maintenance. The data presented in this chapter suggest that BLM acts at telomeres in response to the difficult-to-replicate nature of telomeres. Regardless of the mechanism, both RecQ helicases independently contribute to efficient chromosome end maintenance the loss of both induces an even greater frequency of telomere defects. We also find that BLM activity in telomere maintenance is evidently more significant following drug-induced replication dysfunction or in a genetic background of telomere dysfunction, such as in WS cells. Replication dysfunction led to an increase in BLM foci and in the subset of BLM foci co-localizing with telomeres. In addition, not only do UFBs occur more frequently in WS cells, but these late-replicating intermediates also persisted later in anaphase, which may be indicative of more extreme processing defects.
Results

A deficiency of WRN and BLM RecQ helicases leads to frequent telomere defects and chromosome aberrations

The frequencies of telomere defects in both BS fibroblasts cell lines are remarkably similar to the telomere defects counted in WS cells (Crabbe et al., 2004; Crabbe et al., 2007). Furthermore, the expression of hTERT significantly reduced telomere defects and anaphase bridges in BS cells; as in the un-transformed fibroblast cells, the significant reduction of defects were almost identical to the effect hTERT had on defects in WS cells. We wanted to determine if these helicases act in the same pathway with overlapping activities.

BLM expression was knocked down in WS cells and WRN expression was knocked down in BS cells by retroviral delivery of an shRNA against the target gene and puromycin selection to generate stable cell lines. Western blotting confirmed efficient reduction of WRN and BLM protein expression (Figure 3-1A, top and bottom panels, respectively). Cells were collected and prepared for FISH analysis of metaphase chromosomes, as described in Chapter two. Examination of metaphase spreads revealed a significant increase in telomere defects and chromosomal aberrations in fibroblast cells treated with shRNA against BLM or WRN helicase (Figure 3-1C). Quantification of these events shows a significant increase in telomere defects in cells deficient in both helicases, compared to fibroblasts lacking only BLM or WRN (Figure 3-1B).
Figure 3-1. Loss of both BLM and WRN RecQ helicases exacerbates telomere defects. Werner syndrome fibroblasts (AG05229-E6E7) and Bloom syndrome fibroblasts (GM02548-E6E7 and GM3402B-E6E7) were retrovirally transduced with an shRNA targeting BLM or WRN, respectively, or with a non-coding shRNA. Stable cell lines were generated by puromycin selection and cells were prepared for FISH of metaphase chromosomes, as described previously. (A) Western blot confirming successful reduction of WRN or BLM; the bottom panel represents a tubulin loading control. (B) The percent of chromatids with telomere defects (TDs). We counted at least 2000 chromatids from each cell line and average values were compiled from two experiments; arrow bars indicate standard deviation and \( p \) values were calculated by a student’s t-test (*\( p < 0.05 \) and **\( p < 0.005 \)). (C) Examples of metaphases from AG05229-E6E7 (WS) fibroblasts following BLM knockdown and GM02548-E6E7 (BS) fibroblasts following WRN knockdown. Arrows indicate chromosomes in the magnified panels below each metaphase spread.
**WS cells exhibit an increase in BLM foci and Ultra fine bridge formation**

These data suggest that BLM and WRN independently contribute to telomere maintenance, but do not exclude a possible connection or overlap between both RecQ helicases in the process of chromosome end protection. To explore if BLM recruitment to telomeres responds to the absence of WRN helicase activity, we examined BLM expression and localization in WRN deficient fibroblasts compared to IMR90 cells. IF-FISH was performed to visualize BLM protein and telomeric DNA. WS cells showed a distinct change in BLM localization patterns in WS cells compared to untreated IMR90 cells (Figure 3-2A). Quantification confirmed a significant elevation in BLM foci per cell (Figure 3-2B) and a modest increase in cells exhibiting multiple colocalization events between BLM and telomeric foci (Figure 3-2C).
Figure 3-2. BLM shows distinct localization patterns between IMR90 and WS fibroblasts. IMR90 cells and WS cells, without or without expression of E6 and E7 oncoproteins – were fixed with 4% PFA. IF-FISH was performed with a BLM antibody (red) and a FITC-[CCCTAA]₄ oligonucleotide (green). (A) IF-FISH images from IMR90-E6E7 and AG05229-E6E7 fibroblast cells, representing distinct BLM localization patterns. Arrows indicated colocalization events between BLM and telomeric foci. (B) The calculated average of BLM foci per cell and (C) percent of cells with over two colocalization events between BLM and telomeres. The values for each cell line were averaged from three independent experiments; arrow bars represent standard deviation and p values were calculated by a student’s t-test (*p < 0.05 and **p < 0.005).
In the process of examining BLM localization patterns, we frequently observed UFBs, which have been described by Ian Hickson’s group. UFB formation was assessed by IF-FISH in IMR90 fibroblasts and Werner’s syndrome fibroblasts, with and without the E6 and E7 oncoproteins. Our data confirmed published findings that these late-replicating intermediates are common events even in normal cells (Chan et al., 2007; Chan et al., 2009). Extensive characterization of UFBs in both cell types revealed significant differences between untreated IMR90 fibroblasts and the replication-defective WS cells. We recorded data on features of anaphases, such as number of UFBs, UFB proximity to telomeric foci, and the distance between separating chromosomes.

The final data were generated from five independent IF-FISH experiments, which included at least 20 anaphases from each cell line, per experiment (Figure 3-3A). Initial screening for frequency of anaphases with at least one UFB found that WRN-deficient cells had a higher frequency of UFB-positive anaphases (Figure 3-3B). We next combined the data from all five experiments and grouped anaphases according to the distance between separating DAPI clusters (Figure 3-3C). From these data, we observed that greater number of UFBs per anaphase in WS cells, and furthermore, these UFBs persisted longer through anaphase (Figure 3-3D).

As previously reported, a portion of UFBs was observed to extend (from one or both ends) from telomeric foci (Figure 3-4A). These telomere-derived UFBs were significantly more common in WS cells (Figure 3-4B).
Figure 3-3. Ultra-fine bridges (UFBs) occur in normal fibroblast cell lines, but are more frequent and persist longer in AG05229 WS fibroblasts. IMR90-E6E7 and AG05229-E6E7 cells grown on glass cover slips were fixed and prepared for IF-FISH, as described previously. (A) Representative images of fibroblast anaphases; UFBs are visualized with BLM antibody (red). (B) The percent of anaphases with UFBs. These values were calculated from four independent experiments, where at least 30 anaphases were analyzed from each cell line. Statistical significance was determined by a two-tailed student’s t-test (*p < 0.05 and **p < 0.005). Figure 3-3 continued on next page.
Figure 3-3 (continued). Ultra-fine bridges (UFBs) occur in normal fibroblast cell lines, but are more frequent and persist longer in AG05229 WS fibroblasts. IMR90-E6E7 and AG05229-E6E7 cells grown on glass cover slips were fixed and prepared for IF-FISH, as described previously. (C) DAPI stained anaphases representing the distance between dividing cells in each of the four stages: N.S. (Not Separated), Early, Mid, and Late anaphase. (D) Percent of IMR90-E6E7 anaphases (left panel) and AG05229-E6E7 anaphases (right panel) with the indicated number of UFBs, according to the degree of separation. Anaphase data from each cell line were accumulated from four independent experiments and were grouped into four populations, determined by the distance (in µm) between separating DNA clusters (see panel C). At least 25 anaphases were analyzed for each cell line per experiment.
Figure 3-4. A subset of Ultra fine bridges (UFBs) extend at one or both ends from telomeric regions. IF-FISH was performed as described previously. UFBs were detected by staining with BLM antibody (red) and telomeres were visualized with a FITC-[CCCTAA]₄ (green). (A) The anaphases shown here represent UFBs that do not localize with telomeric foci, UFBs that extend from a telomeric region at one end, and UFBs that colocalize with telomeres at both ends. Graphs illustrating the signal intensities were generated by the ‘profile’ function on AxioVision software. (B) The percent of UFBs extending from telomeres. These values were calculated from four independent experiments, where at least 30 anaphases were analyzed from each cell line. Statistical significance was determined by a two-tailed student’s t-test (*p < 0.05 and **p < 0.005) and arrow bars indicate standard deviation.
As an added measure to confirm that WRN-dependent replication contributes to more frequent UFB formation, we examined anaphases from WRN-complemented WS cells. The same population of WS cells used in the previous experiment were stably transduced with a control vector or the full length WRN helicase. The loss of WRN does particularly affect the requirement for UFB-associated BLM localization to telomeres, since expression of WRN in WS cells significantly reduces the percent of anaphases with UFBs (Figure 3-5A) and the percent of UFBs that extend from telomeric signal (Figure 3-5B).
Figure 3-5. Expression of WRN in AG05229 cells reduces the frequency of UFB formation and suppresses UFB formation at telomeres. AG05229 cells were transduced with a vector control or the full length WRN cDNA. Cells were selected with puromycin to generate stable cell lines. After approximately five-ten population doublings, cells were fixed with 4% PFA. IF-FISH, with a BLM antibody and a fluorescent telomeric probe, was performed in two independent experiments. At least 30 anaphases per cell line were analyzed in each experiment. (A) Quantification of the percent of anaphases with UFBs and (B) the percent of UFBs that extend from telomeres. The values for each cell line were averaged from two independent experiments; arrow bars represent standard deviation and p values were calculated by a student’s t-test (*p < 0.05 and **p < 0.005).
Treatment with replication inhibitor induces more frequent formation of UFBs and changes in cellular localization of the BLM RecQ helicase

Telomeres have recently been shown to behave similarly to genomic fragile sites, which are hypersensitive to treatment with replication inhibitors, such as aphidicolin (Glover et al., 1984). Exposure to this compound induces chromosome breaks and/or chromosome deletions, as well as an increase in the frequency of ultra-fine bridges extending from common fragile sites (Chan et al., 2009). Aphidicolin treatment has also been shown to reduce telomere replication efficiency, particularly in the absence of a key telomeric protein, TRF1. A heightened sensitivity to mild replication stress led to the prediction that telomeres act as fragile sites (Sfeir et al., 2009).

WS cells experience telomere replication defects and have a greater number of UFBs, therefore we asked if these cells were sensitive to aphidicolin exposure and exhibit fragile site behavior at telomeres. The difference in BLM localization patterns in cells treated with EtOH compared to aphidicolin was immediately evident (Figure 3-6A), and quantification revealed a two-fold increase in the number of BLM foci per cell (Figure 3-6B). This significant change in BLM staining pattern was greater in WS cells, suggesting that the replication efficiency in this cell line is more severely compromised, and therefore requires BLM recruitment to areas of replication dysfunction.
Figure 3-6. Treatment with aphidicolin induces changes in BLM localization patterns. IMR90-E6E7 and AG05229-E6E7 cells were incubated with 15% FBS media containing 0.4\(\mu\)M aphidicolin for 24 hours, then washed well and grown in untreated media for five hours. Cells were fixed with 4% PFA and analyzed by IF-FISH with a BLM antibody and a telomeric probe. (A) IF-FISH images from IMR90-E6E7 and AG05229-E6E7 fibroblast cells. Arrows indicate colocalization events at BLM bridges observed in interphase cells. (B) Average BLM foci per cell. At least 100 cells were counted per each condition per experiment. Values were averaged from three independent experiments and \(p\) values were calculated by a two-tailed student’s t-test (\(*p < 0.05\) and \(**p < 0.005\)).
Werner’s syndrome cells without E6 and E7 have such a low mitotic index it was very rare to locate anaphases, therefore only IMR90-E6E7 and WS-E6E7 cell populations were included in IF-FISH analysis of UFB formation (Figure 3-7A). Anaphases with UFBs were more frequent following treatment with the replication inhibitor (Figure 3-7B), which is similar to previous reports that aphidicolin-induced delay of replication increases the number of late-replicating structures that are observed as UFBs (Chan et al., 2009; Naim et al., 2009). It is of note that this replication inhibitor induces expression of fragile sites throughout the genome, therefore a large proportion of the BLM foci in IMR90 and WS fibroblasts form at inter-chromosomal regions. IMR90 and WS fibroblast cells displayed a similar increase in the percentage of UFBs extending from telomeres (Figure 3-7C). This is likely due to the genome-wide effect of aphidicolin treatment, and would not be expected to specifically induce a delay in telomere-specific replication.
Figure 3-7. Treatment with aphidicolin increases UFB formation. IMR90-E6E7 and AG05229-E6E7 cells were incubated with 15% FBS media containing 0.4µM aphidicolin for 24 hours, then washed well and grown in untreated media for five hours. Cells were fixed with 4% PFA and analyzed by IF-FISH with a BLM antibody and a telomeric probe. (A) IF-FISH images from IMR90-E6E7 and AG05229-E6E7 fibroblast cells with UFBs. (B) Percent of anaphases with UFBs and (C) percent of UFBs extending from telomeres in IMR90-E6E7 and AG05229-E6E7 cells treated with DMSO or aphidicolin. Statistical analyses were performed, as described previously. Values were averaged from three independent experiments and $p$ values were calculated by a two-tailed student’s t-test ($^*p < 0.05$ and $^{**}p < 0.005$).
Induction of telomere replication defects by TRF1 knockdown increase UFB formation in fibroblasts

Although loss of the WRN RecQ helicase is detrimental to lagging strand replication, telomere replication defects occur at varying degrees of severity and may be partially resolved by other factors, such as POT1 (Arnoult et al., 2009). The telomere-associated TRF1 protein has a documented role in telomere length regulation (van Steensel and de Lange, 1997). More recently, TRF1 is shown to facilitate efficient telomere replication and prevent unresolved intermediates that persist into mitosis (Sfeir et al., 2009). To more accurately examine if BLM is recruited to chromosome ends in response to a telomere replication delay, we induced an additional replication defect by removing TRF1 in WRN deficient cells. Loss of both proteins should specifically affect telomere replication and we asked if a more severe telomere replication dysfunction correlated with UFB formation and UFBs extending from telomeric DNA.

TRF1 knockdown was achieved by transfecting IMR90-E6E7 and AG05229-E6E7 cells with ON-TARGET plus SMARTpool TERF1 siRNA (Dharmacon, Thermo Scientific). After 72 hours with siRNA, successful knockdown was confirmed by IF and WB with a TRF1 antibody (Figure 3-8A,B). Cells grown on glass cover slips were fixed and subjected to IF-FISH with the BLM antibody and a fluorescent telomeric probe. Reduction of TRF1 indeed led to a significant increase in formation of UFBs in both IMR90-E6E7 and AG05229-E6E7 cell lines (Figure 3-8C). However, we did not observe an increase in UFBs
extending at one or both ends from telomeric foci. This may be due to the significant telomere loss induced by TRF1 and WRN deficiencies; therefore telomeric signal may below detection by FISH method.
TRF1 knockdown increases frequency of UFB formation. TRF1 knockdown was achieved by transfecting IMR90-E6E7 and AG05229-E6E7 cells with siRNA. After 72 hours, successful knockdown was confirmed by IF and WB with a TRF1. (A) Cells grown on coverslips were fixed with 4% paraformaldehyde and before co-immunofluorescence with antibodies staining TRF1 and TRF2. (B) Whole cell lysates were analyzed by SDS-PAGE and immuno-blotted with the indicated antibodies. (C) Frequency of anaphases bridges with BLM-positive ultra fine bridges. (D) Frequency of UFBs that extend from telomeric foci at one or both ends of the bridges. To examine anaphases, cells were fixed with 4% PFA and analyzed by IF-FISH with a BLM antibody and a telomeric probe. Statistical analyses were performed, as described previously. Values were averaged from two independent experiments and p values were calculated by a two-tailed student’s t-test (*p < 0.05 and **p < 0.005).
Figure 3-9. Metaphase chromosomes from (A) IMR90-E6E7 and (B) AG05229-E6E7 fibroblast cell lines with TRF1 knockdown. Telomere defects in BLM and TRF1-deficient fibroblast cells. Fibroblast cells were treated with Dharmacon siRNA. After 72 hours, cells were incubated with colcemide for two hours and then collected for FISH analyses of metaphase chromosomes. White arrows indicate chromosomes in magnification panels below each metaphase spread. Note the telomere bridges that span two chromosome ends and the chromatids with multiple telomere signals.
Table 3-1. Telomere defects in BLM and TRF1-deficient fibroblast cells. Fibroblast cells were treated with Dharmacon siRNA. After 72 hours, cells were incubated with colcemide for two hours and then collected for FISH analyses of metaphase chromosomes. Telomere defects are represented as the percent of chromatids with sister telomere loss or telomere free ends.

<table>
<thead>
<tr>
<th></th>
<th>siNON</th>
<th>siBLM</th>
<th>siTRF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMR90-E6E7</td>
<td>2.8</td>
<td>4.1</td>
<td>5</td>
</tr>
<tr>
<td>AG05229-E6E7</td>
<td>5.1</td>
<td>7.2</td>
<td>12.2</td>
</tr>
</tbody>
</table>
**Discussion**

*WRN and BLM independently contribute to telomere maintenance*

In this chapter, we illustrated that BLM can localize to telomeres independently of WRN. Metaphase analysis clearly shows that telomere defects and chromosome integrity are abrogated when BLM and WRN are absent. Possibly BLM also facilitates lagging strand replication by resolving G-quadruplex structures in the G-rich DNA, and is more frequently recruited to telomeres to compensate for WRN loss. Our CO-FISH data suggested that BLM activity at telomeres is not strand specific (Chapter 2, figure 2-8). BLM may assist in lagging strand replication, but our data indicate another function at telomeres.

As extensively described by this lab and others, WS cells have significant telomere replication defects. In addition to facilitating lagging strand replication, WRN helicase activity suppresses homologous recombination at telomeres, probably due to its role in unwinding inappropriate strand invasion at stalled replication forks (Laud et al., 2005). WRN is also implicated in telomere processing by unwinding t-loop structures that sequester the extreme terminus of chromosome ends and stably protect telomeres from recognition by DNA damage and repair proteins. This secondary level of telomere protection is cell-cycle regulated and must be ‘opened up’ to ensure that telomere length is fully replicated. The loss of WRN helicase function leads to hyper-homologous recombination at telomeres.
We next considered if the presence of BLM at telomeres is regulated by replication efficiency and if BLM recruitment is altered in response to telomere dysfunction, such as in WS cells (Arnoult et al., 2009; Crabbe et al., 2004; Chang et al., 2004). We observed a significant increase in BLM foci per cell, as well as an increase in cells with colocalization events between BLM and telomeric foci. These observations in WRN deficient cells supported our prediction that BLM localization patterns correlate with severity of telomere dysfunction.

**BLM positive UFBs are more frequent in the replication dysfunctional WRN deficient cells**

BLM is a necessary component in the BTR complex (BLM-TOPOIIIα-hRMI1-hRMI2) that binds and dissolves late-replicating intermediates (LRIs); these DNA-protein complexes are observed as UFBs (Chan, et al., 2007). UFBs are commonly observed under ‘normal’ conditions in most cell types, and the reported frequency in unchallenged fibroblasts was quite similar to our observations in IMR90. They are most frequently seen during early anaphase, predictably because a significant proportion is resolved before cells progress into mid-to-late anaphase (Chan et al., 2007; Chan et al. 2009). By organizing my data with particular consideration for the progress through anaphases, I was able to develop more detailed conclusions about BLM-positive bridges. In very early anaphases, before newly replicated DNA separate, both IMR90 and WS fibroblasts contain up to eight BLM-positive bridges, but cells rarely had more than two UFBs by mid-anaphase. I observed that the overall frequency of UFB-
positive anaphases and the extended duration of UFBs into mid and late-anaphase were remarkably greater in WS cells.

Furthermore, a number of BLM-positive bridges appeared to form at – or immediately adjacent to – telomeric foci marked by a fluorescent oligonucleotide. According to previous characterization of UFBs, the presence of these foci at bridge termini indicates that ultra fine DNA is composed of telomeric repeats. We concluded that these events represent delayed replication at telomeres. Telomere replication defects are relatively common and a number of telomere-associated proteins are known to facilitate replication fork progression through this TTAGGG-repeat region of the genome. UFBs that extend from telomeric foci at one or both ends (T-UFBs) were seen in normal and WS fibroblasts, suggesting that even in unchallenged conditions, the replication efficiency at telomeres is compromised (Figure 3-3). However, T-UFBs were significantly greater in WS cells (Figure 3-4). This observation was un-surprising, since WRN-deficient cells have a much higher rate of telomere dysfunction than normal IMR90 fibroblasts.

To confirm that WRN helicase activity prevents the replication dysfunction and UFB formation at telomeres, I performed IF-FISH in WRN-complemented WS cells and analyzed the frequency of UFB formation. WRN deficiency does particularly affect UFB-associated BLM localization to telomeres, since expression of WRN reduces the percent of anaphases with UFBs (Figure 3-5A) and the proportion of UFBs that extend from telomeric signal (Figure 3-5B).
result implicates BLM activity at chromosome ends in response to telomere defects. WRN helicase is required for efficient lagging strand replication and suppression of inappropriate recombination at telomeres (suppresses T-SCEs). WRN deficiency alone can lead to inappropriate and detrimental recombination events. However, WRN deficiency coupled with dysfunctional telomeres induces more frequent aberrant phenotypes (Laud et al., 2005).

These data suggest that changes in BLM activity in WS cells are a response to general telomere dysfunction, rather than a telomere-specific function or a particular shared RecQ helicase activity. BLM and interacting proteins associate with intermediate DNA structures, which appear to originate from genomic regions with intrinsic replication difficulties. If so, we considered that BLM’s participation in chromosome end maintenance is through a ‘back-up’ mechanism to quickly dissolve late-replicating DNA structures without disturbing cell cycle progression. This evidently occurs at normal levels in unchallenged telomeres, due to the frequency of UFBs in early-anaphases from healthy IMR90 fibroblasts (Figure 3-3). However, perhaps these late replicating intermediate structures are more commonly observed when replication is challenged?

*Treatment with replication inhibitor induces more frequent formation of UFBs and changes in cellular localization of the BLM RecQ helicase*

Fragile sites are difficult to replicate regions of the genome that are highly sensitive to treatment with partial replication inhibitors, yet the intrinsic property that makes them so vulnerable to replication challenge remains generally
unknown. The list of these regions continues to grow, but the most well
described regions are referred to as common fragile sites (CFSs). While most
fragile sites experience a delay in replication timing, they do not commonly affect
overall genome stability. However, exposure to a replication inhibitor, such as
aphidicolin, induces chromosome breaks and/or chromosome deletions and
increases the frequency of ultra-fine bridges (Naim et al., 2009). Generally
UFBs extending from fragile sites are observed by IF-FISH, using a DNA
oligonucleotide that binds the sequence of the CFS, FANCD2 (Chan et al., 2009;
Naim et al., 2009).

WS cells experience telomere replication defects, therefore we asked if
these cells would be more sensitive to aphidicolin exposure and thus produce a
greater number of UFBs than normal IMR90 fibroblasts. The difference in BLM
localization patterns in cells treated with EtOH compared to aphidicolin was
immediately evident, and quantification revealed a two-fold increase in the
number of BLM foci per cell (Figure 3-6). A previous report concluded that
endogenous levels of BLM protein were significantly higher in cells treated with a
replication inhibitor, such as aphidicolin or hydroxyurea (Dutertre et al., 2000).
This significant change in BLM staining pattern was more remarkable in WS
cells, suggesting that the replication efficiency in this cell line is more severely
compromised, and therefore require BLM recruitment to areas of replication
dysfunction.
Because WS cells without E6 and E7 have such a low mitotic index, it was very rare to locate anaphases and therefore the untreated IMR90 and WS cell populations were not included in the UFB data sets. Anaphases with UFBs were more frequent following treatment with the replication inhibitor (Figure 3-7B), which is similar to previous reports that aphidicolin-induced delay of replication increases the residual, late-replicating structures that are observed as UFBs (Chan et al., 2009; Naim et al., 2009). IMR90 and WS fibroblast cells displayed a similar increase in the percentage of UFBs extending from telomeres (Figure 3-7C). This is likely due to the genome-wide effect of aphidicolin treatment, and would not be expected to specifically induce a delay in telomere-specific replication.

*Induction of telomere replication defects by TRF1 knockdown increase UFB formation in fibroblasts*

WS cells are an ideal system for examining BLM recruitment to chromosome ends following telomere-specific dysfunction. Telomere defects are predicted to be the most severe damage events that contribute to compromised genome stability in WRN deficient cells. Telomere defects (such as telomeric SCEs or telomere double-minutes) are observed in WRN deficient cells, however telomere dysfunction significantly increases the appearance of these replication defects (Laud et al., 2005). Previous research identified that the telomere-associated protein, TRF1, facilitates telomere replication and prevents unresolved intermediates that persist into mitosis (Sfeir et al., 2009). Loss of
TRF1 cooperates with aphidicolin based replication challenge to further exacerbate the ‘fragile telomere’ phenotype. It is therefore likely that TRF1 contributes to efficient chromosome end replication by recruiting associated proteins that facilitate replication of challenging regions, perhaps by preventing stalling or enabling fork restart (Sfeir et al., 2009). Reduction of TRF1 indeed led to a significant increase in formation of UFBs in both IMR90-E6E7 and AG05229-E6E7 cell lines.
Materials and Methods

Cell Culture and production of cell lines

IMR90 primary lung fibroblasts, GM02548 and GM03402B primary BS fibroblasts, and AG05229 primary WS fibroblasts (Coriell Cell Repositories, Camden, NJ) were grown in Glutamax-DMEM (Gibco/Invitrogen, Carlsbad, CA) supplemented with 15% FBS, 0.1 mM nonessential amino acids and 100 units/ml penicillin/streptomycin. Cells were grown at 7.5% CO$_2$ and 3% O$_2$.

Inhibition of the p53 and pRb pathways was achieved by expression of the E6 and E7 HPV oncoproteins (Smogorzewska et al., 2002). Retroviral infection to generate stable cell lines was performed as previously described (Smogorzewska et al., 2002). Full-length cDNA of hTERT and WRN were cloned into the pBabe-puro retroviral vector (Addgene, Cambridge, MA). Retroviral constructs were stably expressed in fibroblast cells according to established protocols (Crabbe et al., 2004; Hahn et al., 1999). BLM and WRN knockdowns were achieved by introducing shBLM (TGCCAATGACCAGGCGATC) and shWRN (AATTCTCCGAACGTGTCACGT) into the pSuper.retro puromycin retroviral vector (Oligoengine, Seattle, WA); viral production and infection were carried out as described (Crabbe et al., 2004).
Antibodies and Western Blotting

Anti-BLM (Rabbit, #7099) (Orazio et al., 2010), Anti-TRF1 (Rabbit, #6839), Anti-TRF2 (Rabbit, #6841) were produced at the Salk Institute. Commercial antibodies used: anti-γH2AX (Cell Signaling), anti-53BP1 (A300-273A; Bethyl Laboratories, Montgomery, TX), and anti-WRN (ab200, Abcam). Antibody dilutions were determined according to manufacturers’ specifications.

For western blotting, whole cell lysates were counted and lysed in Laemmeli buffer before SDS-PAGE analysis and immunoblotting with the appropriate antibody.

Telomere FISH of metaphase spreads

BS fibroblasts or untreated IMR90 fibroblasts were grown to 75% confluence and incubated with 0.1 ug/ml colcemide for three hours. Cells were harvested by trypsinization and carefully swollen by resuspending pellets in 0.075 M KCl and incubating at 37°C for seven minutes. Cells were then fixed in Methanol: Acetic Acid (3:1) and stored at 4°C overnight. Fixed cells were dropped on a glass microscope slide, washed with Methanol:Acetic Acid and dried on a humidified heat block at 80°C for one minute. Slides were dried at room temperature overnight before proceeding to hybridization. FISH was performed on PBS re-hydrated slides with a fluorescent PNA telomeric probe, FITC-OO-[CCCTAA]₃ (Applied Biosystems) as previously described (Smogorzewska et al., 2002). DNA was DAPI stained and slides were mounted
with Pro-Long Antifade Reagent (Invitrogen). Metaphases were imaged on a Zeiss Axio Imager.Z1.

**Immunofluorescence, and Immunofluorescence – Fluorescence In Situ Hybridization (IF-FISH)**

Fibroblasts were grown on glass cover slips to approximately 75% confluence, washed in PBS and fixed in 4% PFA at room temperature for 10 minutes. Cover slips were washed with three PBS changes in 20 minutes, then incubated in blocking solution (0.3% Triton X-100, 5% Normal Goat Serum, 1mg/ml BSA in 1x PBS) for one hour prior to antibody staining. For BLM detection, cover slips were incubated with BLM antibody at 4°C for 16 hours. Secondary antibody staining was performed with Donkey anti-Rabbit Alexa 594 fluorescent conjugate, according to manufacturer recommended protocol (Invitrogen). For TIF detection, cells were fixed in 2% PFA at room temperature for 10 minutes and immuno-stained with γH2AX or 53BP1 antibodies, according to the manufacturer’s recommendation.

When performing IF-FISH, the secondary antibody fluorescent signal was fixed by a second incubation with 2% or 4% PFA at room temperature for 10 minutes. Cover slips were washed with three PBS changes in 20 minutes, ethanol dried, and stored overnight at room temperature. FISH was performed by applying hybridization solution with fluorescent PNA telomeric probe, FITC-OO-[CCCTAA]₃ (Applied Biosystems). DNA was DAPI stained and slides were mounted with Pro-Long Antifade Reagent (Invitrogen). Interphase cells and
anaphases were imaged with a Zeiss Axio Imager.Z1 and an external Hamamatsu ORCA-ER digital camera.

**Aphidicolin Treatment**

IMR90 and AG05229 cells, with and without E6 and E7 oncoproteins, were treated with 0.3 µM aphidicolin for 24 hours, when the media was replaced with untreated 15% FBS-supplemented media. After an additional five hours of incubation, cells growing on glass cover slips were fixed with 4% PFA and washed in 1x PBS before IF-FISH.

**Anaphase and metaphase analysis following TRF1 knockdown**

TRF1 knockdown was achieved by transfecting IMR90-E6E7 and AG05229-E6E7 cells with ON-TARGET plus SMARTpool TERF1 siRNA (Dharmacon, Thermo Scientific). After 72 hours with siRNA, successful knockdown of TRF1 was confirmed by immunofluorescence and Western blotting (Figure 3-8A,B). Cells grown on glass cover slips were fixed and subjected to IF-FISH with the BLM antibody and a fluorescent telomeric probe. Reduction of TRF1 indeed led to a significant increase in formation of UFBs in both IMR90-E6E7 and AG05229-E6E7 cell lines (Figure 3-8C). However, we did not observe an increase in UFBs extending at one or both ends from telomeric foci. This may be due to the significant telomere loss induced by TRF1 and WRN deficiencies; therefore telomeric signal may below detection by FISH method.
Chapter 4. Conclusions

The data collected in this thesis reflect a shift in our predicted role for BLM in ‘normal,’ unchallenged telomere replication. Research from the Karlseder lab had previously established that WRN RecQ helicase activity facilitated efficient replication of the telomeric lagging strand. Telomere defects in WRN-deficient fibroblasts occur infrequently, suggesting that other proteins can perform a similar function in telomere synthesis. Because RecQ helicases share similarities in cellular localization and targeted DNA substrates, I first proposed that BLM would possess an overlapping or cooperative role with WRN in telomere replication.

Our investigation confirmed that BLM helicase activity is necessary to prevent significant telomere shortening at one or both sister chromatids, and telomere defects in BS metaphases (2.5-3.7% chromatids) occurred at frequency similar to WS metaphase TDs (2.1-5% chromatids) (Table 2-2). In both BS and WS cell lines, dramatic telomere loss – and chromosome fusion events – are prevented by hTERT-mediated telomere elongation (Figure 2-5). This experiment confirmed that telomere-specific defects contribute to a portion of the breaks or deletions that characterize BS and WS cells.

However, my data show that BLM actually participates in chromosome end maintenance through a novel pathway, which employs dissolution of late replicating intermediates. BLM performs this activity in a conserved complex with topoisomerase IIIα and the OB-fold containing proteins, RMI1 and RMI2. This
recently identified mechanism has been demonstrated at fragile sites and centromeres; this thesis is the first to provide evidence that telomeric DNA is another common site for late-replicating intermediates (Figure 4-1).

In summary, these data strongly support a role for BLM in telomere maintenance. Interestingly, the nature of this telomeric function appears to be in response to an intrinsic feature of telomere replication, rather than through a regular, ongoing activity in ‘normal’ telomere maintenance. Recruitment of BLM and its interacting proteins, TOP IIIα-RMI1-RMI2- confirms that telomeric sequence does act as a ‘difficult-to-replicate’ genomic region. We predict that these proteins occasionally localize to telomeres that have experienced delay in replication fork progression; this is a commonly observed defect, but it is not a ‘normal’ event. Furthermore, the severity of the defect varies, depending on the proximity of the replication fork to chromosome ends. A greater delay in replication completion will produce ultra fine bridges that persist later into mitosis. The presence of late replicating intermediates, even as the cells progress through mitosis, indicates that cells are equipped to deal with replication delay without sacrificing efficiency.

The additional processing at telomeres also indicates that telomeres can commonly encounter replication defects, which are resolved through the same process as resolution of late replication intermediates at fragile sites and centromeres. Our thorough investigation of BLM helicase activity in chromosome end protection does contribute to the overall understanding of telomere
maintenance. These findings are most notable for their indications for telomeres as potentially new ‘fragile sites’ or perhaps they only behave as difficult to replicate regions that require special processing to ensure faithful chromosome segregation and maintain an intact genome.
Figure 4-1. Model proposing a role for BLM in processing late replication intermediates that form at telomeres. Delayed replication at telomeres can arise from a minor dysfunction, such as incomplete resolution of a secondary structure and late re-start of the replication fork. DNA is fully replicated, but incompletely unwound, and therefore is generally dissolved early in mitosis. Alternatively, more severe replication defects, such as the deficiency of an important telomere-associated protein, can result in incompletely replicated telomeric sequence and longer LRIs that persist later into anaphase.
References


