THE FANCONI ANAEMIA PROTEIN FANCJ IS INVOLVED IN THE ALTERNATIVE LENGTHENING OF TELOMERES (ALT) MECHANISM IN HUMAN CELLS

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Approximately 15% of human cancers utilize a recombination-based mechanism termed Alternative Lengthening of Telomeres (ALT) to maintain the lengths of their telomeres. The Fanconi anaemia protein FANCJ localizes to telomeric foci in human ALT cells, but not in telomerase-positive or primary cells. Telomere-associated FANCJ frequently localizes with FANCD2 and BRCA1, and primarily localizes to ALT-associated PML nuclear bodies. Depletion of FANCJ in human ALT cells causes the loss of BRCA1 at telomeric foci and a decrease in telomeric repeat DNA content primarily as a result of the loss of the brightest telomeric repeat DNA foci. In contrast, depletion of the FANCD2 results in increased telomeric repeat DNA synthesis and this is suppressed upon the codepletion of FANCJ. Together, data from this study suggest that FANCJ is required for telomeric repeat DNA synthesis in human ALT cells, which may or may not be dependent on BRCA1, and FANCD2 restrains this synthesis.
I would like to express my deep and sincere gratitude to my supervisor, Dr. M. Stephen Meyn, whose expertise, understanding, and patience added considerably to my graduate experience. A very special thanks goes out to Dr. Paul Bradshaw and Dr. Heather Root for your guidance, encouragement, and stimulating discussions. You have made my experience in the lab memorable and helped me become a more mature scientist. I would also like to thank my committee members, Dr. Daniel Durocher and Dr. Christopher E. Pearson, for your constructive suggestions, valuable comments, as well as kind encouragements. To my family, thank you for your support, encouragement, and help throughout the years, and for teaching me the philosophy of life. Shirley, my dear partner, best friend, and companion in life, thank you for your wholehearted understanding, support, and help, for your open mind and patience, and for all the love and happiness you have brought me.
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<th>Description</th>
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<tr>
<td>γ-H2AX</td>
<td>phosphorylated H2AX</td>
</tr>
<tr>
<td>53BP1</td>
<td>p53 Binding Protein 1</td>
</tr>
<tr>
<td>ALT</td>
<td>Alternative Lengthening of Telomeres</td>
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<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
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<td>APB</td>
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<tr>
<td>DSB</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>Full Form</td>
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<tr>
<td>G-rich</td>
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<td>nt</td>
<td>nucleotide</td>
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<td>PALB2</td>
<td>Partner And Localizer of BRCA2</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PML</td>
<td>promyelocytic leukemia protein</td>
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<tr>
<td>PNA</td>
<td>peptide nucleic acid</td>
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<tr>
<td>PNB</td>
<td>PML nuclear body</td>
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<td>repressor/activator protein 1</td>
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<td>small interfering RNA</td>
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<td>TBST</td>
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<td>TERC</td>
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<td>XPD</td>
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CHAPTER 1

INTRODUCTION
CHAPTER 1

1 Introduction

1.1 Human Telomere Biology

1.1.1 Telomere DNA

Unlike the circular genome of most prokaryotes, the human nuclear genome is assembled into discrete linear chromosomes. This poses a biological problem as there are chromosome ends resembling DNA ends generated from double-strand breaks (DSB) in the cellular nucleus. To mask these chromosome ends, cells have evolved specialized nucleoprotein structures, referred to as telomeres, that complete the termini of these linear chromosomes (Figure 1-1 A). In human cells, telomeres consist of double-strand 5’-TTAGGG-3’ basepair (bp) tandem repeats, typically with a length of 5 to 15 kilobases (kb) (Moyzis et al., 1988). The termini of human telomeres are not blunt-ended but consist of single-strand 3’ overhangs of the guanine-rich (G-rich) sequence and vary between 50 and 500 nucleotides (nt) in length (Makarov et al., 1997; Wright et al., 1997). Whereas the 5’ strand nearly always terminates with the sequence ATC-5’, the nucleic acid composition of the 3’ overhang is not precisely defined (Sfeir et al., 2005). Immediately adjacent to the long telomeric repeat tracts are segments of degenerative subtelomeric repetitive elements (Riethman et al., 2001). These tracts have very high sequence similarity to each other and are highly variable in size.

Electron microscopy has revealed that, in vivo, human telomeres may form large duplex lariat structures referred to as telomere-loops (t-loop) (Griffith et al., 1999) (Figure 1-1 B). A t-loop results from the telomere folding back on itself and the single-strand G-rich overhang invading the duplex telomeric repeat DNA. The G-rich overhang forms base-pairs with the complimentary cytosine-rich (C-rich) strand of the duplex, resulting in the displacement of the G-rich strand at this site into a displacement-loop (D-loop). Furthermore, the telomeric repeat DNA sequence predicts the formation of another secondary DNA structure referred to as G-quadruplex (G4) DNA (Figure 1-1 C). G4 DNA structures have the signature motif G3:5N1:3G3:5N1:3G3:5N1:3G3:5 and form by stacking multiple G•G•G•G tetrads to generate a very stable four-stranded helical
Figure 1-1. The DNA composition and structure of human telomeres.

(A) Human chromosome ends are composed of 5’-TTAGGG-3’ bp repeat arrays that vary in length. Proximal to the telomeric repeat tracts are segments of degenerative subtelomeric repetitive elements. The telomere termini contain single-strand 3’ overhangs of the G-rich sequence that vary in length. The 3’ end is not precisely defined whereas the 5’ end nearly always terminates with the sequence ATC-5’. (B) Schematic of the t-loop structure at the telomere. (C) Schematic of G4 DNA structures at the telomere.
Figure 1-1

A

Subtelomeric repeats 5-15 kb (TTAGGG)n 50-500 nt 3' overhang 3'

G-rich strand: TTAGGGTTAGGGTTAGGGTTAGGGTTA 3'
C-rich strand: AATCCCAATCCCAATC 5'

B

D-loop

T-loop

Strand invasion of 3' overhang

C

telomeric G4 DNA structures
structure (Gellert et al., 1962). It has not been fully established whether G4 DNA structures have a biological function at telomeres in vivo. Nevertheless, they preferentially form at the very 3’ end of telomeres and small molecules that stabilize G4 DNA structures have effects on telomere biology (Gomez et al., 2006; Tang et al., 2008).

1.1.2 Telomere Proteins

In addition to its unique DNA sequence, several telomere-specific and non-telomeric proteins complete the nucleoprotein structure of the telomere. The telomere DNA repeats associate with a telomere-specific protein complex referred to as shelterin (reviewed in de Lange, 2005) (Figure 1-2). Shelterin is bound to telomeres throughout the cell cycle and is composed of six proteins, three of which can directly interact with telomeric repeat DNA. Telomere Repeat-binding Factor 1 and 2 (TRF1 and TRF2) bind telomeres at the duplex regions, whereas Protection Of Telomeres 1 (POT1) has high affinity for single-strand regions of the G-rich sequence (Zhong et al., 1992; Broccoli et al., 1997; Baumann and Cech, 2001). These three proteins contribute to the telomere sequence specificity of shelterin. The other three proteins that complete the complex are TRF1-Interacting Nuclear protein 2 (TIN2), repressor/activator protein 1 (Rap1), and TPP1 (Kim et al., 1999; Li et al., 2000; Liu et al., 2004a; Ye et al., 2004a).

TRF1 and TRF2 bind telomeric repeat DNA as either homodimers or oligomers but do not interact directly with each other (Bianchi et al., 1997; Broccoli et al., 1997; Fairall et al., 2001). Both proteins are able to recruit the other four shelterin components to telomeric repeat DNA, making them essential to the formation of the shelterin complex. Independently, TRF1 has the ability to loop and pair stretches of telomeric repeat DNA, whereas TRF2 can form t-loop structures in vitro when a telomeric repeat DNA substrate is provided (Griffith et al., 1998; Bianchi et al., 1999; Stansel et al., 2001). This unique characteristic of TRF2 is probably a result of its ability to preferentially bind the end of a duplex telomeric DNA repeat array adjacent to the single-strand G-rich overhang (Stansel et al., 2001). In addition, TRF2 can unwind duplex telomeric repeat DNA by inducing positive supercoiling (Amiard et al., 2007). This feature may also play a role in t-loop formation as it promotes strand invasion. TRF2 interacts and forms a complex with Rap1, and the localization of Rap1 to telomeric repeat DNA is solely dependent upon its interaction with TRF2 (Li et al., 2000). Although Rap1 is an essential component of shelterin, its role in telomere biology has yet to be fully established.
Figure 1-2. The position of shelterin on human telomere DNA.

TRF1 and TRF2 bind duplex telomeric repeat DNA, while POT1 interacts with single-strand telomeric repeat DNA. Although one of the shelterin complexes may have the depicted structure on the telomere, telomeres contain numerous copies of shelterin bound along the length of each telomere. It is unknown whether all of the shelterin complexes on telomeres are bound as six protein complexes. Figure adapted from Palm and de Lange, 2008.
Figure 1-2

3' GATTGGGATTGGGATTGGGATTGGGATTGGGATTGGGATTGGG
5' CTAACCCTAACCCTAACCCTAACC
POT1 contains domains that enable it to recognize and bind the single-strand G-rich regions of telomeres (Baumann and Cech, 2001; Lei et al., 2004; Loayza et al., 2004). POT1 forms a heterodimer with its binding partner TPP1, and together, they are joined with the rest of the shelterin proteins through a direct interaction between TPP1 and TIN2 (Liu et al., 2004a; Ye et al., 2004a). TIN2 functions as a scaffold to bridge the shelterin proteins into a single complex, as it also has the ability to directly bind TRF1 and TRF2 (Houghtaling et al., 2004; Ye et al., 2004b). Consistent with this, upon the depletion of TIN2 or the expression of mutant TIN2, destabilizing effects on shelterin are observed (Kim et al., 2004; Ye et al., 2004b). However, it has not been fully established whether TIN2 is permanently bound to all three shelterin components or whether it may switch between TRF1, TRF2/Rap1, or TPP1/POT1 bound states. Isolation of shelterin from nuclear cell extracts revealed subcomplexes lacking either TRF1 or TRF2/Rap1 but still retained telomeric repeat DNA binding capacity (Liu et al., 2004b; Ye et al., 2004b; Celli and de Lange, 2005).

Telomerase is a telomere-specific ribonucleoprotein enzyme that is capable of adding telomeric repeat DNA to the ends of linear chromosomes (Greider and Blackburn, 1985). The catalytic core of the enzyme is composed of the protein Telomerase Reverse Transcriptase (TERT) and the Telomerase RNA Component (TERC) (Weinrich et al., 1997). TERT is able to add 5’-TTAGGG-3’ repeats to chromosome ends using TERC as a template through a reverse transcriptase reaction.

Many non-telomeric proteins are also present at telomeres and are primarily recruited by the components of shelterin (Hsu et al., 1999; Hsu et al., 2000; Zhu et al., 2000; Opresko et al., 2002). They are typically less abundant at telomeres compared to the shelterin components and are transiently associated with telomeres throughout the cell cycle. These proteins usually serve primary roles elsewhere in the nucleus such as in DNA replication, DNA damage signaling, and DNA repair, but have been associated with proper telomere function.

1.1.3 Telomere Function

A major drawback of linear chromosomes is that their termini can resemble DNA ends generated from DSBs. DSBs can be catastrophic to the genome if not accurately repaired or if not repaired at all, resulting in aberrant end joining and recombination events that can promote oncogenesis.
In human cells, DNA ends typically elicit the cellular DNA damage response (DDR), which ultimately results in the activation of DNA damage checkpoints (reviewed in Jackson and Bartek, 2009). These checkpoints can be triggered during different phases of the cell cycle leading to cell cycle arrest. This provides the cell time to activate and recruit proteins involved in DNA repair to the site of damage such that the repair process can take place. Therefore, these checkpoints are important defence mechanisms that protect the cell from genome instability when DNA damage arises.

Two central players in the cellular DDR are ataxia telangiectasia mutated (ATM) and ATM and RAD3-related (ATR) (reviewed in Shiloh, 2003). These two protein kinases respond to various DNA stresses and phosphorylate many important proteins, triggering a signal transduction cascade and DNA damage checkpoint activation. ATR primarily responds to the presence of single-strand DNA commonly formed from DNA damage or the stalling of replication forks. On the other hand, ATM activation is triggered by DNA DSBs. Both ATM and ATR phosphorylate the histone H2A variant, H2AX, on Serine 139 within the chromatin that flanks the damaged site. This promotes the local accumulation of DNA damage repair factors at the site of damage. In addition, ATM and ATR also phosphorylate two cell cycle kinases, CHK1 and CHK2. These kinases can block cell cycle progression by inducing G1/S- or G2/M-phase arrest.

The activation of ATM or ATR is incompatible with normal cell cycle progression, and telomeres must ensure that these signal transducers remain dormant. Therefore, one of the essential functions of telomeres is to allow the cell to distinguish natural chromosome ends from the DNA ends generated from DSBs. Consistent with this, the inhibition of proper shelterin function by either the removal or inhibition of TRF2 results in the activation of the cellular DDR (Karlseder et al., 1999; Celli and de Lange, 2005; Denchi and de Lange, 2007). This is evidenced by the presence of factors closely associated with an activated cellular DDR pathway, such as p53 Binding Protein 1 (53BP1) and phosphorylated H2AX (γ-H2AX), at chromosome ends (d’Adda di Fagagna et al., 2003; Takai et al., 2003). These foci are typically referred to as telomere dysfunction-induced foci (TIF) and are very similar to DNA damage foci that occur at DNA DSBs. ATM appears to be the main transducer of the DNA damage signal at dysfunctional telomeres. In cells lacking ATM, the cellular DDR is no longer activated upon the removal or inhibition of TRF2 (Denchi and de Lange, 2007). On the other hand, the phosphorylation and activation of CHK1, a target of ATR, is not detected (Denchi and de Lange, 2007).
It is not clear as to how telomeres normally avert activation of the cellular DDR pathway. One possibility is that telomeres form secondary DNA structures to hide the chromosome termini from being sensed by ATM and ATR. The ATM transduction pathway is dependent on the interaction of the MRE11-RAD50-NBS1 (MRN) complex with DNA ends (reviewed in Lee and Paull, 2007). T-loops and G4 DNA structures at telomere ends may provide a mechanism to restrict the accessibility of MRN to the chromosome ends and therefore suppressing the cellular DDR. Nevertheless, t-loops and G4 DNA structures are likely transient structures as it would be necessary to remove them for DNA replication machinery to pass. In addition, small molecules that stabilize G4 DNA structures, such as telomestatin, result in dysfunction at telomeres (Gomez et al., 2006). Another possibility is that telomere ends do not need to be hidden, but a step that is downstream of DNA end sensing is blocked. Consistent with this, the overexpression of TRF2 can inhibit the activation of ATM even at sites of DNA damage that are not at telomere regions (Karlseder et al., 2004; Bradshaw et al., 2005). In addition, TRF2 can physically interact with both ATM and MRN, and these interactions may contribute to the suppression of the cellular DDR at telomeres (Zhu et al., 2000; Karlseder et al., 2004).

The single-strand 3’ overhangs at the termini of telomeres are long enough to trigger and activate the single-strand DNA sensing kinase ATR. The repression of ATR does not require TRF2, but involves POT1 and its association with TPP1 (Denchi and de Lange, 2007; Hockemeyer et al., 2007). The deletion of both POT1 proteins in mouse cells is sufficient to generate TIFs at all chromosome ends and induce cell cycle arrest through the phosphorylation and activation of CHK1 (Denchi and de Lange, 2007). This response is not dependent on ATM, as it is unaltered in cells deficient of ATM function (Denchi and de Lange, 2007). The formation of TIFs is reduced when ATR signaling is obstructed, establishing POT1 as a repressor of the ATR pathway (Denchi and de Lange, 2007). TPP1 is required probably to recruit POT1 to telomeres and improve its ability to bind single-strand DNA (Wang et al., 2007).

Telomeres that are not properly capped can be recognized as DNA ends generated from DSBs, which are primarily processed by two major repair mechanisms in human cells. Two uncapped telomeres may be joined together through the non-homologous end joining (NHEJ) pathway, generating fused chromosomes that are readily detectable in metaphase spreads. It appears as though shelterin is important in preventing NHEJ at telomeres. The expression of a dominant-negative allele of TRF2 induces chromosome end fusions in human cells (van Steensel et al., 2004).
Similarly, within a few days of TRF2 deletion in mouse cells that lack the ability to activate DNA damage checkpoints, 30-50% of the telomeres become joined (Konishi and de Lange, 2008). Finally, components of the NHEJ pathway have been implicated in chromosome end fusion events upon the inhibition of TRF2 function (Zhu et al., 2003; Celli et al., 2006).

The other repair mechanism that may act on uncapped telomeres is one that involves homologous recombination (HR). The single-strand 3’ overhang at the ends of telomeres is an ideal substrate to undergo HR, especially since there are large regions of telomeric repeat DNA present within the nucleus. In fact, t-loop generation mimics an intermediate event during HR when the single-strand G-rich telomere overhang invades duplex telomeric repeat DNA resulting in a D-loop. During HR-based DSB repair, the invading single-strand 3’ end acts as a primer that would be utilized by DNA repair and replication machinery to synthesize DNA using the complementary strand as a template. This is not observed to occur at t-loops in human cells, suggesting a mechanism that inhibits this process. Once again, it is believed that shelterin is responsible for the prevention of HR-based DSB repair at telomere ends. In human cells, expression of an allele of TRF2 lacking the amino-terminal (N-terminal) basic domain prevents telomere end fusion events but results in disregulated HR at telomeres (Wang et al., 2004). After replication, the telomeres at the ends of a duplicated chromosome are in the vicinity of each other and can undergo HR. When a telomere end undergoes HR with the telomere on the sister chromatid, this is referred to as a telomere-sister chromatid exchange (T-SCE). Therefore, T-SCEs are indicators of relaxed control of DNA repair at telomeres and their frequency can be increased by the removal of TRF2 and the inhibition of NHEJ together (Celli et al., 2006).

Another drawback of linear chromosomes is the inability of the replication machinery to completely replicate chromosome ends. This is because it is not possible to replicate a linear DNA molecule fully through lagging strand synthesis, as the removal of the most terminal RNA primer leaves a gap that cannot be filled (Olovnikov et al., 1973; Ohki et al., 2001). Although there are tissue specific length variations, telomeres are ~10 to 15 kb in length in most human cells at birth (de Lange et al., 1990). During each replication cycle, around 50 to 150 bp of telomeric repeat DNA is lost from each chromosome end due to incomplete replication and processing events that occur after replication to generate the G-rich overhang (Huffman et al., 2000; Martens et al., 2000). Replicative senescence was first described in human primary fibroblast cells before the advent of telomere biology (Hayflick and Moorhead, 1961), and it is
now clear that telomere erosion is responsible. Analysis of human primary cells that have entered replicative senescence revealed that one or more critically short telomeres activate the cellular DDR (Zou et al., 2004). These cells have accumulated TIFs and have activated both ATM and ATR. This results in DNA damage checkpoint activation and ultimately leads to a permanent withdrawal from the cell cycle or programmed cell death through apoptosis. The current model as to why critically short telomeres induce the cellular DDR is that they carry insufficient shelterin to keep ATM and ATR dormant (Smogorzewska et al., 2000; Loayza and de Lange, 2003). Thus, a second function of telomeres is to limit the number of replication cycles a cell can undergo. Consistent with this, the inhibition of checkpoint signaling can extend the life of human cells with critically short telomeres (Brown et al., 1997).

1.1.4 Telomere Length Maintenance Mechanisms

In humans, telomerase activity is restricted to germline and stem cell compartments of tissues that undergo a high cell turnover. On the other hand, somatic cells lack a mechanism to maintain the lengths of their telomeres, resulting in an age related decline in average telomere length (Lindsey et al., 1991). Similarly, human primary cells grown in culture will only cycle a limited number of times until one or a few of the telomeres become critically short (Zou et al., 2004). This triggers the cellular DDR and results primarily in replicative senescence but some cells undergo apoptosis. This stage is referred to as mortality 1 (M1) checkpoint (Figure 1-3). Telomeres that are less than 100 bp in length begin to appear within these cells and are usually associated with DNA repair foci (Zou et al., 2004). This suggests that these cells have activated DNA damage checkpoints and have entered replicative senescence. However, if telomerase activity is induced in human primary cells, their replicative lifespan can be extended beyond the M1 checkpoint (Bodnar et al., 1998; Vaziri and Benchimol, 1998).

If replicative senescence is bypassed by mutations or expression of viral genes, human primary cells continue to cycle past the M1 checkpoint in the absence of a telomere length maintenance mechanism. Telomeres within these cells become extremely short, ultimately triggering high levels of genome instability (Capper et al., 2007). These cells eventually reach a point where they experience a high rate of cell death, referred to as cellular crisis or mortality 2 (M2) crisis. This stage can be visualised by the detection of dicentric chromosomes resulting from telomere end fusions in the nuclei of these cells (Zou et al., 2009). To survive cellular crisis and become
Figure 1-3. The telomere length hypothesis of cellular senescence and immortalization.

Telomerase activity is sufficient to maintain telomere lengths fully in germline cells, but is insufficient to maintain telomere lengths in stem cell compartments over a human lifespan. Human somatic cells and primary cells grown in culture do not have a telomere length maintenance mechanism, which leads to a progressive loss of telomeric repeat DNA upon repeated cellular divisions. At M1 checkpoint, cells have accumulated critically short telomeres and have triggered checkpoint activation resulting in replicative senescence or apoptosis. Bypass of cellular checkpoints allows the cells to continue dividing until they reach M2 crisis. At this stage, cells experience extreme genome instability and cell death. Escape from crisis requires a telomere length maintenance mechanism, either by activating telomerase or the ALT pathway. Figure adapted from Royle et al., 2009.
immortal, cells must activate a telomere length maintenance mechanism. Most immortalized cell lines accomplish this by expressing telomerase. However, ~30% of immortalized cell lines tested use a telomerase-independent mechanism referred to as Alternative Lengthening of Telomeres (ALT) (Bryan et al., 1997). In contrast to telomerase activity, the ALT mechanism has yet to be detected in a primary cell setting (Bryan et al., 1995). However, ALT has been found to maintain the lengths of telomeres in ~15% of human cancers, mostly of mesenchymal origin (Bryan et al., 1997; Henson et al., 2005; Jeyapalan et al., 2008; Subhawong et al., 2009).

The ALT mechanism was first discovered in immortalized human cell lines with no detectable telomerase activity (Murnane et al., 1994). A striking feature of these cells is the heterogeneity in the lengths of their telomeres. In a single cell, some telomeres are less than 1 kb in length, while others are greater than 50 kb (Bryan et al., 1995). In addition, some chromosome ends display a complete lack of a detectable telomeric signal indicating that these telomeres are so short that they are unable to be detected by standard protocol (Perrem et al., 2001). Similar to human somatic cells, immortalized ALT cells show gradual telomere shorting, but their telomeres are also subjected to rapid lengthening and deletion events of several kilobases long.

A similar phenomenon was later described in Saccharomyces cerevisiae and appeared to be dependent on HR (Le et al., 1999; Teng and Zakian, 1999). The removal of telomerase activity from yeast cells results in telomere shortening and cell death. However, rare survivors are able to maintain the lengths of their telomeres in a Rad52-mediated manner, a protein involved in HR. Furthermore, the Rad50-dependent type II survivors most closely resemble the features of telomeres observed in immortalized human ALT cells. The first evidence that the ALT mechanism in human cells is recombination-based came from experiments in which a single telomere was tagged and copied to other chromosome ends in ALT cells after a number of cell cycles (Dunham et al., 2000). However, tags that were placed internal to the telomere did not multiply. In addition, analysis of the degenerative subtelomeric repetitive elements in human telomeres revealed a class of complex telomere mutations only found in ALT cells (Varley et al., 2002). Such mutations are most easily explained to arise by intermolecular recombination-like processes. Since then, several recombination proteins have been discovered to be necessary for telomere length maintenance in human ALT cells (Stavropoulos et al., 2002; Potts and Yu, 2007; Zhong et al., 2007), and it is thought that the ALT mechanism is dependent upon the process of disregulated recombinational activities at telomeres.
Another hallmark of human ALT cells is the presence of extrachromosomal telomeric repeat (ECTR) DNA. This DNA is predominantly double-strand telomeric circles (Cesare and Griffith, 2004; Wang et al., 2004), but can also exist as partially single-strand circles of both the G-rich and C-rich strands (Henson et al., 2009; Nabetani and Ishikawa, 2009), linear single-strand and double-strand DNA (Ogino et al., 1998; Tokutake et al., 1998; Nabetani and Ishikawa, 2009), and high molecular weight branched DNA (Nabetani and Ishikawa, 2009). The origin of ECTR DNA in human ALT cells is currently unknown, but it is probably generated from aberrant resolution and endonucleolytic cleavage of intermediate DNA structures formed during the ALT mechanism.

Human ALT cells also display high rates of T-SCEs without an increase in the frequency of exchange events elsewhere in the genome (Londoño-Vallejo et al., 2004). Although this may explain the rapid lengthening and deletion events observed at telomeres in ALT cells, it would not contribute to the net gain in telomeric repeat DNA. The t-loop at telomere ends contains a D-loop, an intermediate structure formed during HR. Disregulation of the activity that masks the telomere 3’ invading strand from being recognized by the DNA replication machinery may exist in ALT cells, resulting in intratelomeric rolling circle replication (Figure 1-4 A). In addition, intertelomeric replication may occur in which the single-strand 3’ overhang of one telomere invades the sequence of another telomere and uses it as a template for telomeric repeat DNA synthesis (Figure 1-4 B). Finally, both circular and linear forms of ECTR DNA may serve as templates for telomeric repeat DNA synthesis (Figure 1-4 C, D). Particularly, rolling circle replication using a circular ECTR DNA template can occur continuously, contributing to the large net gain of telomeric repeat DNA that is observed in human ALT cells.

Finally, human ALT cells contain a specific form of promyelocytic leukemia protein (PML) nuclear body (PNB), termed ALT-associated PNB (APB) (Yeager et al., 1999). APBs are defined by the colocalization of telomeric repeat DNA, telomere binding proteins, DNA recombination proteins, and PML (Yeager et al., 1999; Stavropoulos et al., 2002; Nabetani et al., 2004). Thus, APBs may be sites of telomeric recombination, elongation, and deletion events in human ALT cells. They are not normally observed in telomerase-positive or primary cells, and arise at around the same time as the activation of the ALT mechanism, suggesting the two phenomena are related (Yeager et al., 1999).
Figure 1-4. Potential mechanisms of recombination-based telomere elongation.

(A) The single-strand 3’ telomere overhang invades the duplex DNA of the same telomere and is used for telomeric repeat DNA synthesis. (B) The single-strand 3’ telomere overhang of one telomere invades the duplex DNA of a different telomere and is used for telomeric repeat DNA synthesis. (C) The single-strand 3’ telomere overhang invades the duplex DNA of linear ECTR DNA and is used for telomeric repeat DNA synthesis. (D) The single-strand 3’ telomere overhang invades the duplex DNA of circular ECTR DNA and is used for telomeric repeat DNA synthesis. Although double-strand ECTR DNA is shown, it is possible that single-strand linear and circular ECTR DNA of the C-rich strand can be used as templates for telomeric repeat DNA synthesis.
Figure 1-4

A

Intratelicomic rolling circle replication

5'  

B

Intertelomic replication between telomeres

5'  

5'  

5'  

C

Telomeric replication using linear ECTR DNA

5'  

5'  

D

Rolling circle replication using circular ECTR DNA

5'  

G-rich strand

C-rich strand
1.2 Fanconi Anaemia

1.2.1 Clinical Features

Fanconi anaemia (FA) is a phenotypically and genetically heterogeneous disorder that was first reported by the Swiss paediatrician Guido Fanconi in 1927 (Fanconi, 1927). Fanconi described three affected brothers between the ages of 5 and 7 that had an unusual combination of physical abnormalities and aplastic anaemia. Since this first description, many more cases have been reported and the disorder is now better characterized. FA is an autosomal recessive or X-linked disorder that has a prevalence of 1 to 5 per million found in all races and ethnic groups. FA patients live to an average age of 20 years with a range of 0 to 50+ years, primarily because these individuals develop life-threatening bone marrow failure and are susceptible to developing malignancies.

Approximately two-thirds of FA patients have detectable congenital abnormalities (reviewed in Auerbach, 2009). The classic and most common features include skeletal defects such as ray, hip, vertebral, and rib anomalies. Other common features are short stature, hyper and hypopigmentation, genitourinary, and ophthalmic abnormalities. FA is also associated with reduced fertility. Male patients may develop manifestations that include testicular hypoplasia or cryptorchidism, while female patients may have hypoplastic ovaries and an infantile uterus. Less frequent birth defects include neurologic, gastrointestinal, and cardiac malformations. It is important to realize that about one-third of FA patients have no congenital abnormalities. This suggests that there is not a strong correlation between genotype and phenotype. Consistent with this, in an analysis of 45 groups of FA siblings, 12 sets contained siblings with and without malformations, while 12 sets contained siblings with malformations of different severity (Giampietro et al., 1993). In addition, two sets of monozygotic FA twins have been identified both with and without malformations, and different malformations (Giampietro et al., 1994).

The haematological abnormalities of FA patients are of great clinical significance. Bone marrow failure often presents by the age of 10 and ~90% of individuals will develop bone marrow failure by the age of 40 (Butturini et al., 1994; Kutler et al., 2003). Although it is currently unknown as to why FA patients develop bone marrow failure, the favoured hypothesis is that it is a direct result of the exhaustion of haematopoietic stem cells (HSC) that repopulate the blood cell...
lineage. Another haematological abnormality that commonly develops in FA patients is acute myeloid leukemia (AML) (Butturini et al., 1994). AML is characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells.

In the first part of the twentieth century, the advent of modern blood banking allowed for the treatment of bone marrow failure through blood transfusion. As FA patients survived into adulthood, the looming issue of developing other malignancies was inevitable. In addition to AML, FA patients are also prone to developing solid tumors, with a cumulative incidence of 28% by the age of 40 (Kutler et al., 2003). The most common malignancy of this type is squamous cell carcinoma of the head and neck.

1.2.2 Molecular Pathway

Cells from FA patients are susceptible to genome instability, especially after being exposed to DNA interstrand crosslink (ICL)-inducing agents (Sasaki, 1975). This linked the cellular pathway obstructed in FA patients to the repair of DNA ICLs, enabling the identification of FA genes. Currently, there are 12 genes that have been discovered, each of which when mutated gives rise to FA (FANCA, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -N) (reviewed in de Winter and Joenje, 2009). With the exception of FANCB, which demonstrates X-linked inheritance, all of the other FA genes function in an autosomal recessive manner (Auerbach, 2009). The FA genes encode proteins that appear to have roles in one common pathway to repair DNA ICLs, a type of DNA damage that effectively blocks replication (Figure 1-5).

The FA proteins are subdivided into three groups. The nuclear core complex is composed of FANCA, -B, -C, -E, -F, -G, and L. Immunoprecipitation of FANCA coupled with mass spectrometry was used to identify other components referred to as FANCA-associated polypeptides (FAAPs) (Meetei et al., 2003). FAAP24 and FAAP100 appear to be important for core complex function, however patients with mutations in these genes alone have yet to be identified (Ciccia et al., 2007; Ling et al., 2007). Mutations in the gene that encodes the third FAAP, FAAP250, have been found in one patient (Meetei et al., 2005). Thus, FAAP250 was renamed to FANCM. However, this patient also has biallelic mutations in the gene that encodes FANCA, making the human FANCM phenotype unclear (Singh et al., 2009).
Figure 1-5. Model of the FA pathway.

Upon DNA ICL damage, FANCM, the core complex, and accessory proteins localize to the site of damage (a). The core complex along with UBE2T monoubiquitinate the ID complex (b), which assembles at the site of DNA damage along with the third subgroup of FA proteins and other DNA repair factors (c). USP1 promotes the deubiquitination and disassembly of the ID complex (d).
Figure 1-5

DNA ICL damage

ID complex

core complex

DNA

FAAP100

FAAP24

UBE2T

USP1

DNA

RADS1

D1/BRCA2

N/PALB2

J/BACH1

BRCA1

BLM

FAN1

FAN1
Given the hypersensitivity of FA cells to DNA ICL-inducing agents, the sequences of the FA proteins that belong to the core complex were studied to search for domains and motifs common to other DNA repair proteins. Nuclear localization signals and scaffold domains were found (Kupfer et al., 1997; Garcia-Higuera et al., 1999; de Winter et al., 2000a; de Winter et al., 2000b; Blom et al., 2004). However, no domains that established a clear connection between FA and DNA repair were revealed. It was not until FANCD2 was discovered that a link between DNA repair and FA was found (Timmers et al., 2001). Upon DNA damage induced by DNA ICL-inducing agents, FANCD2 binds chromatin and forms nuclear foci at sites of DNA damage with breast cancer gene 1 (BRCA1) and RAD51, two key proteins in HR, (Garcia-Higuera et al., 2001; Taniguchi et al., 2002). For this to occur, FANCD2 must be monoubiquitinated on Lysine 561 in S-phase of the cell cycle in a manner that is dependent on the core complex. These observations suggest a role for the FA proteins in an S-phase specific DNA repair pathway that involves HR. Furthermore, FANCI was recently identified as a second protein that forms nuclear foci and binds to chromatin following monoubiquitination by the core complex (Sims et al., 2007; Smogorzewska et al., 2007). FANCD2 and FANCI can interact, and together, they form the second group of FA proteins, referred to as the ID complex. The monoubiquitination of this complex is thought to function as a molecular switch to turn on the FA pathway.

Unlike the other FA core complex components that have been established to be essential for monoubiquitination of the ID complex, there is a debate as to whether FANCM is necessary for this as well. FANCM promotes the association of the FA core complex with chromatin and subsequent monoubiquitination of FANCD2 but it is not absolutely required for these activities (Bakker et al., 2009). Furthermore, a mouse FANCM model has been generated and shows some novel phenotypes not shared with the other FA core complex mouse models, suggesting a more complex role for FANCM (Bakker et al., 2009). This is also the case in human cells when FANCM is depleted (Mosedale et al., 2005).

Monoubiquitination of the ID complex is carried out by FANCL, which has E3 ubiquitin ligase activity (Meetei et al., 2004). Additional identified components of this pathway include UBE2T, which acts as the E2 ubiquitin activating enzyme, and USP1, which promotes deubiquitination of FANCD2 (Nijman et al., 2005; Machida et al., 2006). Recently, a nuclease referred to as FANCD2-associated nuclease 1 (FAN1) was identified to interact with and be recruited by monoubiquitinated FANCD2 to sites of DNA damage (Kratz et al., 2010; Liu et al., 2010;
MacKay et al., 2010; Smogorzewska et al., 2010). Similar to cells from FA patients, the depletion of FAN1 in human cells sensitizes them to DNA ICL-inducing agents.

A further connection between FA and HR-mediated DNA repair was established through the identification of the FA proteins that make up the third subgroup. These include FANCD1, FANCJ, and FANCN, all of which are not required for the monoubiquitination of the ID complex (Howlett et al., 2002; Levitus et al., 2005; Litman et al., 2005; Reid et al., 2007). All three proteins have been previously associated with HR-mediated DNA repair of DSBs. FANCD1 is also referred to as breast cancer gene 2 (BRCA2), a protein that supports the formation of RAD51 filaments essential for strand invasion (Wooster et al., 1995; Yuan et al., 1999). FANCN has previously been identified as Partner And Localizer of BRCA2 (PALB2) (Xia et al., 2006). The clinical phenotypes of FANCD1 and FANCN appear to be significantly different from other FA groups. Unlike other FA patients, FANCD1- and FANCN-deficient patients are severely affected with early childhood cancers, often resulting in mortality at a young age (Offit et al., 2003; Reid et al., 2007). Furthermore, FANCJ is also known as BRCA1-associated Carboxyl-terminal (C-terminal) Helicase 1 (BACH1) or BRCA1 Interacting Protein 1 (BRIP1) and is described in detail below (Cantor et al., 2001). It is currently unknown whether the third subgroup of FA proteins functions in the same pathway as FANCD2 monoubiquitination to repair DNA damage or in an independent but parallel pathway.

Recently, it was discovered that the inhibition of NHEJ in cells with a disrupted core complex suppresses the sensitivity to DNA ICL-inducing agents and reverses defective HR (Adamo et al., 2010; Pace et al., 2010). Therefore, it is now thought that the FA pathway diverts DNA ends formed from DNA ICL damage away from toxic NHEJ repair and towards HR-mediated repair.

### 1.2.3 FANCJ

FANCJ was originally identified as the physiological binding partner of BRCA1 (Cantor et al., 2001). FANCJ contains seven helicase motifs in its N-terminal region that display strong homology with the DEAH family of helicases, most notably including Xeroderma Pigmentosum complementation group D (XPD) (Fan et al., 2008). FANCJ also contains a nuclear localization signal and a conserved iron-sulfur (Fe-S) domain in its N-terminal region similar to XPD (Fan et al., 2008). The C-terminal region of FANCJ includes amino acids that compose the BRCA1-interacting region (Yu et al., 2003).
Biochemical studies revealed that FANCJ has both DNA-dependent ATPase and helicase activities (Cantor et al., 2004). Purified FANCJ can unwind partial DNA duplexes in a 5’ to 3’ manner but requires backbone continuity in both strands of the duplex as it moves along the DNA (Cantor et al., 2004; Gupta et al., 2006). This suggests that FANCJ has the ability to sense both DNA strands during unwinding. The requirement for backbone continuity can be overcome by increasing the amount of single-strand DNA at the 5’ end, which is required for the loading of FANCJ onto the DNA (Gupta et al., 2006). Replication Protein A (RPA), which immunoprecipitates with FANCJ, is one regulatory factor that stimulates FANCJ helicase activity and promotes it to unwind longer DNA duplexes (Gupta et al., 2007). On the other hand, the addition of BRCA1 in vitro has no effect on FANCJ helicase activity (Cantor et al., 2004; Gupta et al., 2007).

FANCJ has the ability to recognize complex DNA structures that are present at replication forks or in intermediate structures formed during HR. First, FANCJ preferentially binds to and unwinds forked duplex substrates that are common to DNA replication forks (Gupta et al., 2005). Second, FANCJ can also efficiently unwind model structures that mimic a D-loop in vitro (Gupta et al., 2005). D-loops are formed during HR when single-strand DNA invades duplex DNA. On the other hand, FANCJ is not able to resolve Holliday junction structures that can also form during HR (Gupta et al., 2005). Third, FANCJ can disrupt protein-DNA complexes similar to those formed during DNA replication and repair (Sommers et al., 2009). Taken together, these results suggest that FANCJ has the ability to recognize and unwind DNA structures that commonly arise during DNA repair and replication.

FANCJ was linked to HR-mediated DNA DSB repair when a genetic interaction with BRCA1 was established. FANCJ is phosphorylated in S-phase of the cell cycle and remains bound to BRCA1 after the generation of DNA DSBs (Peng et al., 2006). Immunofluorescence microscopy revealed that FANCJ forms discrete nuclear foci that localize with BRCA1 at sites of DNA damage in human cells, and the brightness of FANCJ foci is reduced but not completely abolished in BRCA1 null cells (Cantor et al., 2001; Gupta et al., 2007). This suggests that BRCA1 is required for the efficient localization of FANCJ into nuclear foci. Likewise, BRCA1 foci are diminished and delayed in FANCJ deficient cells, and this is corrected by FANCJ overexpression (Peng et al., 2006). Furthermore, when FANCJ is depleted in human cells, ionization radiation-induced γ-H2AX foci persist and defects in DNA DSB repair are observed.
Mutation of Lysine 52 (K52R), a conserved residue among numerous ATPases and helicases, abrogates the ATPase and helicase activities of FANCJ in vitro (Cantor et al., 2001; Cantor et al., 2004). It appears as though the helicase activity of FANCJ is involved in the repair of DNA DSBs as the overexpression of FANCJ<sup>K52R</sup> acts in a dominant-negative manner and results in a delay in the repair process (Cantor et al., 2001). Although a strong genetic connection exists between FANCJ and BRCA1, their functions differ with respect to RAD51 foci. BRCA1-deficient cells display a loss of RAD51 foci formation while cells deficient of FANCJ do not (Litman et al., 2005). This may be explained by the ability of BRCA1 to bind several other adapter proteins, including cell cycle regulators, DNA damage repair factors, tumor suppressors, oncogenes, and others (reviewed in Deng and Brodie, 2000).

Cells from FA patients with biallelic mutations in the gene that encodes FANCJ or human cells with depleted levels of FANCJ have increased sensitivity to DNA ICL-inducing agents (Levitus et al., 2005; Litman et al., 2005). It is unclear whether FANCJ functions in one pathway with the FA core and ID complex proteins or in a separate mechanism to repair DNA ICLs. FANCJ is not needed for FANCD2 monoubiquitination, suggesting that FANCJ plays a role either parallel to, or downstream of FANCD2 function (Levitus et al., 2004). In chickens, FANCJ appears to exist in an additional pathway that is independent of the core complex, as cells with both fancj and fancc mutations are more sensitive to DNA ICL-inducing agents than either of the single mutants (Bridge et al., 2005). However, in Caenorhabditis elegans, FANCJ is placed downstream of FANCD2 in the same pathway, as the fcd-2 and dog-1 double mutant shows similar sensitivity to single mutants (Youds et al., 2008). The dissimilarity in chickens and C. elegans is probably a result of different genetic interactions being tested or different species being used. However, FANCJ may have a role in two different mechanisms in chicken cells but not in C. elegans. Nevertheless, the DNA ICL repair function of FANCJ appears to be independent of BRCA1. In chicken and human cells deficient of FANCJ, the expression of human FANCJ that is unable to bind BRCA1 restores resistance to DNA ICL-inducing agents (Bridge et al., 2005; Xie et al., 2010). Although the interaction between FANCJ and BRCA1 may not be required for DNA ICL repair, it is still needed for proper DNA damage response and timely progression of cells through S-phase of the cell cycle (Kumaraswamy and Shiekhattar, 2007; Xie et al., 2010).

A function independent of DNA ICL repair has also been established for FANCJ. Human cells and C. elegans deficient of FANCJ and DOG-1 respectively accumulate deletions at genome
sequences that can form G4 DNA structures (Kruisselbrink et al., 2008; London et al., 2008). This led to the discovery that human FANCJ efficiently unwinds G4 DNA structures in a 5’ to 3’ direction in vitro (London et al., 2008; Wu et al., 2008). The human genome has more than 360,000 predicted regions that can form G4 DNA structures and it is thought that these structures cause DNA replication to stall (Huppert and Balasubramanian, 2005). FANCJ may be required to unwind these structures to allow the replication fork to proceed past the G-rich DNA. Consistent with this, FANCJ-depleted cells are sensitive to the addition of telomestatin, a small molecule that stabilizes G4 DNA structures, which results in elevated DNA damage and apoptosis (Wu et al., 2008). The ability of FANCJ to unwind G4 DNA structures appears to be independent of its role in the FA pathway to repair DNA ICLs (Kruisselbrink et al., 2008; London et al., 2008). Thus, FANCJ appears to function to preserve genome stability through the resolution of G4 DNA structures formed during DNA replication and to repair DNA damage.
1.3 Fanconi Anaemia Proteins and Telomere Length Maintenance

FA shares several clinical characteristics with another well documented disorder referred to as Dyskeratosis congenita (DC), most notably including extremely high frequencies of bone marrow failure (Knight et al., 1998). DC is associated with short telomere length as a result of a mutation in one of many genes that are involved in proper telomerase activity (Heiss et al., 1998; Mitchell et al., 1999; Vulliamy et al., 2001; Marrone et al., 2004; Marrone et al., 2007; Walne et al., 2007; Vulliamy et al., 2008). HSCs and progenitor cells have an important role in continuously replenishing the haematopoietic cell lineage. One hallmark of HSCs is their ability to maintain the lengths of their telomeres through telomerase expression. Telomere erosion in HSCs from DC patients could induce replicative senescence and exhaust the stem cell pool, resulting in bone marrow failure. Therefore, a potential cause of the clinical features observed in FA patients may be linked to telomere biology. In FA cells, dysfunctional telomeres may arise due to improper telomere capping, a rapid loss of telomeric repeat DNA, reduced telomere length maintenance, or a secondary effect that ultimately results in excessive proliferation and subsequent telomere shortening in HSCs.

In two studies that examined average telomere length of blood cells in FA patients, telomeres were found to be 1.7 kb shorter when compared to control patients of the same age group (Ball et al., 1998; Leteurtre et al., 1999). However, several of the FA patients had normal average telomere length despite developing bone marrow failure. Furthermore, a more recent study that compared average telomere length in both DC and FA patients showed that telomeres were shorter than average in primarily the FA granulocyte population, whereas in DC patients, telomeres were extremely short in all lineages tested (Alter et al., 2007). This suggests that if telomere dysfunction is involved in FA, the mechanism driving it is likely to differ from DC. Consistent with this, telomerase activity is not altered in blood cells from FA patients, and mutations in TERC have not been identified (Calado et al., 2004).

Cellular replicative senescence may not arise from a low average telomere length, but as a result of one or a few critically short telomeres that activate the cellular DDR (Zou et al., 2004). Two studies used metaphase spreads to observe the length of individual telomeres in cells from FA patients (Callén et al., 2002; Franco et al., 2004). One of the studies reported a slight increase in the number of chromosome ends with telomeres that are so short that they are unable to be
detected by standard protocol in FA cells (Callén et al., 2002). On the other hand, the other study found no difference when FA cells were compared to age-matched controls (Franco et al., 2004). Although some evidence exists that there is a correlation between short telomere length and the presence of FA, there is still a debate as to whether altered telomere biology is the driving force behind certain clinical features of FA, such as bone marrow failure.

Given the fact that the FA pathway is linked to HR, FA proteins may be involved in the ALT telomere length maintenance mechanism. Evidence of this came when FANCD2 was found to localize to telomeric foci and APBs in human ALT cells, and that this localization is dependent on FA proteins that make up the core complex (Spardy et al., 2008; Fan et al., 2009; Root and Meyn, unpublished data). In contrast, detectable localization of FANCD2 to telomeres or PML bodies does not occur in cells that use telomerase to maintain the lengths of their telomeres. A role for FANCD2 was further established in human ALT cells when the depletion of FANCD2 altered telomeric recombination. One group reported a decrease in the frequency of T-SCEs upon FANCD2 depletion (Fan et al., 2009), while work from our lab found an increase (Root and Meyn, unpublished data). Furthermore, it appears that FANCD2 restrains Bloom syndrome protein (BLM)-dependent amplification of telomeric repeat DNA in human ALT cells (Root and Meyn, unpublished data). BLM is the helicase that is mutated in Bloom syndrome and the codepletion of BLM suppresses the abnormally high telomeric repeat DNA synthesis in human ALT cells depleted of FANCD2 (Root and Meyn, unpublished data).
1.4 Hypothesis and Objectives

Although FA core and ID complex proteins have been linked to the ALT mechanism, whether the FA proteins that belong to the third subgroup also function in the ALT mechanism has not been extensively studied. It is currently unknown whether the third subgroup of FA proteins functions in the same pathway as FANCD2 monoubiquitination or in an independent but parallel pathway to repair DNA damage. FANCJ is of interest because (1) it is a FA protein that belongs to the third subgroup that can directly interact with DNA, (2) it physically interacts with BRCA1 and RPA, two key proteins in HR (Cantor et al., 2001; Gupta et al., 2007), (3) it plays a role in HR-mediated DNA repair of DSBs (Litman et al., 2005), and (4) it can unwind human telomeric G4 DNA structures in vitro (Wu et al., 2008). I hypothesize that FANCJ plays a role in the ALT mechanism. Thus, my objectives include to explore the role of FANCJ in the ALT mechanism, as well as to use the ALT mechanism as a model system to study what function FANCJ has in recombination.
CHAPTER 2

FANCIJ PLAYS A CRITICAL ROLE IN MODULATING TELOMERIC REPEAT DNA CONTENT IN HUMAN CELLS THAT UTILIZE THE ALTERNATIVE LENGTHENING OF TELOMERES (ALT) MECHANISM
CHAPTER 2

2 FANCl Plays a Critical Role in Modulating Telomeric Repeat DNA Content in Human Cells that Utilize the Alternative Lengthening of Telomeres (ALT) Mechanism

2.1 Experimental Approach

In the present study, the possible role of FANCl in the ALT mechanism was explored. FANCl has been linked to function in DNA replication and recombination-mediated DNA repair, and thus may play a role in ALT-specific telomeric repeat DNA synthesis in human cells. Indirect immunofluorescence microscopy techniques were used to assess the colocalization of FANCl with telomeric foci (TRF1 and TRF2) in two immortalized human ALT fibroblast cell lines. Comparisons were made with a human telomerase-positive fibrosarcoma cell line, an immortalized human telomerase-positive fibroblast cell line, and a primary human fibroblast cell line. One feature of ALT cells is the presence of APBs, which may be sites of telomeric recombination and elongation events. An important question that needed to be addressed in the present study was whether FANCl forms foci that localize to APBs. In addition, indirect immunofluorescence microscopy techniques were utilized to characterize the localization of FANCl with FANCl-interacting proteins and other FA proteins in human ALT cells.

The role of FANCl in human ALT cells was studied using RNA interference. Two independent small interfering RNAs (siRNAs) targeted against FANCl messenger RNA (mRNA) were used to deplete the level of FANCl protein in two ALT cell lines. Indirect immunofluorescence microscopy techniques were performed to study the effect of FANCl depletion on telomeric foci (TRF1 and TRF2), as well as on the localization of FANCl-interacting proteins and other FA proteins to telomeric foci in ALT cells. Finally, telomere-fluorescence in situ hybridization (t-FISH) procedures with a telomere sequence-specific peptide nucleic acid (PNA) probe were utilized to characterize the effect of FANCl depletion on telomeric repeat DNA content in human ALT cells.
2.2 Materials and Methods

Cell culturing. GM00847 (GM847), Wi38-VA13/2RA (VA13), GM00639 (GM639), and HT1080 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin (HyClone). GM05757 (GM5757) cells were maintained in GIBCO Minimum Essential Medium Alpha Medium (MEM Alpha) (Invitrogen) supplemented with 15% FBS and 1% penicillin-streptomycin. GM5757 cells were cultured from passage 13 to 15. All cell lines were cultured in a humidified 5% CO$_2$ incubator at 37°C. For storing purposes, cells were frozen in a freezing container with isopropyl alcohol and kept at -83°C in media containing 10% dimethyl sulfoxide (DMSO), and rapidly thawed in a 37°C water bath. When required, cells were manually counted using a haemocytometer.

siRNA transfection. siRNA oligonucleotides were synthesized (Dharmacon) to target the following sequences: FANCJ (siFANCJ$_1$, 5’-AGCTTACCCGTCAGCTT-3’ (Yu et al., 2003); siFANCJ$_2$, 5’-AAACAGCAAGCAACATTGTT-3’ (Kumaraswamy and Shiekhattar, 2007)), FANCD2 (siFANCD2$_1$, 5’-CCAGGAAGCAACCCTTTC-3’; siFANCD2$_2$, 5’-GGAGATTGATGGTCTACTA-3’ (Zhu and Dutta, 2006)), and control siRNA (siGL2, 5’-AACGTACGCGGAATACTTCGA-3’ (Zhu and Dutta, 2006)). $5 \times 10^4$ to $1 \times 10^5$ cells were seeded in 35 mm well plates containing 25 mm round coverslips or without coverslips in DMEM supplemented with 10% FBS 24 h prior to transfection. Cells were transfected with a final concentration of 25 nM (siFANCJ$_1$, siFANCJ$_2$, siGL2), 100 nM (siFANCD2$_1$, siFANCD2$_2$, siGL2) and 125 nM (siGL2) siRNA using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer’s instructions. Cells were subjected to a second round of siRNA transfection 48 h after the primary transfection. Cells were harvested for immunoblot analysis or subjected to immunofluorescence microscopy 3 days after primary transfection, and 6 days after for t-FISH analysis.

Immunoblot analysis. Cells were harvested and lysed in RIPA buffer (150 mM NaCl, 10 mM Tris pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1.0% Na-deoxycholate, 5 mM EDTA, 1X protease inhibitor). Protein concentration was determined using Lowry Detergent Compatible Protein Assay (Bio-Rad) following manufacturer’s instructions. Lysates were diluted with 3X SDS buffer (150 mM Tris pH 6.8, 15% β-mercaptoethanol, 6% SDS, 30% glycerol, 3 mg/10ml
bromophenol blue), boiled for 5 min, and stored at -20°C. 5-10µg of protein was run out on a NuPAGE 4-12% Bis-Tris Gel (Invitrogen) at 150 V for 15 min and then at 200 V for 45 min. Protein was transferred onto an Immobile-P Transfer Membrane (Millipore) at 100 V for 1.5 h. Membranes were blocked with 5% skim milk in Tris-buffered saline Tween-20 (TBST) (8% NaCl, 0.2% KCl, 3% Tris pH 7.4, 0.05% Tween-20). Blots were probed with rabbit anti-FANCJ (Sigma: B 1310, 1:20,000), rabbit anti-BRCA1 (Santa Cruz: sc-642, 1:200), mouse anti-FANCD2 (Santa Cruz: sc-20022, 1:500), and mouse anti-α-tubulin (Santa Cruz: sc-32293, 1:20,000) primary antibodies for 1 h at room temperature. Blots were washed in TBST and probed with horseradish peroxidase-conjugated donkey secondary antibodies (Jackson ImmunoResearch, 1:7,500) for 1 h at room temperature. Amersham ECL Plus Western Blotting Detection System (GE Healthcare) was used for chemiluminescent detection following the manufacturer’s instructions. Maximum Sensitivity Imaging films (Bioflex) were exposed to the membrane and developed using a Kodak X-OMAT 2000A film processor.

**Immunofluorescence microscopy.** Cells were seeded onto 25 mm round coverslips in 35 mm well plates 24 h prior to fixation. The coverslips were washed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde, 0.2% Triton X-100 at pH 8.2 for 20 min at room temperature, followed by permeabilization with 0.5% Nonidet P-40 for 10 min at room temperature. After washing with PBS, the coverslips were blocked with 3 mg/ml donkey serum (Jackson ImmunoResearch), 0.2% fish gelatin, 0.5% bovine serum albumin (BSA) in PBS for 30 min at 37°C. Coverslips were washed with PBS and probed with rabbit anti-FANCJ (Sigma: B 1310, 1:4,000), mouse anti-FANCD2 (sc-20022 Santa Cruz, 1:100), rabbit anti-FANCD2 (Novus Biologicals: NB100-182, 1:3,000), mouse anti-BRCA1 (Calbiochem: OP93-100UG, 1:100), rabbit anti-53BP1 (Novus Biologicals: NB100-304, 1:4,000), mouse anti-PML (gift from Dr. Roel van Driel: 5E10, 1:10), rabbit anti-PML (Chemicon International: AB1370, 1:2,000), rabbit anti-TRF1 (Abcam: ab1423, 1:2,000), goat anti-TRF1 (Santa Cruz: sc-6165, 1:100), and mouse anti-TRF2 (Imgenex: IMG-124A, 1:1,000) primary antibodies for 1 h at room temperature. After washing with blocking solution, fluorescein isothiocyanate (FITC)-, tetramethyl rhodamine isothiocyanate (TRITC)-, and Cy-5-conjugated donkey secondary antibodies (Jackson ImmunoResearch, 1:500) were added to the coverslips for 30 min at room temperature. After washing with PBS, DNA was stained with 0.1µg/ml 4’,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature and the coverslips were mounted on slides using SlowFade Antifade
mounting medium (Invitrogen). 12-bit grayscale widefield images were captured with a Zeiss Axioplan 2 microscope equipped with a Hammamatsu Orca ER camera using Openlab software versions 4.0.2 and 5.5.1 (Improvision) under 63x and 40x magnification. Samples from a single experiment were all processed and imaged at the same time, using identical exposure times. Foci number and colocalization were manually determined by analysis of Openlab images. Only foci with a 2-fold intensity over background nucleoplasmic staining intensity were considered.

**Cell Cycle Profiling.** Bromodeoxyuridine (BrdU) was added to cells growing on 25 mm round coverslips in 35mm well plates to a final concentration of 10µM, and incubated in a humidified 5% CO₂ incubator at 37°C for 30 min. Coverslips were washed with PBS and fixed with 2% paraformaldehyde, 0.2% Triton X-100 at pH 8.2 for 20 min at room temperature, followed by permeabilization with 0.5% Nonidet P-40 for 10 min at room temperature. After washing with PBS, coverslips were blocked with 3 mg/ml donkey serum (Jackson ImmunoResearch), 0.2% fish gelatin, 0.5% BSA in PBS for 30 min at 37°C. Coverslips were washed with PBS and probed with rabbit anti-Centromere Protein F (CENPF) (Abcam: ab5, 1:2,000) primary antibody for 1 h at room temperature. After washing with blocking solution, Cy-5-conjugated donkey secondary antibody (Jackson ImmunoResearch, 1:500) was added to the coverslips for 30 min at room temperature. Cells were subjected to a second round of fixation with 4% paraformaldehyde at pH 8.2 for 5 min at room temperature, washed again with PBS, and then the DNA was denatured by incubating the coverslips in 2 N HCl for 30 min at 37°C to allow antibody access to the BrdU. After washing and blocking as described above, mouse anti-BrdU (BD Pharmingen: 555627, 1:3,000) antibody was added for 1 h at room temperature. Coverslips were washed with blocking solution and FITC-conjugated donkey secondary antibody (Jackson ImmunoResearch, 1:500) was added for 30 min at room temperature. After washing the coverslips with PBS, the DNA was stained with 0.1µg/ml DAPI for 5 min at room temperature and the coverslips were mounted using SlowFade Antifade mounting medium (Invitrogen). 12-bit grayscale widefield images were captured with a Zeiss Axioplan 2 microscope equipped with a Hammamatsu Orca ER camera using Openlab software versions 4.0.2 (Improvision) under 20x magnification. Samples from a single experiment were all processed and imaged at the same time, using identical exposure times. Nuclei positive for BrdU or CENPF staining were categorized as being in S-phase or G2-phase respectively. Nuclei in M-phase were determined by morphology with DAPI staining. The remaining nuclei were categorized as being in G1/G0-phase.
**Telomere-fluorescence in-situ hybridization.** Cells were harvested and subjected to hypotonic swelling in 75 mM KCl for 30 min at 37°C, followed by fixation in a 3:1 methanol/acetic acid solution. Cells were dropped on slides, rapidly dried on a slide plate heated to 45°C, and left for 24 h at room temperature to fully air dry. After washing in PBS, slides were fixed with 4% paraformaldehyde in PBS for 2 min, washed again with PBS and treated with 1 mg/ml pepsin at pH 2.0 for 10 min at 37°C. After washing in PBS, the paraformaldehyde fixation and washing steps were repeated. Slides were dehydrated in ethanol and air dried. Hybrization mixture containing 70% formamide, 0.5ug/ml Cy-3-conjugated (C3TA3)3 telomere peptide nucleic acid probe, 10mM Tris pH 7.2, 0.1% blocking reagent (Boehringer), 5% MgCl2 buffer (82 mM Na2HPO4, 9 mM citric acid, 20 mM MgCl2) was preheated for 5 min at 86°C and added to the surface of the slides. Coverslips were placed on the surface to spread the hybridization mixture and the slides were incubated for 3 min at 83°C. After hybridization for 2 h at room temperature, slides were washed with 70% formamide, 10 mM Tris pH 7.2, 0.1% BSA two times for 15 min, and then three times for 5 min with 0.1 M Tris pH 7.2, 0.15 M NaCl, 0.08% Tween-20. DNA was stained with 0.1µg/ml DAPI for 5 min and the slides were dehydrated in ethanol and air dried. Coverslips were mounted on the slides using SlowFade Antifade mounting medium (Invitrogen). 12-bit grayscale widefield images were captured with a Zeiss Axioplan 2 microscope equipped with a Hammamatsu Orca ER camera using Openlab software version 5.5.1 (Improvision) under 40x magnification. Slides from a single experiment were all processed and imaged at the same time, using 200 ms, 100 ms, 50 ms, 25 ms, 15 ms, and 10 ms exposure times. Images were analyzed using Volocity software version 3.7.0 (Improvision). The number and intensity values of telomeric DNA foci were initially analyzed from images taken with 200 ms exposure time using the lasso tool at 95% threshold. Foci with a pixel count of less than 5 were discarded. If a particular focus contained pixels that exceeded the camera’s maximum capacity, an image that was taken with a lower expose time was analyzed to measure the intensity of that focus. The intensity values obtained from images taken with lower exposure times were standardized against the intensity values obtained from images taken at 200 ms.

**Statistical analysis.** Error bars represent standard deviations. For graphs that display percentages, a general linear model with a Poisson distribution was used to determine statistical significance using R software version 2.10.1. For graphs that display number of foci, a general linear model with a normal error distribution was used to determine statistical significance using R software version 2.10.1.
2.3 Results

2.3.1 FANCJ localizes to telomeric foci and APBs in human ALT cells

FANCJ forms discrete nuclear foci in human cells spontaneously during S- and G2-phases of the cell cycle and in response to agents that induce DNA damage (Gupta et al., 2007; Zhang et al., 2010). Indirect immunofluorescence microscopy techniques were utilized to investigate whether FANCJ is present at telomeric foci in human cells that maintain the lengths of their telomeres by using the ALT mechanism. GM847 and VA13 are human skin and lung fibroblast cell lines respectively, each transformed with the SV40 large T antigen and immortalized by a spontaneous development of the ALT pathway. FANCJ forms spontaneous foci that frequently colocalize with foci of TRF1 and TRF2 in GM847 and VA13 cells (Figure 2-1 A). TRF1 and TRF2 bind telomeric repeat DNA and are commonly used as markers for telomeres (de Lange et al., 2005).

In randomly selected nuclei from asynchronous populations of GM847 and VA13 cells, 32.8% and 43.0% of the detectable FANCJ foci localize to foci of TRF2 respectively (Figure 2-1 B). In contrast, the presence of FANCJ at telomeric foci is rarely observed in a human telomerase-positive fibrosarcoma cell line (HT1080), an immortalized human telomerase-positive fibroblast cell line (GM639), and a primary human fibroblast cell line (GM5757) (Figure 2-1 A). Only 3.2%, 2.7%, and 2.5% of the detectable FANCJ foci localize to foci of TRF2 in randomly selected nuclei from asynchronous populations of HT1080, GM639, and GM5757 cells respectively (Figure 2-1 B). In addition, there is a ~2-fold increase in the total number of spontaneous non-telomeric FANCJ foci detected in ALT cell lines compared to telomerase-positive cell lines and a primary cell line (Figure 2-1 B).

Not all of the nuclei in asynchronous populations of ALT cells display FANCJ colocalization with telomeric foci. Although more than 60% of the GM847 and VA13 nuclei display at least one detectable FANCJ focus, only 37.5% and 39.8% of the nuclei display at least one detectable FANCJ-TRF2 colocalization event respectively (Figure 2-1 C). In these nuclei, one to four colocalization events are commonly detected. However, a small fraction of the nuclei have five or more detectable FANCJ-TRF2 colocalization events but account for a large fraction of the total number of colocalization events detected. In an asynchronous population of GM847 cells, 6.3% of the randomly selected nuclei display at least five FANCJ-TRF2 colocalization events and account for 38.8% of the total number of colocalization events detected (Figure 2-1 C).
Figure 2-1. FANCJ frequently localizes with TRF2 in ALT cells.

(A) Representative images of GM847, VA13, GM639, HT1080, and GM5757 nuclei with TRF2 and FANCJ foci. (B) The number of FANCJ foci that either localize or do not localize to foci of TRF2 in 200 randomly selected nuclei from asynchronous populations of GM847, VA13, GM639, HT1080, and GM5757 cells. (C) The percentage of nuclei that contain at least one FANCJ focus, at least one FANCJ-TRF2 colocalization event, and at least five FANCJ-TRF2 colocalization events in 600 randomly selected nuclei from asynchronous populations of GM847 and VA13 cells. Data obtained from three independent experiments. Error bars represent standard deviations.
Figure 2-1

A

<table>
<thead>
<tr>
<th>Sample</th>
<th>DAPI</th>
<th>TRF2</th>
<th>FANCJ</th>
<th>MERGE</th>
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<tr>
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<td>![Image]</td>
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<tr>
<td>GM639 (tel +ve)</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>GM5757 (1°)</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
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</tr>
</tbody>
</table>

Scale: 5 μm
Figure 2-1

B

Number of FANCD2 foci in 200 nuclei

- not localized to TRF2
- localized to TRF2

GM847  VA13  HT1080  GM639  GM5757

C

% of nuclei

- at least 1 FANCD2 focus
- at least 1 FANCD2-TRF2 colocalization event
- at least 5 FANCD2-TRF2 colocalization events

GM847  VA13
Similarly, 8.7% of the randomly selected nuclei from an asynchronous population of VA13 cells have at least five FANCJ-TRF2 colocalization events and account for 53.6% of the total number of colocalization events detected (Figure 2-1 C).

APBs are nuclear bodies found in human ALT cells and are characterized by the colocalization of PML with telomeric repeat DNA, telomere binding proteins, and DNA recombination proteins (Yeager et al., 1999). Thus, APBs may be sites of ALT-specific telomeric recombination and elongation events. FANCJ displays strong localization to APBs in ALT cells (Figure 2-2 A). APBs were identified by visualizing TRF1 or TRF2 foci within a PML body. In randomly selected nuclei with at least one detectable FANCJ focus from asynchronous populations of GM847 and VA13 cells, 97.9% and 95.7% of the telomere-associated FANCJ foci detected colocalize with PML respectively (Figure 2-2 B). Although the majority of the telomere-associated FANCJ foci are detected at APBs, there is a substantial number of APBs that do not colocalize with FANCJ. 44.0% and 25.2% of the APBs detected in these GM847 and VA13 nuclei do not colocalize with FANCJ respectively (Figure 2-2 B).

### 2.3.2 Depletion of FANCJ impairs expansion of populations of human ALT cells

To study the function of FANCJ in human ALT cells, RNA interference was used to deplete FANCJ protein level. FANCJ was transiently depleted in both GM847 and VA13 cells using two independent siRNAs targeted against FANCJ mRNA (siFANCJ_1 and siFANCJ_2) (Figure 2-3 A). FANCJ foci formation is rarely detected in one hundred randomly selected nuclei from FANCJ-depleted asynchronous populations of GM847 and VA13 cells using either siRNA (Figure 2-3 B, C). Furthermore, the depletion of FANCJ impairs expansion of populations of both ALT cell lines 3 days after initial transfection (Figure 2-4 A). However, by indirect immunofluorescence microscopy, the cell cycle profiles of both ALT cell lines are not altered upon FANCJ depletion (Figure 2-4 B).

### 2.3.3 Depletion of FANCJ causes the loss of large APBs in human ALT cells

Since FANCJ frequently localizes to a subset of APBs in human ALT cells, it was important to determine whether FANCJ is required for APB formation. The depletion of FANCJ in ALT cells results in a small but significant decrease in the total number of detectable APBs. In one hundred randomly selected nuclei from control asynchronous populations of GM847 and VA13 cells,
Figure 2-2. Telomere-associated FANCJ primarily localizes to APBs in ALT cells.

(A) Representative images of GM847 and VA13 nuclei with colocalization between TRF1, FANCJ, and PML foci. (B) Venn diagrams displaying colocalization between FANCJ, PML, and TRF1 foci in 50 randomly selected nuclei with at least one FANCJ focus from asynchronous populations of GM847 and VA13 cells.
Figure 2-2
Figure 2-3. FANCJ protein level is depleted in ALT cells.

(A) Immunoblot analysis of FANCJ protein level in GM847 and VA13 cells treated with control, siFANCJ_1, or siFANCJ_2 siRNA. The level of α-tubulin represents protein loading. (B) Representative images of control siRNA-treated and FANCJ-depleted GM847 and VA13 nuclei with and without FANCJ foci respectively. (C) The number of FANCJ foci in 100 randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells. Data obtained from three independent experiments. Error bars represent standard deviations.
Figure 2-3

A

<table>
<thead>
<tr>
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<th>GM847</th>
<th></th>
<th>VA13</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>non-treated</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>control siRNA</td>
<td>+</td>
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<td></td>
</tr>
<tr>
<td>siFANCJ_2</td>
<td></td>
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<td></td>
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</tbody>
</table>

1 2 3 4

FANCJ

α-tubulin

B

control siRNA

siFANCJ_1

siFANCJ_2

DAPI  GM847  FANCJ

DAPI  VA13  FANCJ

10 μm
Figure 2-3

C

![Bar chart showing the number of FANCJ foci in 100 nuclei for GM847 and VA13 cell lines under different conditions. The chart includes data for non-treated, control siRNA, siFANCJ-1, and siFANCJ-2 treatments. The p-values are indicated for each comparison.](image)
Figure 2-4. Depletion of FANCJ impairs expansion of populations of ALT cells without altering cell cycle profile.

(A) Representative images of cell confluency of control and FANCJ-depleted GM847 and VA13 cells 3 days after initial transfection. (B) Cell cycle profiles of control and FANCJ-depleted GM847 and VA13 cells. Data obtained from 3,000 randomly selected nuclei from asynchronous populations of cells.
Figure 2-4

A

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<tr>
<th>GM847</th>
<th>non-treated</th>
<th>control siRNA</th>
<th>siFANCJ_1</th>
<th>siFANCJ_2</th>
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Legend:
- M-phase
- G2-phase
- S-phase
- G1/G0-phase
~375 and ~462 APBs are detected respectively (Figure 2-5 A). The total number of detectable APBs in GM847 and VA13 nuclei depleted of FANCJ using either siRNA decreases by ~20% (Figure 2-5 A). Although the total number of detectable APBs is affected upon FANCJ depletion, the percentage of TRF2 foci detected that colocalize with PML is not significantly altered. In randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells, ~32% and ~41% of the total number of TRF2 foci detected colocalize with PML respectively (Figure 2-5 B). The decrease in the total number of detectable APBs in FANCJ-depleted ALT cells is probably a preferential loss of the APBs with large TRF2 foci as these are no longer detected in GM847 and VA13 nuclei (Figure 2-5 C).

2.3.4 FANCJ localizes with FANCD2 at telomeric foci in human ALT cells

FANCD2 monoubiquitination is considered to be a switch that turns on the FA pathway (de Winter and Joenje, 2009). FANCJ functions either downstream of FANCD2 monoubiquitination or in a completely independent but parallel mechanism with respect to DNA ICL repair. In human ALT cells, FANCJ displays strong colocalization with FANCD2 at telomeric foci (Figure 2-6 A). In randomly selected nuclei with at least one detectable FANCJ focus from asynchronous populations of GM847 and VA13 cells, 93.7% and 86.4% of the telomere-associated FANCJ foci colocalize with FANCD2 respectively (Figure 2-6 B). Although the majority of the telomere-associated FANCJ foci detected colocalize with FANCD2, there is a substantial number of telomere-associated FANCD2 foci detected that do not colocalize with FANCJ. 23.4% and 32.1% of the telomere-associated FANCD2 foci detected in these GM847 and VA13 nuclei do not colocalize with FANCJ respectively (Figure 2-6 B).

As FANCJ and FANCD2 colocalize frequently at telomeric foci in human ALT cells, it was important to determine whether the localization of one FA protein to telomeric foci is dependent on the other. The depletion of FANCJ in ALT cells results in a small but significant decrease in the total number of detectable TRF2-FANCD2 colocalization events. In one hundred randomly selected nuclei from control asynchronous populations of GM847 and VA13 cells, ~151 and ~211 TRF2-FANCD2 colocalization events are detected respectively (Figure 2-7 A). The total number of detectable TRF2-FANCD2 colocalization events in GM847 and VA13 nuclei depleted of FANCJ using either siRNA decreases by ~30% and ~20% respectively (Figure 2-7 A). Although the total number of detectable TRF2-FANCD2 colocalization events is affected
Figure 2-5. ALT cells depleted of FANCJ contain less APB.

(A) The number of APBs in 100 randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells. (B) The percentages of TRF2 foci colocalizing with foci of PML in 300 randomly selected nuclei from control and FANCJ-depleted synchronous populations of GM847 and VA13 cells. Data obtained from three independent experiments. Error bars represent standard deviations. (C) Representative images of control siRNA-treated and FANCJ-depleted GM847 and VA13 nuclei with TRF2 and PML foci.
Figure 2-5

A

Number of APBs in 100 nuclei

GM847

- Control: blue bar
- siFANC1-1: blue bar
- siFANC1-2: green bar

p < 0.05

VA13

- Control: blue bar
- siFANC1-1: blue bar
- siFANC1-2: green bar

p < 0.05

B

% TRF2 colocalized with PML

GM847

- Control: blue bar
- siFANC1-1: blue bar
- siFANC1-2: green bar

p > 0.05

VA13

- Control: blue bar
- siFANC1-1: blue bar
- siFANC1-2: green bar

p > 0.05
Figure 2-5

C

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5μm

VA13

control siRNA |  |  |  |  |
| siFANCJ_1 |  |  |  |  |
| siFANCJ_2 |  |  |  |  |
Figure 2-6. Telomere-associated FANCJ primarily localizes with FANCD2 in ALT cells.

(A) Representative images of GM847 and VA13 nuclei with colocalization between TRF1, FANCJ, and FANCD2 foci. (B) Venn diagrams displaying colocalization between FANCJ, FANCD2, and TRF1 foci in 50 randomly selected nuclei with at least one FANCJ focus from asynchronous populations of GM847 and VA13 cells.
Figure 2-6

A

GMB47

VA13

5 μm

B

GM847

FANCJ

290

FANCD2

389

FANCJ

571

FANCD2

712

TRF1

302

TRF1

316

TRF1

545

TRF1

927

375

59

177

12

40

118

12

61

118

37

100

60

177

54

118

60
Figure 2-7. ALT cells depleted of FANCJ display less TRF2-FANCD2 colocalization events.

(A) The number of TRF2-FANCD2 colocalization events in 100 randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells. (B) The percentage of TRF2 foci colocalizing with foci of FANCD2 in 300 randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells. Data obtained from three independent experiments. Error bars represent standard deviations.
Figure 2-7

A

![Bar chart showing the number of TRF2-FA NCD2 in 100 nuclei.](chart)

B

![Bar chart showing the percentage of TRF2 colocalized with FA NCD2.](chart)
upon FANCJ depletion, the percentage of TRF2 foci detected that colocalize with FANCD2 is not significantly altered. In randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells, ~13% and ~19% of the total number of TRF2 foci detected colocalize with FANCD2 respectively (Figure 2-7 B).

FANCD2 was transiently depleted in both GM847 and VA13 cells using two independent siRNAs targeted against FANCD2 mRNA (siFANCD2_1 and siFANCD2_2) (Figure 2-20). The depletion of FANCD2 in human ALT cells results in a small but significant increase in the total number of detectable TRF2-FANCJ colocalization events. In one hundred randomly selected nuclei from control asynchronous populations of GM847 and VA13 cells, ~99 and ~141 TRF2-FANCJ colocalization events are detected respectively (Figure 2-8 A). The total number of detectable TRF2-FANCJ colocalization events in GM847 and VA13 nuclei depleted of FANCD2 using either siRNA increases by ~20% and ~25% respectively (Figure 2-8 A). Although the total number of detectable TRF2-FANCJ colocalization events is affected upon FANCD2 depletion, the percentage of TRF2 foci detected that colocalize with FANCJ is not significantly altered. In randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells, ~9% and ~13% of the total number of TRF2 foci detected colocalize with FANCJ respectively (Figure 2-8 B).

### 2.3.5 FANCJ localizes with BRCA1 at telomeric foci in human ALT cells and telomere-associated BRCA1 foci are lost upon the depletion of FANCJ

BRCA1 has an established role in HR-mediated DNA damage repair and is a physiological binding partner of FANCJ. As a result, it was important to determine whether BRCA1 localizes with FANCJ at telomeric foci in human ALT cells. FANCJ displays frequent colocalization with BRCA1 at telomeric foci in ALT cells (Figure 2-9 A). In randomly selected nuclei with at least one detectable FANCJ focus from asynchronous populations of GM847 and VA13 cells, 60.1% and 56.6% of the telomere-associated FANCJ foci detected colocalize with BRCA1 respectively (Figure 2-9 B). However, the majority of the telomere-associated BRCA1 foci detected colocalize with FANCJ. 87.1% and 94.2% of the telomere-associated BRCA1 foci detected in these GM847 and VA13 nuclei colocalize with FANCJ respectively (Figure 2-9 B).
Figure 2-8. ALT cells depleted of FANCD2 display more TRF2-FANCJ colocalization events.

(A) The number of TRF2-FANCJ colocalization events in 100 randomly selected nuclei from control and FANCD2-depleted asynchronous populations of GM847 and VA13 cells. (B) The percentage of TRF2 foci colocalizing with foci of FANCJ in 300 randomly selected nuclei from control and FANCD2-depleted asynchronous populations of GM847 and VA13 cells. Data obtained from three independent experiments. Error bars represent standard deviations.
Figure 2-8

A

Number of TRF2-FANCJ in 100 nuclei

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B

% TRF2 colocalized with FANCJ

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<td>siFANC2-2</td>
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Figure 2-9. Telomere-associated FANCJ localizes with BRCA1 in ALT cells.

(A) Representative images of GM847 and VA13 nuclei with colocalization between TRF1, FANCJ, and BRCA1 foci. (B) Venn diagrams displaying colocalization between FANCJ, BRCA1, and TRF1 foci in 50 randomly selected nuclei with at least one FANCJ focus from asynchronous populations of GM847 and VA13 cells.
A report demonstrated a reduction in the number of BRCA1 foci in FANCJ-deficient human cells (Peng et al., 2006). Consistent with this, a ~2-fold decrease in the total number of detectable BRCA1 foci is observed in one hundred randomly selected nuclei from FANCJ-depleted asynchronous populations of GM847 and VA13 cells compared to cells from control populations (Figure 2-10 A). However, BRCA1 protein level is not significantly altered upon the depletion of FANCJ (Figure 2-10 B). The subset of BRCA1 foci that is lost in FANCJ-depleted ALT cells encompasses the foci that are also found at telomeric foci. In randomly selected nuclei from control asynchronous populations of GM847 and VA13 cells, ~6% and ~11% of the TRF1 foci detected colocalize with BRCA1 respectively (Figure 2-10 C). In contrast, TRF1-BRCA1 colocalization events are rarely detected in GM847 and VA13 cells upon the depletion of FANCJ using either siRNA (Figure 2-10 C).

2.3.6 Human ALT cells depleted of FANCJ display an elevated level of TIF formation

If telomeres are not properly capped, they typically associate with cellular DDR proteins. 53BP1 is a DNA damage checkpoint mediator that localizes to dysfunctional telomeres, and this localization is commonly used to detect telomere dysfunction in human cells (Takai et al., 2003). A small number of TIFs are detected in human ALT cells, as defined by the colocalization of 53BP1 with foci of TRF2. In randomly selected nuclei from control asynchronous populations of GM847 and VA13 cells, ~4% and ~7% of the total number of TRF2 foci detected colocalize with 53BP1 respectively (Figure 2-11). Upon the depletion of FANCJ using either siRNA, there is a ~2-fold increase in the percentage of detectable TRF2 foci that colocalize with 53BP1 in the nuclei of GM847 and VA13 cells compared to cells from control populations (Figure 2-11).

2.3.7 Human ALT cells depleted of FANCJ display reductions in the numbers and intensities of TRF1 and TRF2 foci

Indirect immunofluorescence microscopy reveals a telomeric staining pattern where 5 to 10% of the nuclei in asynchronous populations of GM847 and VA13 cells display very large TRF1 and TRF2 foci (Figure 2-12 A). Upon the depletion of FANCJ using either siRNA, very large telomeric foci are no longer detected in GM847 and VA13 nuclei (Figure 2-12 A). Furthermore, the total numbers of detectable TRF1 and TRF2 foci is reduced by ~18% in randomly selected nuclei from FANCJ-depleted asynchronous populations of GM847 and VA13 cells compared to cells from control populations (Figure 2-12 B).
Figure 2-10. BRCA1 localization to telomeric foci is lost in ALT cells depleted of FANCJ.

(A) The number of BRCA1 foci in 100 randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells. (B) Immunoblot analysis of FANCJ and BRCA1 protein level in GM847 and VA13 cells treated with control, siFANCJ_1, or siFANCJ_2 siRNA. The level of α-tubulin represents protein loading. (C) The percentage of TRF2 foci colocalizing with foci of BRCA1 in 300 randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells. Data obtained from three independent experiments. Error bars represent standard deviations.
Figure 2-10

A

Number of BRCA1 foci in 100 nuclei

GM847

- non-treated
- control siRNA
- siFANCJ1
- siFANCJ2

VA13

- non-treated
- control siRNA
- siFANCJ1
- siFANCJ2

p < 0.01

p < 0.001

B

GM847

- non-treated
- control
- siRNA
- siFANCJ1
- siFANCJ2

VA13

- non-treated
- control
- siRNA
- siFANCJ1
- siFANCJ2

1 2 3 4

FANCJ

BRCA1

α-tubulin

C

GM847

- non-treated
- control siRNA
- siFANCJ1
- siFANCJ2

p < 0.001

% TRF2 co-localized with BRCA1

VA13

- non-treated
- control siRNA
- siFANCJ1
- siFANCJ2

p < 0.001

% TRF2 co-localized with BRCA1
Figure 2-11. ALT cells depleted of FANCJ display an elevated level of TIF formation.

The percentage of TRF2 foci colocalizing with foci of 53BP1 in 300 randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells. Data obtained from three independent experiments. Error bars represent standard deviations.
Figure 2-11

The bar charts show the percentage of TRF2 colocalized with 53BP1 in GM847 and VA13 cells under different conditions. The charts indicate significant differences in the percentage of TRF2 colocalization, with p-values less than 0.001 for both GM847 and VA13 cells when comparing treated and non-treated conditions.
Figure 2-12. ALT cells depleted of FANCJ display reductions in the numbers and intensities of TRF1 and TRF2 foci.

(A) Representative images of control siRNA-treated and FANCJ-depleted GM847 and VA13 nuclei with TRF2 foci. (B) The numbers of TRF1 and TRF2 foci in 100 randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells. Data obtained from three independent experiments. Error bars represent standard deviations.
Figure 2-12

A

controls/siRNA

B

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<td>p &lt; 0.05</td>
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Number of foci in 100 nuclei
2.3.8 Human ALT cells depleted of FANCJ have less telomeric repeat DNA content primarily as a result of the loss of the brightest telomeric repeat DNA foci

Since FANCJ has been linked to DNA repair and replication, it was tempting to speculate that FANCJ has a role in the generation of telomeric repeat DNA in human ALT cells, as opposed to a role in the localization of TRF1 and TRF2. To investigate this idea, telomeric repeat DNA content was measured in ALT cells depleted of FANCJ by t-FISH with a telomere sequence-specific PNA probe. Similar to the decrease in the total numbers of TRF1 and TRF2 foci observed in GM847 and VA13 nuclei depleted of FANCJ, there is a ~30% and ~25% decrease in the total number of telomeric repeat DNA foci detected in one hundred randomly selected nuclei from FANCJ-depleted asynchronous populations of GM847 and VA13 cells respectively compared to cells from control populations (Figure 2-13 A). Furthermore, the mean telomeric repeat DNA content per nucleus was found to be ~2.5-fold less in GM847 and VA13 cells depleted of FANCJ compared to cells from control populations (Figure 2-13 B, C).

The depletion of FANCJ in human ALT cells preferentially affects nuclei with high telomeric repeat DNA content. In one hundred randomly selected nuclei from non-treated and control siRNA-treated asynchronous populations of GM847 cells, 30% and 32% of the nuclei exceed a PNA intensity of 10,000 respectively (Figure 2-14 A, B). In contrast, only 5% and 6% of the nuclei exceed a PNA intensity of 10,000 when these cells are treated with siFANCJ_1 and siFANCJ_2 siRNAs respectively (Figure 2-14 C, D). Similarly, in one hundred randomly selected nuclei from non-treated and control siRNA-treated asynchronous populations of VA13 cells, 33% and 29% of the nuclei exceed a PNA intensity of 10,000 respectively (Figure 2-15 A, B). On the other hand, only 10% and 6% of the nuclei exceed a PNA intensity of 10,000 when these cells are treated with siFANCJ_1 and siFANCJ_2 siRNAs respectively (Figure 2-15 C, D).

When considering individual telomeric repeat DNA foci, the depletion of FANCJ in human ALT cells does not have a major affect on the population of foci with low telomeric repeat DNA content. The distributions of foci with PNA intensities of less than 1,000 in one hundred randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells are almost identical (Figure 2-16, Figure 2-17). However, when considering only the foci with high telomeric repeat DNA content, ALT cells depleted of FANCJ contain fewer of these foci compared to control ALT cells. The distributions of telomeric repeat DNA foci that exceed a PNA intensity of 1,000 in one hundred randomly selected nuclei from
Figure 2-13. ALT cells depleted of FANCJ display a reduction in telomeric repeat DNA content.

(A) The number of telomeric repeat DNA foci in 100 randomly selected nuclei from control and FANCJ-depleted asynchronous populations of GM847 and VA13 cells. (B) Representative images of control siRNA-treated and FANCJ-depleted GM847 and VA13 nuclei with telomeric repeat DNA foci. (C) The mean telomeric repeat DNA content per nucleus from 100 randomly selected nuclei from control and FANCD2-depleted asynchronous populations of one hundred GM847 and VA13 cells.
Figure 2-13

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B

[Images of fluorescence microscopy showing telomeric DNA localization in GM847 and VA13 cells treated with control siRNA, siFANCJ-1, and siFANCJ-2.]
Figure 2-13

C

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Mean telomeric repeat DNA content/nucleus (PNA intensity)
Figure 2-14. The depletion of FANCJ in GM847 cells reduces the number of nuclei that have high telomeric repeat DNA content.

The distributions of 100 randomly selected nuclei from (A) non-treated, (B) control siRNA-treated, (C) siFANCJ_1-treated, and (D) siFANCJ_2-treated asynchronous populations of GM847 cells. The percentage of nuclei that exceed a PNA intensity of 10,000 is displayed for each population of cells.

Figure 2-15. The depletion of FANCJ in VA13 cells reduces the number of nuclei that have high telomeric repeat DNA content.

The distributions of 100 randomly selected nuclei from (A) non-treated, (B) control siRNA-treated, (C) siFANCJ_1-treated, and (D) siFANCJ_2-treated asynchronous populations of VA13 cells. The percentage of nuclei that exceed a PNA intensity of 10,000 is displayed for each population of cells.
Figure 2-14

**A**
GM847 (non-treated)
30% of nuclei exceed 10,000 PNA intensity

**B**
GM847 (control siRNA-treated)
32% of nuclei exceed 10,000 PNA intensity

**C**
GM847 (siFANCI_1-treated)
5% of nuclei exceed 10,000 PNA intensity

**D**
GM847 (siFANCI_2-treated)
6% of nuclei exceed 10,000 PNA intensity

Figure 2-15

**A**
VA13 (non-treated)
33% of nuclei exceed 10,000 PNA intensity

**B**
VA13 (control siRNA-treated)
29% of nuclei exceed 10,000 PNA intensity

**C**
VA13 (siFANCI_1-treated)
10% of nuclei exceed 10,000 PNA intensity

**D**
VA13 (siFANCI_2-treated)
6% of nuclei exceed 10,000 PNA intensity
Figure 2-16. The depletion of FANCJ in GM847 cells does not have a major affect on the distribution of telomeric repeat DNA foci with low telomeric repeat DNA content.

The distributions of telomeric repeat DNA foci with PNA intensities of less than 1,000 in 100 randomly selected nuclei from (A) non-treated, (B) control siRNA-treated, (C) siFANCJ_1-treated, and (D) siFANCJ_2-treated asynchronous populations of GM847 cells. The mean and median focus intensities are displayed for each population of cells.

Figure 2-17. The depletion of FANCJ in VA13 cells does not have a major affect on the distribution of telomeric repeat DNA foci with low telomeric repeat DNA content.

The distributions of telomeric repeat DNA foci with PNA intensities of less than 1,000 in 100 randomly selected nuclei from (A) non-treated, (B) control siRNA-treated, (C) siFANCJ_1-treated, and (D) siFANCJ_2-treated asynchronous populations of VA13 cells. The mean and median focus intensities are displayed for each population of cells.
control populations of GM847 and VA13 cells differ greatly compared to the distributions from FANCJ-depleted populations of ALT cells (Figure 2-18, Figure 2-19). In one hundred randomly selected nuclei from non-treated and control siRNA-treated asynchronous populations of GM847 cells, telomeric repeat DNA foci with PNA intensities of greater than 1,000 contain 33.9% and 35.8% of the total telomeric repeat DNA content respectively (Figure 2-18 A, B). In contrast, only 1.2% and 12.9% of the total telomeric repeat DNA content is contained in the telomeric repeat DNA foci with PNA intensities of greater than 1,000 in the nuclei from siFANCJ_1-treated and siFANCJ_2-treated populations of GM847 cells respectively (Figure 2-18 C, D). Similarly, in one hundred randomly selected nuclei from asynchronous non-treated and control siRNA-treated populations of VA13 cells, telomeric repeat DNA foci with PNA intensities of greater than 1,000 contain 49.3% and 50.5% of the total telomeric repeat DNA content respectively (Figure 2-19 A, B). On the other hand, only 3.2% and 7.5% of the total telomeric repeat DNA content is contained in the telomeric repeat DNA foci with PNA intensities of greater than 1,000 in the nuclei from siFANCJ_1-treated and siFANCJ_2-treated populations of VA13 cells respectively (Figure 2-19 C, D).

2.3.9 The telomeric phenotypes in human ALT cells depleted of FANCD2 are suppressed upon the codepletion of FANCJ

It was previously discovered that FANCD2 restrains telomeric repeat DNA synthesis in human ALT cells (Root and Meyn, unpublished data). Consistently, there is >2-fold increase in the mean telomeric repeat DNA content per nucleus in one hundred randomly selected nuclei from FANCD2-depleted asynchronous populations of GM847 and VA13 cells compared to cells from control populations (Figure 2-21 A). FANCJ is considered to function downstream of FANCD2 monoubiquitination. If FANCJ is required for telomeric repeat DNA synthesis in ALT cells and FANCD2 restrains this synthesis, then the codepletion of FANCJ should suppress the telomeric phenotypes observed in ALT cells depleted of FANCD2. To explore the relationship between FANCJ and FANCD2 function, the levels of both proteins were codepleted in GM847 and VA13 cells using two independent sets of siRNA targeted against FANCJ and FANCD2 mRNA (siFANCJ_1/siFANCD2_1 and siFANCJ_2/siFANCD2_2) (Figure 2-20). Consistent with the above hypothesis, the elevated telomeric repeat DNA content per nucleus in ALT cells depleted of FANCD2 is suppressed upon the codepletion of FANCJ using either sets of siRNA (Figure 2-21 A). Furthermore, in one hundred randomly selected nuclei from FANCD2-depleted
Figure 2-18. The depletion of FANCJ in GM847 cells reduces the number of telomeric repeat DNA foci with high telomeric repeat DNA content.

The distributions of telomeric repeat DNA foci with PNA intensities that exceed 1,000 in 100 randomly selected nuclei from (A) non-treated, (B) control siRNA-treated, (C) siFANCJ_1-treated, and (D) siFANCJ_2-treated asynchronous populations of GM847 cells. The percentage of total telomeric DNA content that these foci contain is displayed for each population of cells.

Figure 2-19. The depletion of FANCJ in VA13 cells reduces the number of telomeric repeat DNA foci with high telomeric repeat DNA content.

The distributions of telomeric repeat DNA foci with PNA intensities that exceed 1,000 in 100 randomly selected nuclei from (A) non-treated, (B) control siRNA-treated, (C) siFANCJ_1-treated, and (D) siFANCJ_2-treated asynchronous populations of VA13 cells. The percentage of total telomeric DNA content that these foci contain is displayed for each population of cells.
Figure 2-18

A
GM847 (non-treated)
foci > 1,000 PNA intensity contain 33.9% of total telomeric DNA

B
GM847 (control siRNA-treated)
foci > 1,000 PNA intensity contain 35.8% of total telomeric DNA

C
GM847 (siFANCJ_1-treated)
foci > 1,000 PNA intensity contain 1.2% of total telomeric DNA

D
GM847 (siFANCJ_2-treated)
foci > 1,000 PNA intensity contain 12.9% of total telomeric DNA

Figure 2-19

A
VA13 (non-treated)
foci > 1,000 PNA intensity contain 49.3% of total telomeric DNA

B
VA13 (control siRNA-treated)
foci > 1,000 PNA intensity contain 50.5% of total telomeric DNA

C
VA13 (siFANCJ_1-treated)
foci > 1,000 PNA intensity contain 3.2% of total telomeric DNA

D
VA13 (siFANCJ_2-treated)
foci > 1,000 PNA intensity contain 7.5% of total telomeric DNA
Figure 2-20. FANCJ and FANCD2 protein levels are codepleted in ALT cells.

Immunoblot analysis of FANCD2 and FANCJ protein levels in GM847 and VA13 cells treated with control, siFANCJ_1, siFANCJ_2, siFANCD2_1, siFANCD2_2, siFANCJ_1/siFANCD2_1, or siFANCJ_2/siFANCD2_2 siRNA. The level of α-tubulin represents protein loading.
Figure 2-20

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GM847</th>
<th>VA13</th>
<th>GM847</th>
<th>VA13</th>
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<tbody>
<tr>
<td>non-treated control</td>
<td>+</td>
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<tr>
<td>siFANCD2_1</td>
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<tr>
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Western blots for FANCD2 and α-tubulin in GM847 and VA13 cell lines.
Figure 2-21. ALT cells depleted of FANCD2 have elevated telomeric repeat DNA content and this is suppressed upon the codepletion of FANCJ.

(A) The mean telomeric repeat DNA content per nucleus from 100 randomly selected nuclei from control, FANCD2-depleted, and FANCJ/FANCD2-codepleted asynchronous populations of GM847 and VA13 cells. (B) The number of telomeric repeat DNA foci in 100 randomly selected nuclei from control, FANCD2-depleted, and FANCJ/FANCD2-codepleted asynchronous populations of GM847 and VA13 cells.
Figure 2-21

A

Mean telomeric repeat DNA content/nucleus (FRA intensity)

GM847

- non-treated
- control
- siRNA
- siFANC2-1
- siFANC2-2
- siFANC1-1 + siFANC2-1
- siFANC1-2 + siFANC2-2

VA13

- non-treated
- control
- siRNA
- siFANC2-1
- siFANC2-2
- siFANC1-1 + siFANC2-1
- siFANC1-2 + siFANC2-2

B

Number of telomeric repeat DNA per 100 nuclei

GM847

- non-treated
- control
- siRNA
- siFANC2-1
- siFANC2-2
- siFANC1-1 + siFANC2-1
- siFANC1-2 + siFANC2-2

VA13

- non-treated
- control
- siRNA
- siFANC2-1
- siFANC2-2
- siFANC1-1 + siFANC2-1
- siFANC1-2 + siFANC2-2
asynchronous populations of GM847 and VA13 cells, a small increase in the total number of telomeric repeat DNA foci is detected compared to cells from control populations (Figure 2-21 B). This increase is also suppressed upon the codepletion of FANCJ using either sets of siRNA, and the total number of detectable telomeric repeat DNA foci is similar to that of populations of ALT cells depleted of FANCJ alone (Figure 2-21 B; compare with Figure 2-13 A).

FANCD2 depletion in human ALT cells results in an increase in the number of nuclei with high telomeric repeat DNA content. In randomly selected nuclei from siFANCD2_1-treated and siFANCD2_2-treated asynchronous populations of GM847 cells, 41% and 39% of the total nuclei exceed a PNA intensity of 10,000 respectively (Figure 2-22 A, B; compare with Figure 2-14 A, B). Similarly, in randomly selected nuclei from siFANCD2_1-treated and siFANCD2_2-treated asynchronous populations of VA13 cells, 55% and 60% of the total nuclei exceed a PNA intensity of 10,000 respectively (Figure 2-22 C, D; compare with Figure 2-15 A, B). This increase in the number of nuclei with high telomeric repeat DNA content in FANCD2-depleted ALT cells is suppressed upon the codepletion of FANCJ. In randomly selected nuclei from siFANCJ_2/siFANCD2_1-treated and siFANCJ_2/siFANCD2_2-treated asynchronous populations of GM847 cells, only 10% and 17% of the nuclei exceed a PNA intensity of 10,000 respectively (Figure 2-23 A, B; compare with Figure 2-22 A, B). Similarly, in randomly selected nuclei from siFANCJ_2/siFANCD2_1-treated and siFANCJ_2/siFANCD2_2-treated asynchronous populations of VA13 cells, only 25% and 23% of the nuclei exceed a PNA intensity of 10,000 respectively (Figure 2-23 C, D; compare with Figure 2-22 C, D).

Similar to what is observed when FANCJ is depleted, the depletion of FANCD2 in human ALT cells does not have a major affect on the population of telomeric repeat DNA foci with PNA intensities of less than 1,000 (Figure 2-24; compare with Figure 2-16 A, B and Figure 2-17 A, B). However, the number of very bright foci with PNA intensities that exceed 1,000 is significantly increased compared to control ALT cells (Figure 2-25; compare with Figure 2-18 A, B and Figure 2-19 A, B). In randomly selected nuclei from siFANCD2_1-treated and siFANCD2_2-treated asynchronous populations of GM847 cells, telomeric repeat DNA foci with PNA intensities that exceed 1,000 contain 77.6% and 80.2% of the total telomeric repeat DNA content respectively (Figure 2-25 A, B; compare with Figure 2-18 A, B). Similarly, in randomly selected nuclei from siFANCD2_1-treated and siFANCD2_2-treated asynchronous populations of VA13 cells, telomeric repeat DNA foci with PNA intensities that exceed 1,000
Figure 2-22. The depletion of FANCD2 in ALT cells increases the number of nuclei with high telomeric repeat DNA content.

The distributions of 100 randomly selected nuclei from (A) siFANCD2_1-treated and (B) siFANCD2_2-treated asynchronous populations of GM847 cells. The distributions of 100 randomly selected nuclei from (C) siFANCD2_1-treated and (D) siFANCD2_2-treated asynchronous populations of VA13 cells. The percentage of nuclei that exceed a PNA intensity of 10,000 is displayed for each population of cells.

Figure 2-23. The codepletion of FANCJ in ALT cells depleted of FANCD2 suppresses the increase in the number of nuclei with high telomeric repeat DNA content.

The distributions of 100 randomly selected nuclei from (A) siFANCJ_1/siFANCD2_1-treated and (B) siFANCJ_2/siFANCD2_2-treated asynchronous populations of GM847 cells. The distributions of 100 randomly selected nuclei from (C) siFANCJ_1/siFANCD2_1-treated and (D) siFANCJ_2/siFANCD2_2-treated populations of VA13 cells. The percentage of nuclei that exceed a PNA intensity of 10,000 is displayed for each population of cells.
Figure 2-22

A

GM847 (siFANCD2_1-treated)
41% of nuclei exceed 10,000 PNA intensity

B

GM847 (siFANCD2_2-treated)
39% of nuclei exceed 10,000 PNA intensity

C

VA13 (siFANCD2_1-treated)
55% of nuclei exceed 10,000 PNA intensity

D

VA13 (siFANCD2_2-treated)
60% of nuclei exceed 10,000 PNA intensity

Figure 2-23

A

GM847 (siFANCD1_1/siFANCD2_1-treated)
10% of nuclei exceed 10,000 PNA intensity

B

GM847 (siFANCD1_2/siFANCD2_2-treated)
17% of nuclei exceed 10,000 PNA intensity

C

VA13 (siFANCD1_1/siFANCD2_1-treated)
25% of nuclei exceed 10,000 PNA intensity

D

VA13 (siFANCD1_2/siFANCD2_2-treated)
23% of nuclei exceed 10,000 PNA intensity
Figure 2-24. The depletion of FANCD2 in ALT cells does not have a major affect on the distribution of telomeric repeat DNA foci with low telomeric repeat DNA content.

The distributions of telomeric repeat DNA foci with PNA intensities of less than 1,000 in 100 randomly selected nuclei from (A) siFANCD2_1-treated and (B) siFANCD2_2-treated asynchronous populations of GM847 cells. The distributions of telomeric repeat DNA foci with PNA intensities of less than 1,000 in 100 randomly selected nuclei from (C) siFANCD2_1-treated and (D) siFANCD2_2-treated asynchronous populations of VA13 cells. The mean and median focus intensities are displayed for each population of cells.

Figure 2-25. The depletion of FANCD2 in ALT cells increases the number of telomeric repeat DNA foci with high telomeric repeat DNA content.

The distributions of telomeric repeat DNA foci with PNA intensities that exceed 1,000 in 100 randomly selected nuclei from (A) siFANCD2_1-treated and (B) siFANCD2_2-treated asynchronous populations of GM847 cells. The distributions of telomeric repeat DNA foci with PNA intensities that exceed 1,000 in 100 randomly selected nuclei from (C) siFANCD2_1-treated and (D) siFANCD2_2-treated asynchronous populations of VA13 cells. The percentage of total telomeric DNA content that these foci contain is displayed for each population of cells.
Figure 2-24

A

GM847 (siFANCD2_1-treated)
mean focus intensity: 457
median focus intensity: 68

B

GM847 (siFANCD2_2-treated)
mean focus intensity: 499
median focus intensity: 64

C

VA13 (siFANCD2_1-treated)
mean focus intensity: 544
median focus intensity: 124

D

VA13 (siFANCD2_2-treated)
mean focus intensity: 629
median focus intensity: 110

Figure 2-25

A

GM847 (siFANCD2_1-treated)
foci > 1,000 PNA intensity contain 77.6% of total telomeric DNA

B

GM847 (siFANCD2_2-treated)
foci > 1,000 PNA intensity contain 80.2% of total telomeric DNA

C

VA13 (siFANCD2_1-treated)
foci > 1,000 PNA intensity contain 70.6% of total telomeric DNA

D

VA13 (siFANCD2_2-treated)
foci > 1,000 PNA intensity contain 76.1% of total telomeric DNA
contain 70.6% and 76.1% of the total telomeric repeat DNA content respectively (Figure 2-25 C, D; compare with Figure 2-19 A, B). This increase in the number of very bright telomeric repeat DNA foci in ALT cells depleted of FANCD2 is suppressed upon the codepletion of FANCJ (Figure 2-26; compare with Figure 2-25). In randomly selected nuclei from siFANCJ_2/siFANCD2_1-treated and siFANCJ_2/siFANCD2_2-treated asynchronous populations of GM847 cells, telomeric repeat DNA foci with PNA intensities that exceed 1,000 only contain 38.7% and 56.3% of the total telomeric repeat DNA content respectively (Figure 2-26 A, B; compare with Figure 2-25 A, B). Similarly, in randomly selected nuclei from siFANCJ_2/siFANCD2_1-treated and siFANCJ_2/siFANCD2_2-treated asynchronous populations of VA13 cells, telomeric repeat DNA foci with PNA intensities that exceed 1,000 only contain 59.5% and 45.2% of the total telomeric repeat DNA content respectively (Figure 2-26 C, D; compare with Figure 2-25 C, D).

Finally, it was previously observed that human ALT cells depleted of FANCD2 display a reduction in the rate of growth and increased cell death (Root and Meyn, unpublished data). Upon the codepletion of FANCJ, populations of GM847 and VA13 cells depleted of FANCD2 are observed to expand more efficiently (Figure 2-27).
Figure 2-26. The codepletion of FANCJ in ALT cells depleted of FANCD2 suppresses the increase in the number of telomeric repeat DNA foci with high telomeric repeat DNA content.

The distributions of telomeric repeat DNA foci with PNA intensities that exceed 1,000 in 100 randomly selected nuclei from (A) siFANCJ_1/siFANCD2_1-treated and (B) siFANCJ_2/siFANCD2_2-treated asynchronous populations of GM847 cells. The distributions of telomeric repeat DNA foci with PNA intensities that exceed 1,000 in 100 randomly selected nuclei from (C) siFANCJ_1/siFANCD2_1-treated and (D) siFANCJ_2/siFANCD2_2-treated asynchronous populations VA13 cells. The percentage of total telomeric DNA content that these foci contain is displayed for each population of cells.

Figure 2-27. The codepletion of FANCJ suppresses impaired expansion of populations of ALT cells depleted of FANCD2.

Representative images of cell confluency of control, FANCD2-depleted, and FANCJ/FANCD2-depleted GM847 and VA13 cells 6 days after initial transfection.
Figure 2-26

A

GM847 (siFANCJ_1/siFANCD2_1-treated)

foci > 1,000 PNA intensity contain 38.7% of total telomeric DNA

B

GM847 (siFANCJ_2/siFANCD2_2-treated)

foci > 1,000 PNA intensity contain 56.3% of total telomeric DNA

C

VA13 (siFANCJ_1/siFANCD2_1-treated)

foci > 1,000 PNA intensity contain 59.5% of total telomeric DNA

D

VA13 (siFANCJ_2/siFANCD2_2-treated)

foci > 1,000 PNA intensity contain 45.2% of total telomeric DNA

Figure 2-27

non-treated control siRNA siFANCD2_1 siFANCJ_1 + siFANCD2_1

GM847

VA13
2.4 Summary and Conclusions

The present study investigates whether the DNA helicase FANCJ has a role in the ALT mechanism in human cells. Data obtained using indirect immunofluorescence microscopy and t-FISH techniques coupled with siRNA interference suggest that this may be the case. FANCJ frequently localizes to telomeric foci (TRF1 and TRF2) with FANCD2 and BRCA1 in two human ALT cell lines, but not in cell lines that do not utilize the ALT mechanism. Furthermore, telomere-associated FANCJ foci primarily localize to APBs, which may be sites of telomeric recombination and elongation activities in ALT cells. Depletion of FANCJ using two independent siRNAs targeted against FANCJ mRNA yields consistent phenotypes in two ALT cell lines. Interestingly, the depletion of FANCJ causes a small but significant decrease in the total numbers of APBs and TRF2-FANCD2 colocalization events, but does not alter the percentage of TRF2 that colocalizes with either PML or FANCD2. On the other hand, the depletion of FANCJ results in a significant loss of BRCA1 foci in ALT cells, including those that normally localize to telomeric foci. Furthermore, the depletion of FANCJ results in a moderate but significant increase in TIF formation in human ALT cells.

Most interestingly, FANCJ-depletion in human ALT cells results in both a decrease in the numbers and intensities of TRF1 and TRF2 foci and a loss of APBs that contain large TRF2 foci. Telomeric repeat DNA was analyzed by t-FISH and it was found that the depletion of FANCJ preferentially affects nuclei of ALT cells with high levels of telomeric repeat DNA. A reduction in the telomeric repeat DNA content in these FANCJ-depleted nuclei is detected, primarily as a result of the preferential loss of the brightest telomeric repeat DNA foci. The telomeric repeat DNA phenotypes observed in FANCJ-depleted ALT cells are in direct contrast to what is observed in ALT cells depleted of FANCD2. These cells display an increase in telomeric repeat DNA content, primarily a result of an increase in the number of abnormally bright telomeric repeat DNA foci. Finally, the codepletion of FANCJ is able to suppress the telomeric repeat DNA phenotypes observed in FANCD2-depleted ALT dependent cells. This finding was of particular interest because it links FANCJ and FANCD2 function together to ALT-specific telomeric repeat DNA synthesis. Taken together, these data suggest a role for FANCJ in modulating telomeric repeat DNA content in human ALT cells.
CHAPTER 3

DISCUSSION AND FUTURE DIRECTIONS
3 Discussion and Future Directions

3.1 Discussion

The present study utilized indirect immunofluorescence microscopy techniques to characterize the localization of FANCJ in human ALT cells. FANCJ forms discrete nuclear foci in human cells spontaneously during S- and G2-phases of the cell cycle and in response to agents that induce DNA damage (Gupta et al., 2007; Zhang et al., 2010). In two immortalized human fibroblast cell lines that utilize the ALT mechanism to maintain the lengths of their telomeres, FANCJ spontaneously forms nuclear foci that frequently colocalize with telomeric foci (TRF1 and TRF2). This frequent telomeric localization of FANCJ in ALT cells is in accord with a recent study and is specific to ALT cell lines (Déjardin and Kingston, 2009). FANCJ-TRF2 colocalization events are only detected at a low level in a human telomerase-positive fibrosarcoma cell line, an immortalized human telomerase-positive fibroblast cell line, and a primary human fibroblast cell line.

The low level of FANCJ-TRF2 colocalization events observed in human cells that do not utilize the ALT mechanism can be explained in multiple ways. First, FANCJ has been linked to DNA replication and FANCJ foci formation has been observed at stalled replication forks following treatment with hydroxyurea (Zhang et al., 2010). It is possible that FANCJ may form foci at telomeric regions as a result of a replication fork passing through and stalling at telomeric DNA. Second, FANCJ has been linked to DNA damage repair and forms foci in response to induced DNA damage (Gupta et al., 2007). Therefore, FANCJ may concentrate at sites of DNA damage located in telomeric regions. Third, FANCJ may have unknown roles in general telomere biology in human cells that do not use the ALT mechanism. If this is the case, FANCJ may in fact be commonly found at telomeres in cells that do not utilize the ALT mechanism, but in such low quantities that telomere-associated localization is not readily detected by indirect immunofluorescence microscopy. Finally, the low level of FANCJ-TRF2 colocalization events detected in non-ALT human cells may be a direct result of the method that was used to obtain images for colocalization analysis. Only single-plane images were captured and analyzed for colocalization events. In such images, two foci that appear to colocalize on the XY-plane may in
fact occupy different positions and may not colocalize with respect to the Z-axis. These would be falsely classified as positive colocalization events.

In a recent study, FANCJ was found to bind telomeric repeat DNA in a human ALT cell line but not in a human telomerase-positive cell line (Déjardin and Kingston, 2009). Briefly, proteins bound to telomeric repeat DNA in ALT VA13 cells and telomerase-positive HeLa cells were analyzed using the proteomics of isolated chromatin segments protocol. Telomeric DNA was purified from both cell lines and the associated proteins were identified by mass spectrometry. Using this method, FANCJ was detected to bind telomeric repeat DNA in VA13 cells but not in HeLa cells. The binding of FANCJ to telomeric repeat DNA in telomerase-positive cells may be transient and too weak to be detected by the proteomics of isolated chromatin segments protocol. Nevertheless, this study demonstrates that FANCJ more frequently binds telomeric repeat DNA in ALT cells compared to telomerase-positive cells.

There is a ~2-fold increase in the number of detectable FANCJ foci that are not associated with a telomeric focus in the nuclei of human ALT cell lines compared to telomerase-positive and primary cell lines. Although the significance of this is unknown, it may be a result of FANCJ foci formation at telomeric foci in ALT cells that are too small to be detected by indirect immunofluorescence microscopy techniques. Consistent with this, ~4-fold more telomeric repeat DNA foci per nucleus are detected by t-FISH compared to the numbers of TRF1 or TRF2 foci per nucleus as detected by indirect immunofluorescence microscopy techniques. In addition, it is common to find chromosome ends without a detectable telomeric PNA signal in metaphase spreads of ALT cells (Perrem et al., 2001). FANCJ may localize and form detectable foci at these very short telomeres as well.

Human ALT cells contain APBs, which are defined by the colocalization of telomeric repeat DNA, telomere binding proteins, DNA recombination proteins, and PML (Yeager et al., 1999; Stavropoulos et al., 2002; Nabetani et al., 2004). Thus, APBs may be sites of telomeric recombination, elongation, and deletion events that are common to ALT cells. In the present study, telomere-associated FANCJ foci were found to primarily localize to APBs, suggesting a possible role for FANCJ in the ALT mechanism. APBs are readily detected in ALT cells during S-phase of the cell cycle and are enriched in G2-phase when cells are synchronized (Grobelny et al., 2000). The cell cycle profiles of asynchronous populations of ALT cells show that ~40% of
the cells are present in S- and G2-phases. Thus, this is the most likely explanation as to why ~40% of the nuclei from asynchronous populations of ALT cells account for all of the FANCJ-TRF2 colocalization events detected. Furthermore, the function of FANCJ appears to be restricted to S- and G2-phases of the cell cycle. FANCJ is detected to be phosphorylated on Serine 990 in S- and G2-phases, but not in G1-phase, to facilitate its interaction with BRCA1 (Yu et al., 2003). In addition, the FANCJ-BRCA1 complex remains tightly associated with chromatin during S-phase progression (Yu et al., 2003; Kumaraswamy and Shiekhattar, 2007). Finally, FANCJ is phosphorylated early in G1-phase at a yet unidentified residue, which inactivates its ATPase activity (Kumaraswamy and Shiekhattar, 2007). Taken together, the function of FANCJ appears to be restricted to S- and G2-phases of the cell cycle and FANCJ foci formation at APBs in ALT cells may be limited to these phases. Additionally, the majority of telomere-associated FANCJ foci are localized to APBs, but a significant fraction of APBs do not contain FANCJ. This suggests that APB formation occurs independently and prior to FANCJ localization.

Human ALT cells depleted of FANCJ display a similar reduction in the numbers of APBs and TRF2-FANCD2 colocalization events. These two phenomena are possibly related as the majority of telomere-associated FANCD2 foci localize to APBs (Root and Meyn, unpublished data). Although marked decreases in the numbers and intensities of TRF1 and TRF2 foci are also detected in FANCJ-depleted ALT cells, the percentage of TRF2 foci colocalizing with PML is not significantly altered. This suggests that FANCJ is not required for efficient localization of PML to telomeric foci. Similarly, the percentage of TRF2 foci colocalizing with FANCD2 is not affected upon FANCJ depletion, suggesting that FANCJ is also not required for efficient formation of FANCD2 foci at telomeric foci. However in a recent study, FANCJ-deficient cells with exogenous wild-type FANCJ expression showed marked increases in the number and brightness of FANCD2 foci (Zhang et al., 2010). This suggests that FANCJ is needed for efficient FANCD2 foci formation but is not absolutely required.

FANCJ foci formation has been established to be independent of FANCD2 monoubiquitination and FANCD2 foci formation (Levitus et al., 2004). Consistent with this, the depletion of FANCD2 in human ALT cells does not alter the percentage of TRF2 foci colocalized with FANCJ. However, the depletion of FANCD2 results in a minor but significant increase in the number of TRF2-FANCJ colocalization events. This can be explained by secondary effects of
FANCD2 depletion, which includes an increase in the number of bright telomeric repeat DNA foci. These foci are commonly associated with APBs, which are the preferential sites of TRF2-FANCJ colocalization events.

FANCJ is a DNA helicase that has been linked to DNA replication and repair. Thus, a hypothesis was proposed in the present study that the preferential loss of the most intense TRF1 and TRF2 foci in FANCJ-depleted human ALT cells was a direct result of a decrease in telomeric repeat DNA content as opposed to an altered localization of the telomere binding proteins. To investigate this idea, the present study utilized t-FISH techniques to visualize telomeric repeat DNA in ALT cells. It was found that FANCJ depletion does indeed result in a decrease in the total amount of telomeric repeat DNA in ALT cells, preferentially affecting nuclei with high telomeric repeat DNA content. In addition, a decrease in the total number of detectable telomeric repeat DNA foci was observed similar to the decrease in the numbers of TRF1 and TRF2 foci. Finally, the depletion of FANCJ preferentially affects the formation of the brightest telomeric repeat DNA foci. The distributions of foci with high PNA intensity values are greatly altered upon FANCJ depletion, while the distributions of foci with low PNA intensity values are not. Taken together, these data suggest that FANCJ modulates telomeric repeat DNA content in human ALT cells.

The loss of telomeric repeat DNA in human ALT cells depleted of FANCJ suggests that FANCJ may be necessary for telomeric repeat DNA synthesis or to restrain the degradation of telomeric repeat DNA in ALT cells. To date, there is no evidence of a role for FANCJ in DNA degradation or its prevention. Thus, the involvement of FANCJ in telomeric repeat DNA synthesis in ALT cells is the favoured hypothesis. FANCJ may be involved in ALT-specific telomeric repeat DNA elongation in several ways. First, FANCJ can efficiently unwind human telomeric G4 DNA structures in vitro (Wu et al., 2008). G4 DNA structures are very stable four-stranded helical structures formed by stacking multiple G•G•G•G tetrads and preferentially form at the very end of the single-strand overhang of the telomere (Tang et al., 2008). These telomere termini are normally hidden to keep the cellular DDR at chromosome ends dormant. It is possible that the ability to mask the chromosome ends is dysfunctional in ALT cells, and FANCJ may be required to unwind G4 DNA structures at the 3’ overhangs to initiate strand invasion and promote ALT-specific telomere elongation through HR-mediated DNA repair mechanisms. In addition, FANCJ may also be essential to facilitate the unwinding of G4 DNA structures throughout telomeric
regions to allow replication forks to proceed during ALT-specific telomeric DNA synthesis. Consistent with this idea, human cells and *C. elegans* deficient of FANCJ and DOG-1 respectively accumulate deletions at G-rich genome sequences (Kruisselbrink *et al.*, 2008; London *et al.*, 2008). The favoured hypothesis for this is that FANCJ is required to unwind G4 DNA structures in these regions to allow the replication fork to proceed past the G-rich DNA during replication. Second, FANCJ has also been found to be able to disrupt model protein-DNA complexes *in vitro* (Sommers *et al.*, 2009). In ALT cell lines, FANCJ may be required to clear telomeric repeat DNA of bound proteins such as RPA, TRF1, TRF2, or POT1 to promote ALT-specific telomeric elongation. Finally, FANCJ can unwind intermediate structures formed during HR *in vitro*, such as D-loop models (Gupta *et al.*, 2005). Thus, FANCJ may be needed to resolve t-loops or other structures formed during HR-mediated events that occur in the ALT mechanism.

It has not been established whether BRCA1 and its interaction with FANCJ is required for telomeric repeat DNA synthesis in human ALT cells. Consistent with data in the present study, a previous report discovered BRCA1 foci localization to telomeric foci in human ALT cells (Wu *et al.*, 2003). However, further experimentation to determine whether BRCA1 has a function in the ALT mechanism was not undertaken. BRCA1 has an established role in other HR-mediated events, most notably in DNA DSB repair. While a significant number of telomere-associated FANCJ foci do not colocalize with BRCA1, telomere-associated BRCA1 foci almost always colocalize with FANCJ. These data fit the model that FANCJ localizes to telomeric repeat DNA in ALT cells first and then recruits BRCA1. In support of this, the depletion of FANCJ results in a loss of roughly half of the BRCA1 foci present in ALT cells, including the foci that normally localize to telomeric foci. The loss of BRCA1 foci upon FANCJ depletion was not surprising as it was previously reported that FANCJ-deficient human cells display a reduction in the number of BRCA1 foci (Peng *et al.*, 2006). However, whether BRCA1 is required for FANCJ function is up for debate. The expression of human FANCJ either with a missing C-terminal BRCA1 binding region or a point mutation at the residue that is phosphorylated to facilitate the FANCJ-BRCA1 interaction restores DNA ICL resistance in cells deficient of FANCJ (Bridge *et al.*, 2005; Xie *et al.*, 2010). This suggests that the FANCJ-BRCA1 interaction is not required for efficient DNA ICL repair. However, FANCJ displays a strong genetic interaction with BRCA1 with respect to HR-mediated DNA DSB repair (Cantor *et al.*, 2001; Litman *et al.*, 2005). These findings suggest that FANCJ may have multiple roles in the cellular nucleus that are not all dependent on BRCA1.
Previous work led to the hypothesis that FANCD2 restrains ALT-specific telomeric repeat DNA synthesis (Root and Meyn, unpublished data). In the present study, the increase in telomeric repeat DNA synthesis in human ALT cells depleted of FANCD2 was quantified through t-FISH analysis. A larger fraction of ALT cells depleted of FANCD2 display high telomeric repeat DNA content compared to control populations of ALT cells. This is primarily a result of an increase in the number of foci that contain abnormally high levels of telomeric repeat DNA. For example, the brightest telomeric repeat DNA focus in one hundred randomly selected nuclei from the siFANCD2_2-treated population of GM847 cells has a PNA intensity value of 95,716. When this is compared to the mean PNA intensity per nucleus values of 7,710 and 8,881 from non-treated and control siRNA-treated populations of GM847 cells respectively, this single focus contains more than 10-fold telomeric repeat DNA than the total amount of telomeric repeat DNA per nucleus on average in the control populations of ALT cells. This single focus also contains ~10-fold telomeric repeat DNA compared to the foci with the maximum PNA intensities of 11,119 and 10,604 in one hundred randomly selected nuclei from the non-treated and control siRNA-treated populations of GM847 cells respectively.

Both FANCD2 and FANCJ are required for the proper repair of DNA ICLs. Thus, the depletion of FANCJ in human ALT cells was not expected to yield opposite telomeric effects compared to the depletion of FANCD2. Furthermore, it is unknown whether FANCJ functions in the same pathway as FANCD2 monoubiquitination or in an independent but parallel pathway to repair DNA damage. In ALT cells, FANCJ appears to be required for telomeric repeat DNA synthesis while FANCD2 restrains telomeric repeat DNA synthesis. Since telomere-associated FANCJ foci almost exclusively localize with FANCD2, experiments were undertaken in the present study to investigate whether FANCD2 restrains FANCJ-dependent telomeric repeat DNA synthesis in ALT cells. This appears to be the case as the codepletion of FANCJ is able to suppress the telomeric phenotypes of FANCD2 depletion in ALT cells. The slight increase in the number of telomeric repeat foci detected in the nuclei from FANCD2-depleted populations of ALT cells is suppressed upon the codepletion of FANCJ, and the total number of detectable telomeric repeat DNA foci is similar to that of the nuclei from populations of ALT cells depleted of FANCJ alone. Similarly, the increase in the average telomeric repeat DNA content per nucleus from FANCD2-depleted populations of ALT cells is also suppressed upon the codepletion of FANCJ to a level that is between that of the nuclei from control and FANCJ-depleted populations of ALT cells. This suppression is primarily due to the loss of telomeric
repeat DNA foci with abnormally high telomeric repeat DNA content. Finally, the favoured hypothesis explaining why FANCD2-depleted human ALT cells have reduced growth rates is that the abnormally high quantities of telomeric repeat DNA that is generated within the nuclei is toxic (Root and Meyn, unpublished data). If this is the case, then it is possible that the codepletion of FANCJ in FANCD2-depleted ALT cells suppresses the increase in the amount of toxic telomeric repeat DNA enabling these cells to grow more efficiently. However, only a partial suppression of the impaired expansion of populations of ALT cells depleted of FANCD2 is observed when FANCJ is codepleted. This is possibly a result of the kinetics and efficiency of protein depletion using the multiple siRNAs. Furthermore, the depletion of FANCJ alone impairs the expansion of populations of ALT cells, probably a result of the loss of secondary FANCJ functions such as in general DNA replication and repair.

It was previously reported that the BLM helicase plays a role in the synthesis of telomeric repeat DNA in human ALT cells (Stavropoulos et al., 2002). As with FANCJ, the codepletion of BLM can suppress the ALT-specific telomeric phenotypes detected in FANCD2-depleted cells (Root and Meyn, unpublished data). This suggests that FANCJ and BLM have distinct functions in the ALT mechanism and both are required for ALT-specific telomeric repeat DNA synthesis. Although BLM shares features with FANCJ, such as the ability to unwind G4 DNA structures, there are many dissimilarities between the helicases. For example, while FANCJ unwinds DNA duplexes in a 5’ to 3’ manner, BLM unwinds DNA substrates in the opposite orientation (Karow et al., 1997; Gupta et al., 2006). Furthermore, BLM is efficient in resolving Holliday junction structures that can form during HR-mediated events, while FANCJ is not (Karow et al., 2000; Gupta et al., 2005). These unique properties of FANCJ and BLM together may be required for telomeric repeat DNA synthesis in human ALT cells.

ECTR DNA is commonly found in human ALT cells and it is unclear as to whether the changes in telomeric repeat DNA content upon the depletion of FA proteins is primarily a result of changes in the amount of ECTR DNA, DNA at telomeres, or both. In addition, it is important to know whether APBs are associated with either telomeres, ECTR DNA, or both, as the brightest telomeric repeat DNA foci that are preferentially affected upon the depletion of FA proteins are almost always associated with APBs. Recently, abnormally large APBs were generated by expressing a modified form of Herpes simplex virus protein ICP0 and their structures were studied (Draskovic et al., 2009). Subtelomeric sequences were used as markers for telomeres and
these markers were commonly found to associate with these modified APBs. However, it was never tested whether these marked subtelomeric sequences are also present in ECTR DNA normally found in ALT cells, raising concern regarding their conclusion that telomeres are physically associated with APBs. Furthermore, analysis of these modified APBs suggests that the bodies are solid protein structures with telomeric repeat DNA clustering around the periphery. However, the analysis of unmodified APBs demonstrates that telomeric repeat DNA is primarily located within the body (Root and Meyn, unpublished data). The discrepancy may be a result of the expression of modified ICP0 protein disrupting normal APB structure.

The idea that APBs are primarily composed of ECTR DNA is supported by several studies. First, in metaphase spreads of human ALT cells, most APBs present are clearly not associated with chromosome ends (Nabetani et al., 2004). Second, when telomerase is overexpressed in human cell lines that already use endogenous telomerase to maintain the lengths of their telomeres, these cells accumulate ECTR DNA and are able to form APB-like structures, suggesting a trimming mechanism of very long telomeres (Pickett et al., 2009). Third, induced DNA damage amplifies the amount of linear and circular ECTR DNA in ALT cells and increases the proportion of cells that contain APBs (Fasching et al., 2007). Finally, a subclone of the ALT VA13 cell line was isolated that does not contain ECTR DNA or APBs but still maintains the lengths of their telomeres independent of telomerase (Cerone et al., 2005). The above studies suggest a close connection between the generation of ECTR DNA and the formation of APBs.

If APBs are not the sites of recombination activities between telomeric sequences, they may still play important roles in human ALT cells. The presence of ECTR DNA poses a biological problem as there are single-strand DNA regions and exposed DNA ends in the nucleus that normally should activate the cellular DDR pathway. Although the ALT mechanism appears to be used exclusively by cancer cells and immortalized cell lines that are frequently associated with having disrupted DNA checkpoint signaling, several reports demonstrated that multiple ALT cell lines have an intact cellular DDR and DNA checkpoints upon the induction of DNA damage (Cliby et al., 1998; Wang et al., 2002). One group proposed that APBs may interestingly serve as sites in the nucleus to sequester ECTR DNA and keep the cellular DDR pathway dormant within human ALT cells (Fasching et al., 2007).
3.2 Future Directions

To verify the requirement of FANCJ in ALT-specific telomeric repeat DNA synthesis, wild-type FANCJ should be overexpressed in human ALT cells. It is expected that this will lead to an increase in telomeric repeat DNA synthesis similar to that of FANCD2-depleted ALT cells. Furthermore, to verify the requirement of the helicase activity of FANCJ in ALT-specific telomeric DNA synthesis, helicase-dead FANCJ (FANCJ\textsuperscript{K52R}) should be overexpressed in ALT cells and the effect on telomeric repeat DNA content should be compared to the outcome of wild-type FANCJ overexpression. Constructs to overexpress wild-type and helicase-dead FANCJ in human cells have been previously described (Cantor \textit{et al.}, 2001).

One concern that the present study did not address is whether the reduced amounts of telomeric repeat DNA in FANCJ-depleted human ALT cells is primarily a result of the loss of DNA at telomeres, ECTR DNA, or both. To detect changes in telomere lengths, quantitative-FISH should be performed on metaphase spreads from control and FANCJ-depleted ALT dependent cells. PNA intensities of only the telomeric repeat DNA foci that are found at chromosome ends should be measured. To detect changes in ECTR DNA content, Hirt lysates should be subjected to Southern blot analysis with a probe specific for telomeric DNA. If a change in ECTR DNA content is detected, 2D gel electrophoresis should be performed to analyze whether linear or circular ECTR DNA is affected.

Data from the present study suggest that FANCJ is required for the efficient localization of BRCA1 to telomeric foci in human ALT cells. However, the question of whether BRCA1 and its interaction with FANCJ are required for ALT-specific telomeric repeat DNA synthesis was not addressed. To tackle this concern, BRCA1 protein level should be depleted through siRNA interference in human ALT cells to observe whether telomeric phenotypes arise that mimic those of FANCJ-depleted cells. To further verify the requirement for the FANCJ-BRCA1 interaction in ALT-specific telomeric DNA synthesis, FANCJ\textsuperscript{S990A} should be overexpressed in ALT cells and the effect on telomeric repeat DNA content should be compared to the outcome of wild-type FANCJ overexpression. FANCJ\textsuperscript{S990A} possesses a functional helicase domain, but is unable to bind BRCA1. A construct to overexpress FANCJ\textsuperscript{S990A} in human cells has been previously described (Peng \textit{et al.}, 2007).
3.3 Concluding Remarks

Cancer cells rely on the ability to maintain the lengths of their telomeres to proliferate rapidly and grow continuously. Approximately 85% of human cancers utilize telomerase to accomplish this. The remaining 15% use the ALT mechanism. Several pre-clinical approaches are undertaken to determine a method to inhibit the activity of telomerase in hopes of developing a novel therapeutic to treat malignancy (reviewed in Phatak and Burger, 2007). Although telomerase inhibition in cancer cells may lead to a reduction in tumor growth, it is possible that this would impose a selective pressure on the cancer cells to activate the ALT mechanism. In fact, in a recent study, the inhibition of telomerase in squamous carcinoma cells alters the telomere length maintenance mechanism in these cells (Chen et al., 2010). Cancer cells that survive begin to display typical ALT-specific characteristics and their telomeres appear to be maintained by the ALT mechanism. Therefore, preventing telomere length maintenance to fight cancer should include the inhibition of telomerase-independent mechanisms such the ALT pathway. Additionally, the idea of hindering the ALT mechanism in telomerase-negative cancers must also be explored as a therapeutic.

The present study builds upon the exploration of the FA pathway and the role it plays in HR-mediated events, such as DNA ICL repair and the ALT mechanism. In addition to inhibiting telomerase activity, altering the FA pathway may prove to be an efficient therapeutic to treat malignancy. Furthermore, the present study is the first to describe a role for a FA protein that belongs to the third subgroup, FANCJ, in the ALT mechanism. The strengths of the present study include the use of two human ALT cell lines and two independent siRNAs targeted against FANCJ mRNA to study FANCJ function, yielding very consistent results. The working model from the data obtained in the present study is that FANCJ is required for telomeric repeat DNA synthesis in human ALT cells, which may or may not be dependent on BRCA1, and FANCD2 restrains this synthesis. The present study contributes to the understanding of the role of FANCJ in recombination, the role of FANCJ with respect to other FA proteins, and the role of FANCJ in the ALT mechanism.
REFERENCES


