Characterization of the Role of RecQ helicases in human DNA replication

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ABSTRACT

Cellular and biochemical studies support a role for all five human RecQ helicases in DNA replication, however their specific functions during this process are unclear. In my thesis, I investigated the *in vivo* association of the five human RecQ helicases with three well-characterized human replication origins. I showed that only RECQ1 and RECQ4 associate with replication origins in a cell cycle-regulated fashion in unperturbed cells, while other RecQ helicases interact with replication origins only under replication perturbed conditions. Under endogenous conditions, RECQ4 is recruited to origins at late G1 after ORC and MCM complex assembly, while RECQ1 and additional RECQ4 are loaded at origins at the onset of S phase when licensed origins begin firing. Both proteins are lost from origins after DNA replication initiation, indicating either disassembly or tracking with the newly formed replisome. Cell proliferation, DNA synthesis, nascent origin DNA synthesis and the frequency of origin firing are reduced after RECQ1 depletion, and to a greater extent after RECQ4 depletion. Depletion of RECQ1, though not RECQ4, also suppresses replication fork rates in otherwise unperturbed cells. Loading of PCNA during S phase is affected by RECQ1 depletion while the RECQ4 depleted cells show defect in RPA and PCNA loading during S phase of the cell cycle. These results indicate that RECQ1 and RECQ4 are integral components of the human replication complex, and play distinct roles in DNA replication initiation and replication fork progression *in vivo*.

I. Introduction

1.DNA helicases

The nucleus is an essential organelle of the mammalian cell which hosts the genome of an organism. Each genome replicates, transcribes, and expresses its genetic information. The DNA present in the genome is in the double stranded form. However, for many important DNA transactions, such as replication, repair, recombination, and in some cases transcriptions, the duplex DNA has to be partially unwound for a short time to create single stranded DNA intermediates, through which some of the proteins responsible for the above mentioned processes can load on the DNA. A class of enzymes, called DNA helicases, catalyzes the transient unwinding of duplex DNA in an adenosine triphosphate (ATP)-dependent manner and play an essential role in all aspects of DNA metabolism. DNA helicases are processive molecular motors which utilize chemical energy from nucleoside triphosphate (NTP) hydrolysis to translocate along the single stranded DNA. The importance of DNA helicases in cellular processes is underlined by the numerous human diseases like Xeroderma pigmentosum, Cockayne syndrome or Trichothiodystrophy, Bloom syndrome, Werner syndrome, and Rothmund-Thomson syndrome that are associated with defective helicases (Ellis, 1997; Matson et al., 1994; Mohaghegh and Hickson, 2001; von Hippel, 2004).

Classification of DNA helicases

The DNA helicases are classified based on their conservation of characteristic amino acid sequences (helicases motifs) and by their direction of translocation along nucleic acid substrates (West, 1996). At the sequence level, based on the presence of different

'signature motifs,' DNA helicases have been divided into six main groups (Singleton et al., 2007) (figure 1.1, table 1.1), the largest being superfamily I and II (SF1 and SF2) helicases. Both SF1 and SF2 helicases contain seven conserved helicase motifs, I, Ia, II, III, IV, V, and VI. Motifs I and II are the highly conserved Walker A and B sequences characteristic of ATPases. The other motifs are generally less conserved and differ between the SF1 and SF2 proteins. PcrA from *Bacillus stearothermophilus* and NS3 from hepatitis C virus are probably the best characterized SF1 and SF2 proteins, respectively (Kim et al., 1998; Velankar et al., 1999). SF3 enzymes are usually from DNA or RNA viruses and contain only four conserved motifs termed A, B, B', and C. The A and B motifs correspond to Walker A and Walker B sequences, whereas motif C is SF3 specific. BPV E1 is a good example of SF3 proteins (Hickman and Dyda, 2005). SF4 helicases contain five conserved motifs, H1, H1a, H2, H3, and H4. The H1 and H2 motifs correspond to Walker A and Walker B sequences, whereas H1a, H3 and H4 motifs are specific to SF4 helicases. T7 gp4D protein belongs to SF4 helicases (Singleton et al., 2000). SF5 helicases contains enzymes such as the bacterial transcription termination factor Rho with three conserved motifs 1, 1a ,and 2, while SF6 helicases contain four conserved motifs A, B, S1 and S2. SF6 helicases are also known as AAA^+ family - ATPases associated with various cellular activities. RuvB from *T.maritima* is an example of an SF6 helicase that has been crystallized and characterized(Singleton et al., 2007).

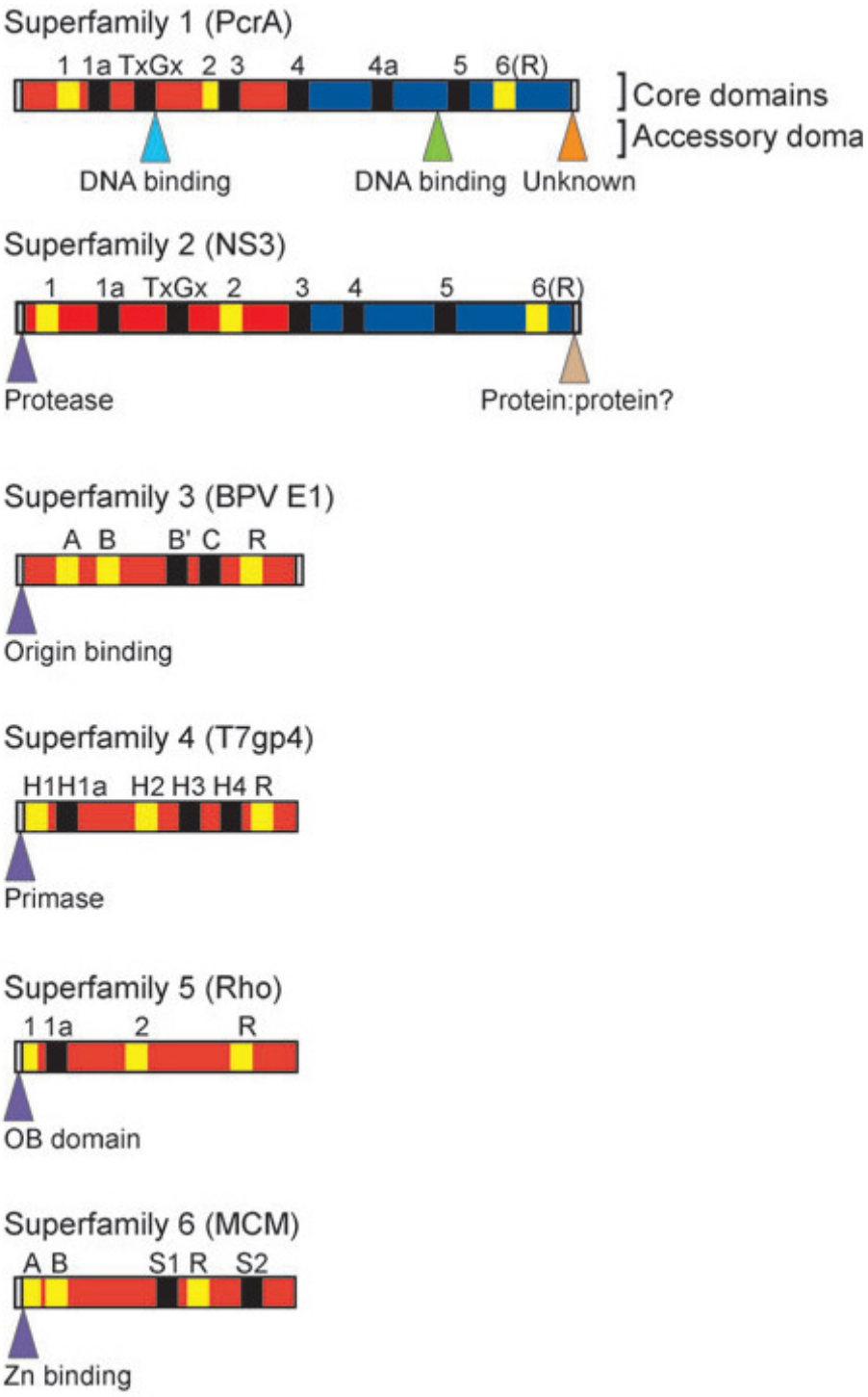


Figure 1.1 : Classification of DNA helicases based on conserved aminoacid sequences.The name of one representative member of each of the six superfamilies is given in parentheses. The domains and the positions of the signature motifs therein are shown for each class of helicase. Precise position of each motif is based on the example family member and is representative for the whole family. Motifs colored yellow represent universal structural elements in all helicases. The positions and functions of accessory domains in each example protein are also shown. Model adapted from (Singleton et al., 2007).

DNA Helicases can be also classified based on the direction of translocation along the nucleic acid substrate. The *in vitro* experiments using partial DNA duplex substrates with a linear single strand DNA (ssDNA) tail have shown that helicases can translocate either 3' to 5' or 5' to 3' along ssDNA, in respect to the binding strand (Figure1.2). SF1 and SF2 helicases contain examples of helicases with 3' to 5' and 5' to 3' processivity. All characterized SF3 helicases show 3' to 5' activity, whereas SF4 and SF5 helicases show 5' to 3' activity. SF6 helicases contains examples of both 3' to 5' and 5' to 3' processivity (Tuteja and Tuteja, 2004; West, 1996).

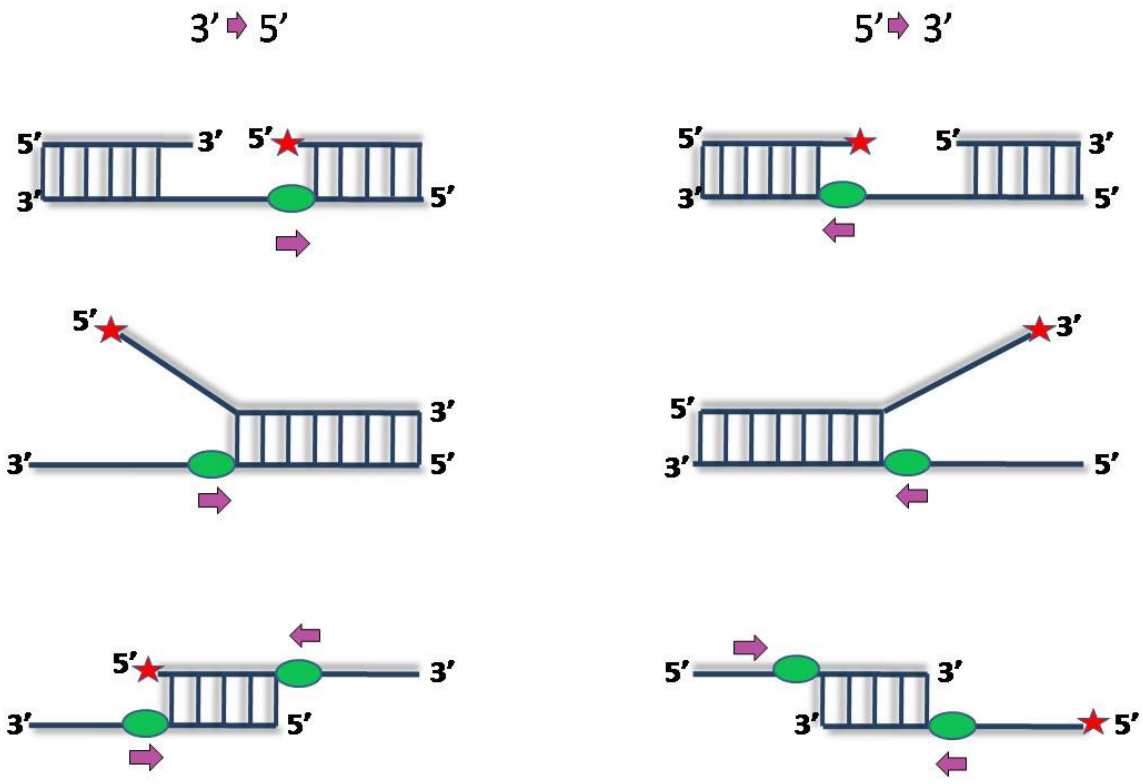


Figure 1.2 : Structures of the partial duplex substrates commonly used to determine the direction of translocation of the helicase. The 3' to 5' directional substrates are on the left and 5' to 3' directional substrates are on the right. Red asterisks denote the 32P-labeled end.

Superfamily	Protein fold	Assembly state	Polarity	Functions	Example members
Superfamily 1 (SF1)	RecA (tandem pair)	Monomer (dimer/ multimer)	3'-5' 5'-3'	DNA unwinding, repair and degradation	Bacterial PcrA, Rep, UvrD, RecBCD, Pif1 eukaryotic Rrm3,
Superfamily 2 (SF2)	RecA (tandem pair)	Monomer (dimer/ multimer)	3'-5' 5'-3'	RNA-melting, RNA-binding protein displacement, NA unwinding and translocation, melting and migration of Holliday junctions or branced substrates	Prp2, Ski2,NS3 of hepatitis C, Rad54, bacterial RecQ, RecG, UVrB
Superfamily 3 (SF3)	AAA+	Hexamer	3'-5'	DNA unwinding / replication	Papilloma virus E1, Simian virus 40 large T-antigen, Adeno-associated virus Rep 40
Superfamily 4 (SF4)	RecA	Hexamer	5'-3'	DNA unwinding / replication, ssRNA packaging	Bacterial Dna B, Phage T7 gp4, T4 gp41
Superfamily 5 (SF5)	RecA	Hexamer	5'-3'	RNA translocation,RNA/ DNA heteroduplex unwinding, transcription termination	Bacterial Rho
Superfamily 6 (SF6)	AAA+	Hexamer	3'-5'	DNA unwinding / replication	Eukaryotic/archeal MCMs

Table 1: Classification of DNA helicases. DNA helicases superfamily are_shown with respective characteristics and examples.

1.2 Functional mechanism of DNA helicases

DNA helicases have two important activites: unwinding the duplex nucleic acids and translocating along the ssDNA. The existence of mutants that show no defect in either nucleic acid (NA) binding or NTP binding and hydrolysis activities, but with impaired double stranded DNA (dsDNA) unwinding activities, confirms that NA binding and NTP hydrolysis must be coupled. The mechanism by which the helicases couple their

translocation along a single stranded nucleic acid to the unwinding of a double stranded nucleic acid is unclear. Two competing mechanisms known as the inchworm mechanism and active rolling mechanism have been a subject of debate during the past few years.

The inchworm model has been proposed for monomeric helicases. A cycle of DNA binding, release, and translocation events begins with one helicase site bound tightly to the DNA and the second helicase site bound weakly to the DNA. The weak site dissociates from the DNA and in a power stroke motion moves away from the tight site to bind at a position ahead. After the weak site has moved and made tight interactions ahead, the original tight site becomes weak, and, as it dissociates from the DNA, in a power stroke motion it moves forward to get close in distance to the site ahead (fig 1.3). The crystal structures of a *B. stearothermophilus* PcrA monomer in complex with a 3'-$(dN)_7$ – 10 bp dsDNA helped to propose an inchworm type stepping model for a monomeric helicase. The crystal structures of *E.coli* UvrD monomer proved to be another good example of the inchworm model (Lee and Yang, 2006; Velankar et al., 1999).

The active rolling model has been proposed for dimeric helicases. In this model, each of the two subunits of the helicase alternate their binding to single-stranded and duplex DNA as they change their NTP ligation states. In contrast to the inchworm model, where the subunits maintain their relative positioning along the DNA, the subunits in the rolling model take turn in being the trailing or the leading subunit. X-ray structures of two forms of Rep helicase (open and closed forms) bound to a short piece of ssDNA and the structure of a ternary complex containing both ssDNA and

adenosine diphosphate (ADP) helped to propose the active rolling model (Korolev et al., 1997; Waksman et al., 2000).

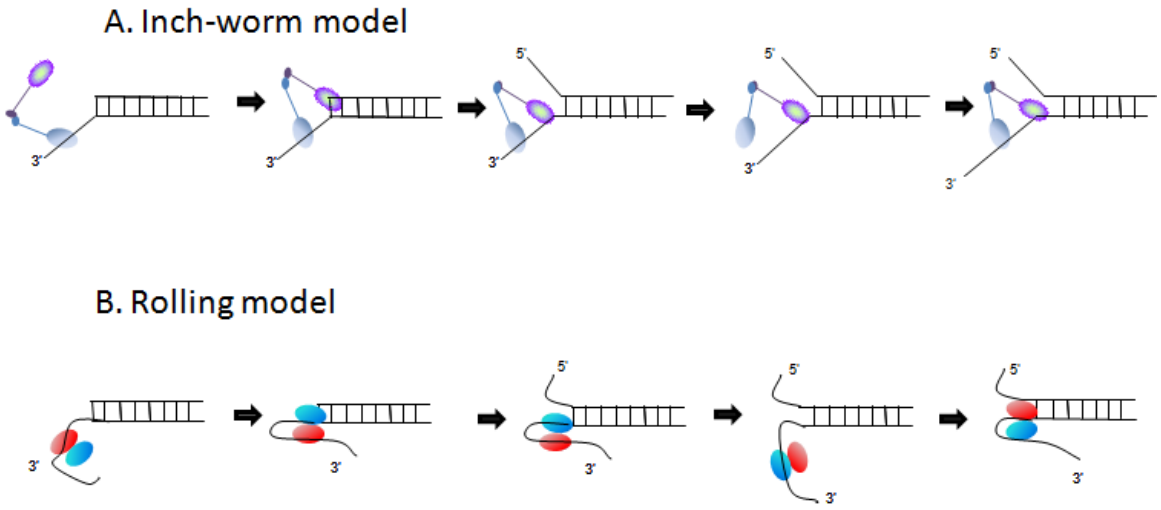


Figure 1.3: Models for DNA helicase translocation and unwinding. (A) **Inchworm model**, involves two binding sites, a single and a double-stranded DNA binding site, either belonging to a single monomeric enzyme (inch-worm monomeric enzyme, D. B. Wigley), or present on two different subunits of a dimeric enzyme (inchworm dimeric enzyme, T. M. Lohman).(B) **Active rolling model**, involves two subunits of a dimeric enzyme which alternate binding of single and double-stranded DNA (T. M.Lohman).

1.3 Functional forms of DNA helicases

DNA helicases exists in different oligomeric forms. Previous studies have shown that some of these enzymes are monomers, while other form dimers or higher-order oligomers. DNA helicases are functionally activated/regulated by their assembly state. However, the assembly state responsible for the " functionally active" DNA helicases differs within the helicase family itself. Pre-steady – state kinetic analysis has shown that helicases like T4 Dda, HCV NS3h and *E.coli* RecQ could function as monomers (Dou et al.,

2004; Nanduri et al., 2002; Xu et al., 2003). A recent study based on fluorescence cross-correlation spectroscopy has shown that *E.coli* RecQ monomers are sufficient to perform the unwinding of short DNA substrates and binding of multiple helicase monomers are required to unwind long DNA substrates (Li et al.). Some helicases need a structural interaction to form homodimers (UvrD) or heterodimers (RecBCD) in order to be functionally active. The different assembly states of DNA helicases are shown in the figure 1.4. The assembly state can also regulate different functions as in the case of human RECQ1 helicases in which monomers or dimers perform unwinding while higher order oligomers perform annealing (Muzzolini et al., 2007).

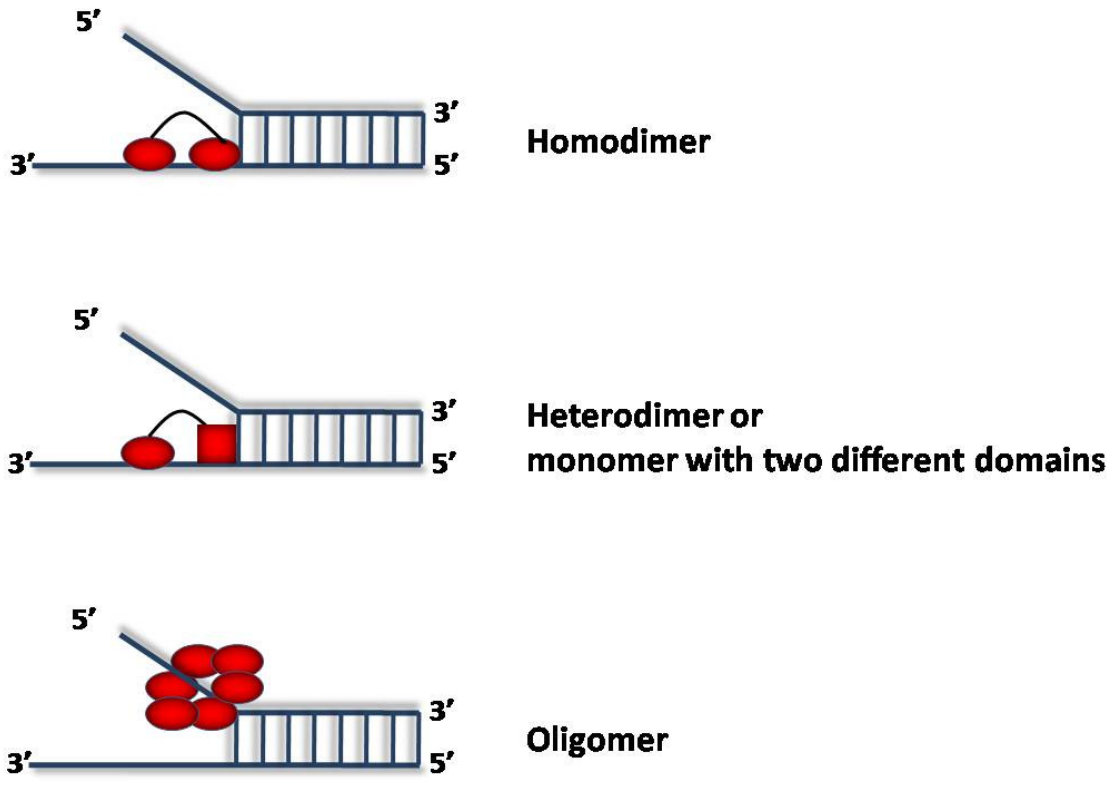


Figure 1.4: Assembly states of DNA helicases. Three possible helicase quaternary structures that makes DNA helicases functionally active. (a) A homodimer, (b) A monomeric protein or a multi-protein complex (illustrated as a heterodimer) containing at least two separate domains. (c) An oligomeric ring-like structure .

2. RecQ helicases

The RECQ protein family is a highly conserved group of SF2 DNA helicases that have been identified in organisms ranging from bacteria to humans. The prototypical member of the family, RecQ of *Escherichia coli*, was identified in a study aimed at clarifying the mechanism of thymineless death, a phenomenon underlying the mechanism for the cytotoxicity of the anticancer drug 5-fluorouracil (Nakayama et al., 1984). Unicellular organisms contain only one representative of the family, such as *E.coli* RecQ and *Saccharomyces cerevisiae* Sgs1, while numbers of RecQ genes vary from species to species in multicellular organisms. For example, fruit flies (*Drosophila melanogaster*) and vertebrates have three and five RecQ genes, respectively

(Bennett et al., 1998; Gray et al., 1997; Karow et al., 1997). Human cells express at least five members; RECQ1, BLM, WRN, RECQ4, and RECQ5 (Figure 2.1). All the RecQ helicases studied so far have been found to unwind DNA with a 3'- 5' polarity.

The RecQ helicases are characterized by the conserved helicase domain, the **R**ec**Q C**-terminal domain (RQC), and the **H**elicase **R**Nase**D C**-terminus (HRDC) domain. The helicase domain is present in all RecQ family proteins, while RQC and HRDC are missing in some representatives of RecQ family. Helicase domain is important for NTP binding and nucleic acid unwinding, while the RQC domain, which is unique to RecQ helicases, is also important in mediating protein-protein interactions. The less conserved HRDC domain assists in recognizing DNA substrates. Nuclear localization signals (NLS) have been identified at the C-terminal end of RECQ1, BLM, and WRN. N-terminal extensions of approximately 600 amino acids that contain regions rich in acidic amino acids have

been identified in Sgs1p, Rqh1, BLM, WRN, and RECQL4. In addtion, WNR is characterized by the presence of a exonuclease domain at the N-terminus
(Vindigni and Hickson, 2009).

In humans, defects in three RecQ family proteins are associated with rare autosomal recessive disorders characterized by genomic instability and increased cancer susceptibility. Mutations in WRN, BLM, and RECQ4 give rise to the disorders Werner syndrome (WS), Bloom syndrome (BS), and Rothmund-Thomson syndrome(RTS), respectively (Ellis et al. 1995, Yu et al.1996, Kitao et al.1999). The clinical features of these disorders differ for each syndrome. Briefly, BS patients are predisposed to many types of cancer with the mean age of onset of 24. WS patients are especially predisposed to sarcomas, premature aging, and age- associated diseases. RTS patients have a characteristic rash, poikiloderma, and are predisposed to osteosarcomas and some features of premature aging.

In vitro biochemical experiments combined with knock out strategies and with the inference from patients with mutated RecQ genes, have shown that RecQ helicases play a key role in protecting and stabilizing the genome. RecQ helicases have been shown to participate in DNA replication, recombination, repair and in cell cycle control events. Deficiencies in RecQ helicases can lead to high levels of genomic instability and to premature aging and increased susceptibility to cancer. These diverse cellular functions support the notion that the RecQ helicases are guardians of the genome
(Chu and Hickson, 2009).

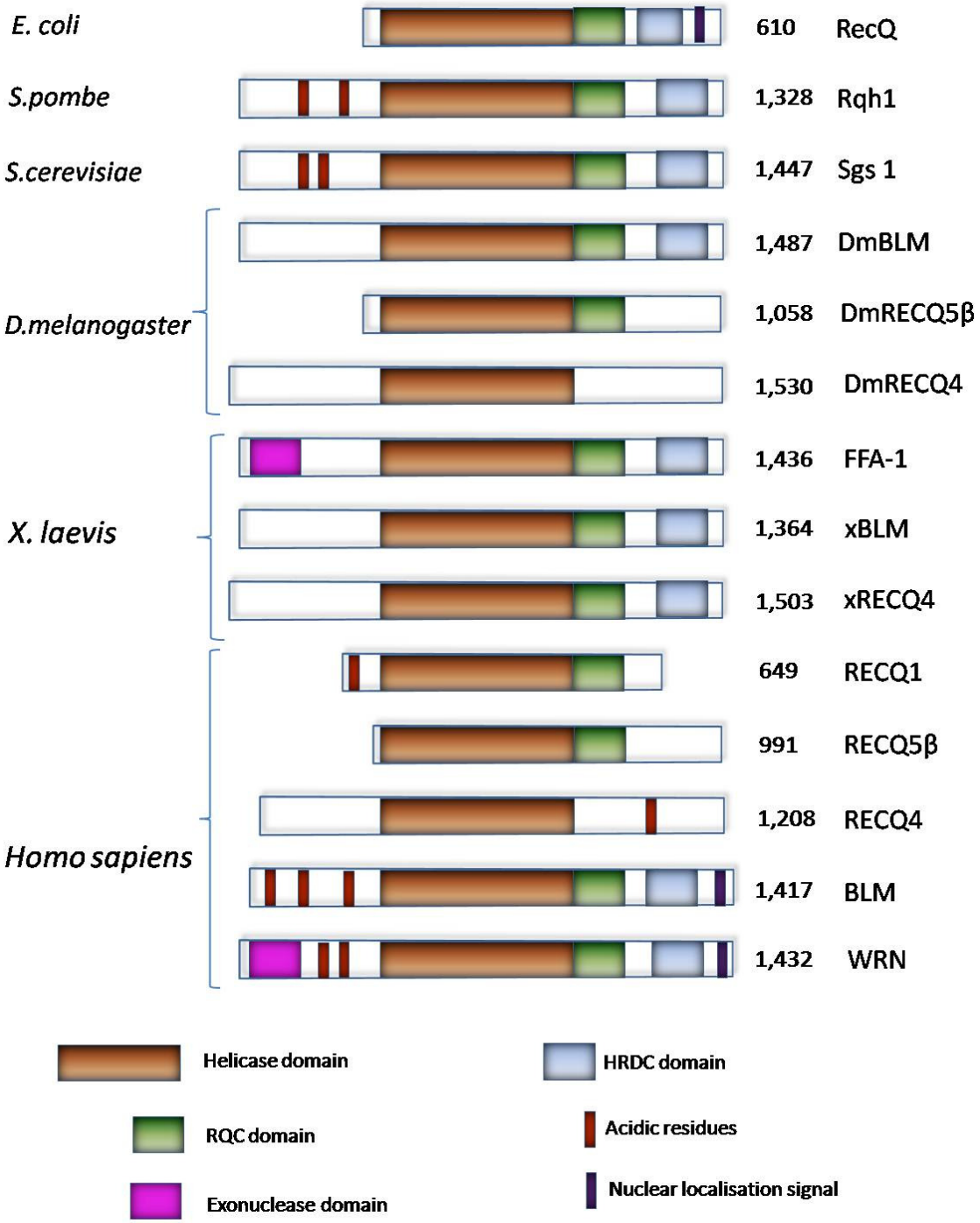


Figure 2.1: Schematic representation of the RecQ helicases of selected organisms. The family is named after the bacterial (*Escherichia coli*) DNA helicase RecQ. Family members have been also identified in fission yeast (Rqh1p), budding yeast (Sgs1p), flies (DmBLM, DmRECQ5β, DmRECQ4),), amphibians (FFA-1, xBLM, xRECQ4) and humans (WRN, BLM, RECQ4, RECQ1, RECQ5β. Proteins are aligned by their conserved helicase domains (brown boxes). At least three splice variants of the human RECQ5 protein are expressed, only one of which is shown. The size of each protein in amino acids is indicated on the right.

2.1 BLM helicase

The Bloom syndrome helicase (BLM) is a DNA helicase of 1417 amino acid residues (Karow et al., 1997). Positional cloning experiments revealed that the BLM helicase is encoded by the locus for Bloom syndrome at 15q26.1 and it contains amino acid motifs that are homologous to motifs present in other RecQ helicases (Ellis et al., 1995). The BLM helicase is expressed in all the tissues, however it shows a high expression pattern in testis and ovary. At cellular level the BLM helicase is expressed during the S and G_2 phases of the cell cycle and it is localized in the nucleus of human cells within discrete foci termed promyelocytic leukemia (PML) nuclear bodies (Ababou et al., 2000; Dutertre et al., 2000; Gharibyan and Youssoufian, 1999). Defects in the *blm* gene are the cause of Bloom Syndrome (BS) (Ellis et al., 1995).

2.1.1 Clinical manifestations of BLM deficiency

A) Bloom syndrome patients

BS is a rare autosomal recessive disorder and persons with BS are characterized by proportionate pre- and postnatal growth deficiency, sun-sensitive telangiectatic hypo- and hyper-pigmented skin, immune deficiency, predisposition to malignancy and chromosomal instability (Figure 2.2). BS patients also show a very high incidence of cancers of various types including leukaemias, lymphomas, and carcinomas (Ellis and German, 1996).

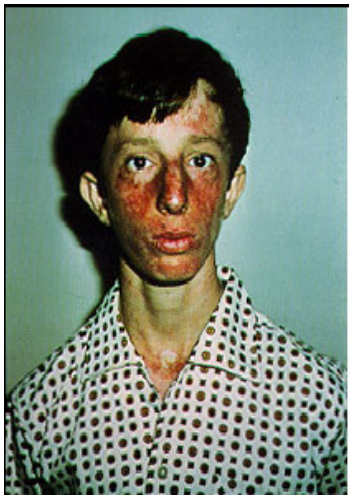


Figure 2.2: A Bloom syndrome patient showing sun sensitive telangiectatic erythema.

B) Murine model

Four BS mice models have been generated. Mice in which part of the *blm* gene upstream of the helicase domain is replaced with neomycin resistance cassette showed growth defect and micronuclei similar to human BS patients(Chester et al., 1998). The second mice in which exons 10–12 were replaced with HPRT (hypoxanthine–guanine phosphoribosyltransferase) showed a slight increase in the frequency of micronuclei (Goss et al., 2002). However, both knockout mice had a short life span. Given the fact that BLM is not essential for viability in humans, Allan Bradley's group generated two more knockout models by replacing exon 2 using an Embryonic stem cell (ES) method. The first mice again showed lethality. However, the second mice where integrated vector sequences were removed, gave rise to viable homozygous mutant mice. These mice served as better model and closely recapitulated the cellular phenotype of Bloom syndrome patients (Luo et al., 2000).

Cellular phenotype

Cells from patients with BS and the cells derived from Blm knockout mice exhibit chromosome instability. Particularly BS patient's cell showed increased numbers of chromatid gaps, breaks, chromosome structural rearrangements and sister chromatid exchanges (SCE). It has been reported that BS cells as well as *BLM-/+* fibroblasts show a tendency for the accumulation of micronuclei (Frorath et al., 1984; Rosin and German, 1985). Moreover, these cells display a significantly lower rate of chain elongation during DNA synthesis (Hand and German, 1975). In addition, BS cells are sensitive to UV radiation and hydroxyurea (HU) (Ababou et al., 2002a). Blm deficient mouse embryonic fibroblast (MEF) cells showed elevated mitotic recombinations with the increased SCE (Luo et al., 2000). Such elevated level of chromosal disruptions observed in BS cells and Blm deficient MEF cells could likely be the reason for high incidence of cancer in Bloom Syndrome patients.

2.1.2 Structural aspects of BLM protein

A) Domain architecture

The BLM helicase consists of seven domains. Starting from the amino terminus, these domains are the poly-aspartate domain (PD1), the poly-serine domain (PS), the poly-aspartate domain (PD2), the DEAH helicase domain (DEAH), the RecQCt, the HRDC, and the NLS. The C-terminal domain is essential for the strand annealing activity of BLM (Cheok et al., 2005). Although many BS mutations truncate the BLM protein upstream of helicase domain, four single amino acid substitutions in helicase domain namely Q672R, I841T, G891E, C901Y, and three point mutations, K703A, Q680P, and I849T have been

reported in the human BLM and in the mouse BLM, respectively (Figure 2.3). The above mentioned substitutions and point mutations abolish both ATPase and DNA unwinding activities of the BLM protein (Rong et al., 2000).

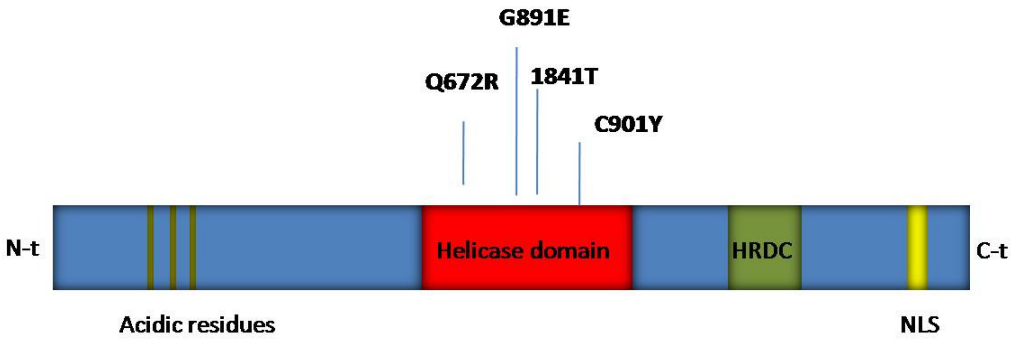


Figure 2.3 : Schematic depiction of wild-type human BLM protein with characterised mutations.

B) Assembly state

Different assembly states have been reported for the BLM protein. Electron microscopy and size exclusion chromatography analysis have shown that the full-length BLM protein forms ring-like oligomers in the absence of ATP and DNA, and the higher oligomeric state is responsible DNA strand annealing activity (Cheok et al., 2005). Interestingly, biochemical studies with the deletion mutant BLM $^{642\text{-}1290}$ revealed that monomers are responsible for DNA unwinding (Cheok et al., 2005; Janscak et al., 2003).

2.1.3 Biochemical characteristics of the BLM protein

A) Enzymatic activities

BLM helicase is an ATP- and Mg^{2+} dependent enzyme that unwinds DNA with a 3' to 5' polarity. BLM also promotes ssDNA annealing. Single strand annealing activity does not require Mg^{2+}, but it is inhibited by ssDNA binding proteins and ATP. In addition, the annealing activity is regulated by the length of the DNA (Cheok et al., 2005).

Apart from conventional fork like structures, BLM helicases can unwind linear duplexes, mobile holliday junctions, G4-DNA, G2-DNA, RNA:DNA hetero duplexes, R-loops, D-loops and model stalled replication forks (Popuri et al., 2008).

2.1.4 Functional role of the BLM protein

BLM helicase plays a vital role in regulating recombination events. The suppression role of BLM in recombination is evident by the hyper-recombination phenotype of BS patient cells and of cells derived from Blm knockout mice. This increased recombination events is connected to the tumour susceptibility in Blm knocout mice (Stenner-Liewen et al., 2000). BLM together with the hTOPO III, RMI1, and RMI2 complex can also process recombination intermediates that contain double Holliday junctions into noncrossover products by a mechanism known as dissolution of Holliday junctions (Wu et al., 2006). In addition, BLM has been shown to participate in various DNA double strand break repair pathways by regulating homologus recombination events. BLM has also been shown to participate in cellular response pathways to ionizing radiations by acting as an ATM kinase downstream effector (Ababou et al., 2000).

Most importantly, BLM's role in DNA replication is well documented. The hyper sensitivity of BS cells and BLM knockout MEF cells to replication inhibitors (Ababou et al., 2002a, b), the blocked S phase progression in HU treated BS cells (Davies et al., 2004), and the stalled replication forks observed in HU treated BS cells (Davies et al., 2007) support a role of BLM role in replication fork restart. Mechanistically, BLM was suggested to restart replication forks by transporting p53 to RAD51 sites at the stalled replication forks (Sengupta et al., 2003). In addition, to fork restart BLM has also shown to suppress origin firing during replication stress (Davies et al., 2007).

2.1.5 Regulation of BLM function

A) Post translational modifications

At least two post translational modifications have been reported for the BLM helicase. The BLM helicase appear to be phosphorylated during mitosis and during replication insults. The ATM kinase phosphorylates the BLM protein and the phosphorylated form loses its association with the nuclear matrix, and it is found preferentially in the nucleoplasmic fraction. This observation suggests that phosphorylation might assist BLM to the sites of DNA damage, thus helping in DNA repair or recombination (Beamish et al., 2002; Dutertre et al., 2000).

BLM helicase is also reported to be "sumoylated" and it has been demonstrated that sumoylated BLM resides in PML-NBs suggesting that sumoylation regulates the nuclear trafficking of BLM (Eladad et al., 2005).

B) Protein protein interactions

Several proteins that are involved in different DNA metabolic pathways have been reported to interact with BLM. Weidong Wang lab has reported the presence of BLM in at least three multiprotein complexes in HeLa cells (Meetei et al., 2003).

Fanconi's anemia proteins (FA) , Replication Protein A (RPA), and MLH1 are components of some of these complexes (Meetei et al., 2005; Meetei et al., 2003), Two proteins are present in all BLM associated complexes: Topo 3α, and a protein first named as BLAP75 (Yin et al., 2005), but later renamed RMI1 (RecQ-mediated genome instability) (Wu et al. 2006). RMI1 contains an oligonucleotide-binding (OB)-fold domain (Yin et al. 2005), and regulates BLM function by interacting with BLM and TOP3α. This complex specifically "dissolves" the Holliday junctions that arise during double strand break repair. Evidence from both human and yeast studies suggests that BLM (or its ortholog Sgs1), Topo 3α, and RMI1 form an evolutionarily conserved complex that works coordinately to process a diverse array of DNA structures in eukaryotes (Raynard et al., 2006; Wu et al., 2006; Xu et al., 2008; Yin et al., 2005) (Figure 2.4). Interestingly, RPA has shown to increase the processivilty of the BLM catalysed DNA unwinding activity by interacting with the BLM protein. Conversely, BLM interaction with FA complex proteins resolves the interstrand crosslinks of DNA. BLM also interacts with p53 and RAD51, and these interactions regulate the localization of BLM during homologous recombination repair in replication insulted cells (Bischof *et al.*, 2001; Wu *et al.*, 2001; Sengupta *et al.*, 2003). Moreover, the interaction of BLM with MLH1 was suggested to regulate the mismatch repair process (Langland et al., 2001).

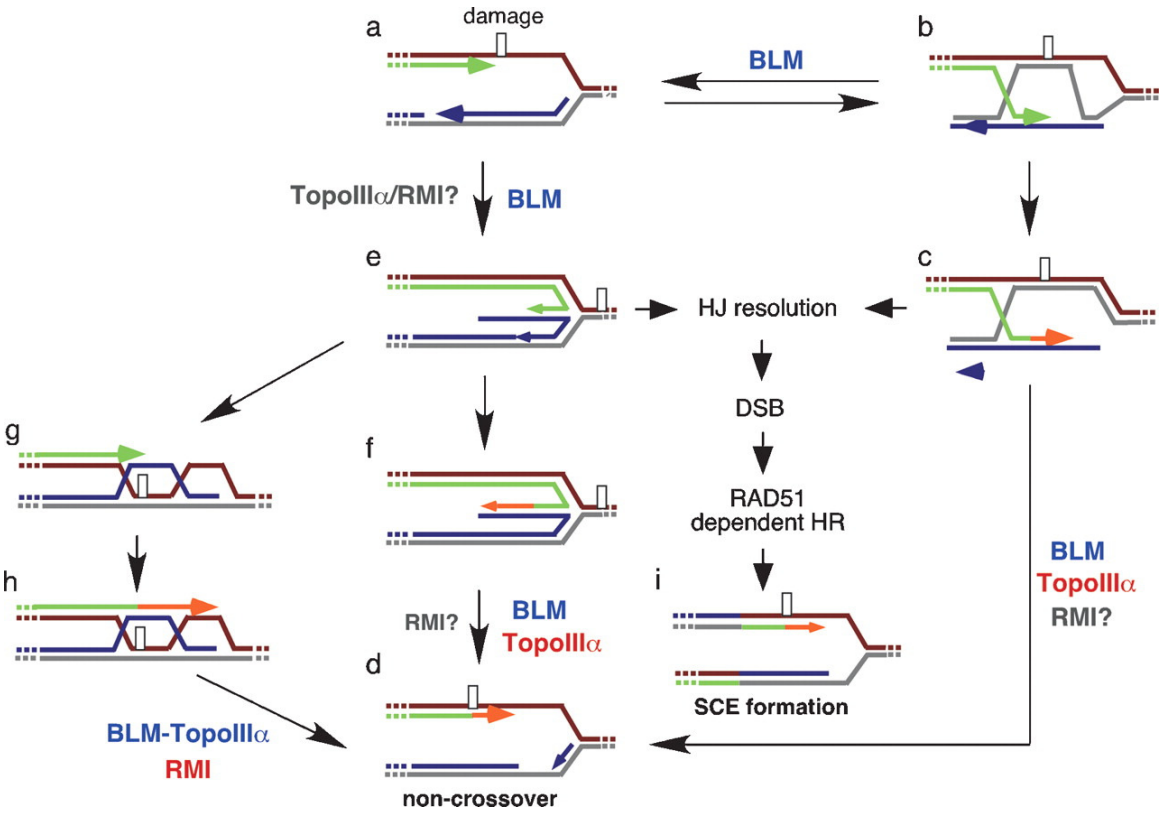


Figure 2.4 : Potential roles of BLM and its interaction partners in human cells. Schematic diagram indicating alternative pathways for the repair of stalled replication forks that arise as a result of DNA damage. Picture adopted from (Liu and West, 2008).

2.1 WRN helicases

The WRN helicase is a DNA helicase and exonuclease of 1432 amino acid residues. Positional cloning experiments revealed that the WRN helicase is encoded by the locus for Werner syndrome at p11-p12 region of chromosome 8, encompassing 180 kb (Matsumoto et al., 1997; Yu et al., 1996). Sequence homology marks WRN as a member of the RecQ family of DNA helicases. WRN is highly expressed in the testis, ovary and pancreas with a cell cycle regulated expression that peaks during the G_2 /M phases

(Kitao et al., 1998). WRN is localized in the nucleus of human cells within discrete foci termed promyelocytic leukemia (PML) nuclear bodies (Ababou et al., 2000; Dutertre et al., 2000; Gharibyan and Youssoufian, 1999). Importantly, defects in the *wrn* gene are found to be the cause of Werner Syndrome (WS) (Ellis, Groden et al., 1995).

2.2.1 Clinical manifestations of WRN deficiency

A) Werner syndrome patients

Werner syndrome is an autosomal recessive condition characterized by an early onset of age-related symptoms that include ocular cataracts, premature graying and loss of hair, arteriosclerosis and atherosclerosis, diabetes mellitus, osteoporosis, and a high incidence of cancers. The predominant cancers in WS are soft-tissue sarcoma, osteosarcoma, myeloid disorders, thyroid cancer, and benign meningiomas (Figure 2.5).

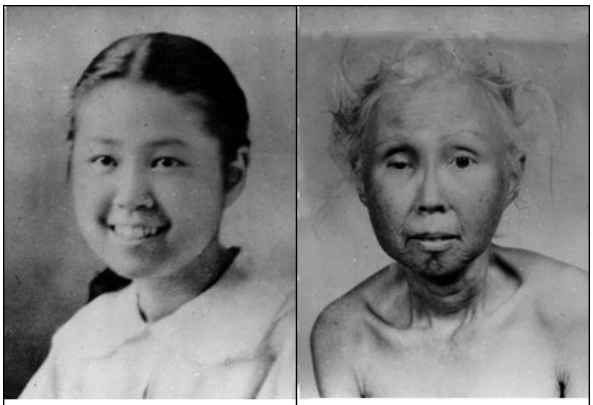


Figure 2.5: A Werner syndrome patient showing ageing phenotype.

B) Murine model

Two WS mice model have been generated so far. The first mice, where exons that encode motifs III and IV of the helicase domain were targeted, shared phenotypes

similar to those of human WS patients. Similar to WS patients, the knockout mice acquired myocardial fibrosis, T cell lymphoma, and were prone to cancer (Lebel and Leder, 1998). The second mice with a mutation that eliminates expression of the C terminus of the helicase domain din't show any organismal phenotype that resembles WS patients. However, *p53−/− WRN−/−* double-knockout mice died earlier and the lack of p53 accelerated the mortality of *WRN−/−* or *WRN−/+*mice (Lebel et al., 2001; Lombard et al., 2000).

C) Cellular phenotype

Primary Cells from WS patients undergo replicative senescence more rapidly than normal cells and are highly sensitive to cross linking agents, especially to 4-nitroquinoline 1-oxide (4-NQO). They also lose their proliferative capacity at an accelerated rate. Additionaly, cultured fibroblasts from such patients have an extended S phase of the cell cycle (Salk et al., 1981). Telomere dysfunction likely contributes the premature senescence because, serially passaged Werner syndrome fibroblasts shorten telomeres more rapidly than controls and senesce prematurely. The expression of telomerase hTERT in WS cells leads to the immortalization of cells and reduces their sensitivity towards cross linking agents (Choi et al., 2001; Salk et al., 1981). At chromosome level, the cells carry increased number of chromosomal translocations and deletions. Such chromosomal aberrations observed in WS cells and WRN deficient MEF cells could likely be the reason for the cancer phenotype of WS patients and WRN knockout mice.

2.2.2 Structural aspects of WRN protein

A) Domain architecture

WRN contains four major domains comprising an exonuclease, a helicase, a RecQCt, and a HRDC domain. The 3'-5' exonuclease region of the protein is 171 amino acids long and is located at the N-terminal end of the protein. It is highly similar to the exonuclease regions of other proteins such as the DNA polymerases that have DNA proofreading activity. When exposed to double stranded DNA, the exonuclease domain forms a hexamer around the end of the strand and remove bases toward the 5' end. This region is essential to the DNA repair function of WRN (Xue *et. al.,* 2001). *Kitano et al* reported the crystal structure of the human WRN HRDC domain. They demonstrated that HRDC domain forms a bundle of α-helices and has a novel looping motif.Moreover, the same authors showed that this domain is thermostable and resistant to protease digestion (Kitano et al., 2007). The HRDC domain consists of 80 amino acids at the C-terminal end of the protein. It contains five helices connected by short turns or hydrophobic loops. Certain hydrophobic residues within the loops are necessary for the packing of this domain into a bundle. A patch near helix a4 has been shown to interact with DNA in a non-sequence specific manner, suggesting that this domain may contribute to the interaction between the WRN protein and the double stranded DNA (Liu *et. al.,* 1999). The RQC domain has also been crystallized. The crystal structure of the WRN RQC-DNA complex represented the first DNA bound structure of a RecQ family member and revealed that the winged-helix motif acts as a strand separating knife during the unwinding reaction (Kitano et al., 2009).

Although mutations throughout the *wrn* gene have been observed in the homozygous state, homozygosity for a mutation very near the 3' end of the *wrn* open reading frame is sufficient to lead to the WS (Figure 2.6). Some of the mutant WRN proteins from WS patients lack the nuclear localization element, thus they are not translocated to the nucleus. Interestingly, mice bearing a targeted mutation in the catalytic helicase domain of *wrn* are viable and fertile revealing that mutations in helicase domains are not responsible for WS (Perry et al., 2006).

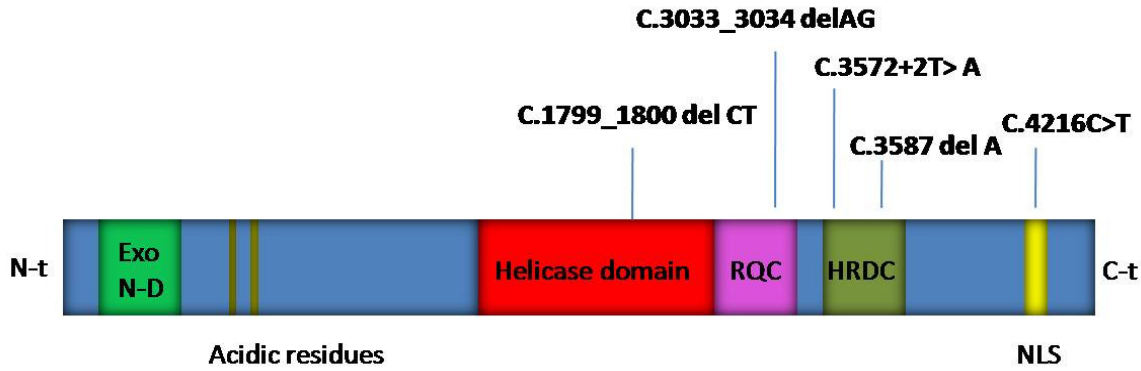


Figure 2.6 : Schematic depiction of wild-type WRN protein with location of few mutations that could contribute to WS. Figure drawn based on Prof. Raymond Monnat lab webpage information. Ref : http://www.pathology.washington.edu/research/werner/database/

C) Assembly state

The assembly states of full length WRN as well as of some fragments of WRN have been characterized. Gel filteration chromatography and atomic force microscopy analysis showed that exonuclease fragment of WRN forms hexamers. Consistently biochemical data obtained with recombinant N-terminal WRN fragments and full-length WRN

suggest oligomerization (possibly trimeric) of WRN *in vivo* (Huang *et al.,* 2000*;* Xue *et al.,* 2002). WRN trimers may provide the necessary quaternary structure for interaction with PCNA. However, recent electron microscopy studies indicated that WRN binds to replication fork like structure and HJ as a tetramer (Perry et al., 2006) suggesting that tetramer formation might enhance WRN exonuclease and/or helicase activities.

2.2.3 Biochemical characteristics of the WRN protein

A) Enzymatic properties

The WRN helicase is an ATP- and Mg^{2+}-dependent enzyms that unwind DNA with a 3' to 5' polarity with poor processivity. WRN can also promote the single-stranded DNA (ssDNA) annealing and possess a characteristic 3'-5' exonuclease activity (Orren et al., 2002). The WRN helicase coordinates its helicase and exonuclease activities to unwind model fork DNA. WRN degrades the 3′ recessed strands of double-stranded DNA or a DNA–RNA heteroduplex and efficiently removes a mismatched nucleotide at a 3′ recessed terminus. It can unwind bubble substrates, bubble-containing double stranded DNA, DNA with a single stranded loop, and G-quadruplex DNA. In addition, WRN can branch migrate Holliday junctions (Mohaghegh et al., 2001).

2.2.4 Functional role of WRN protein

The primary function of Werner helicase can considered to be at telomeres on the basis of the reports that show defective replication of G-rich telomeric DNA in WS cells (Crabbe et al., 2007; Crabbe et al., 2004), cellular senescence phenotype of WS cells and

WRN knockout MEF, ageing phenotype of WS patients (Davis et al., 2007; Fukuchi et al., 1989), (Davis et al., 2007; Fukuchi et al., 1989), and on the basis of the molecular analysis showing a functional interaction of WRN with TRF (Multani and Chang, 2007).

Like BLM, WRN was also shown to participate in DNA replication. The efficient removal of a terminal mismatched nucleotide raises the possibility that WRN may provide 3′→5′ proofreading activity for DNA polymerases that lack such activity. In line with these findings, WS cells are known to be hypermutable, accumulating cytogenetic abnormalities consistent with deletions and rearrangements. Additionaly, WRN is homologous to FFA-1, a *Xenopus laevis* protein required for replication focus formation during DNA replication. In addition, the finding that WRN interacts with PCNA support the notion that WRN may function in DNA replication. In line with these observations, WRN has been proposed to reset reversed forks or other replication intermediates arising after fork stalling (Khakhar et al., 2003). Alternatively, WRN has also been implicated in the resolution of recombination intermediates arising after RAD51-dependent strand invasion (Saintigny et al., 2002). Additional studies have shown that WRN promotes rRNA transcription (Shiratori et al., 2002) and stimulates DNA polymerase δ (Lebel *et al*., 1999). WRN has also been reported to participate in several DNA repair pathways. Mismatch repair involves an incision on the damaged DNA strand followed by removal by an exonuclease. WRN can potentially fulfill the exonuclease requirement in this process. In line with it, SV40-transformed WS cells are deficient in mismatch repair (Saydam et al., 2007). However, WRN may also participate in the repair of other types of DNA damage such as interstrand crosslinks (ICL) mediated DNA

damages as WS cells are hypersensitive to 4NQO, which leads to elevated rates of chromosome breaks and exchanges (Ogburn et al., 1997).

Moreover, WRN was also shown to participate in long patch base excision repair (BER) by cooperating with pol β (Harrigan et al., 2006).

2.2.5 Regulation of the WRN protein function

A) Post-translational modifications of WRN

WRN is acetylated in a DNA damage dependent manner by p300 acetyltransferase and this acetylation helps the WRN helicase to localize in nucleoplasmic fraction (Blander et al., 2002). A recent report from Bohr's group suggests that WRN function during Base Excision Repair (BER) is regulated by acetylation. The WRN helicase is also phosphorylated upon treatment with DNA damaging agents and, in contrast to acetylation, phosphorylation inhibits its helicase and exonuclease activities (Yannone et al., 2001). Pichierri et al have shown that WRN is phosphorylated in an ATR/ATM dependent manner following replication arrest (Pichierri et al., 2003). Additionaly, WRN is sumoylated *in vivo*. WRN helicase interacts and co-localize with SUMO-1 (small ubiquitin-related modifier 1), but the functional significance of this modification is not clear (Suzuki et al., 2001).

B) Protein protein interactions

Consistent with its proposed role in different metabolic process WRN has been identified to interact with several proteins. WRN helicase function at telomeres through its interaction with TRF2 and co-localization with the ALT pathway proteins TRF1, TRF2,

PML, RAD52, and NBS1 (Opresko et al., 2002) (Figure 2.7). Consistent with a role of the WRN helicase role in transcription, it was shown that WRN is associated with the RNA polymerase I complex (Balajee et al., 1999). The role of WRN in DNA replication is evident by the purification of WRN with a 17S multiprotein DNA replication complex containing PCNA, topoisomerase I and RPA (Lebel and Leder, 1998; Lebel et al., 1999). However, the functional significance of this interaction during the DNA replication process is not clear. Additionally, the C-terminus of WRN interacts with FEN1 and stimulates the endonucleolytic activity of FEN1 on a flap substrate (Brosh et al., 2001). Interestingly, p53 interacts with WRN and inhibits the unwinding of a synthetic Holliday junction by WRN (Blander et al., 1999). Finally, WRN also interacts with several proteins involved in the DNA non-homologus end-joing (NHEJ) double strand repair pathway and its enzymatic activity is regulated by phosphorylation events promoted by the DNA-PKcs kinase suggesting a pontential role of WRN in NHEJ (Karmakar et al., 2002; Orren et al., 2001).

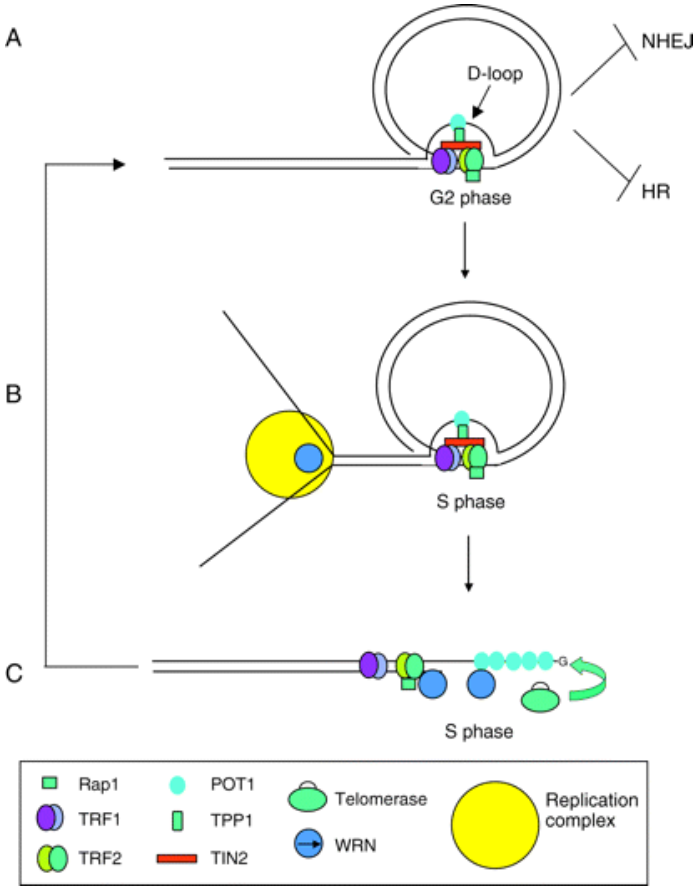


Figure 2.7 : Potential role of WRN and few of its interaction partners in human cells. (A) Telosome complex and telomere structure. The telomere folds back onto itself to form a double-stranded t-loop and a single-stranded D-loop. This complex protects telomeres at the G2 phase of the cell cycle from inappropriate NHEJ- and HR-mediated processing of telomeric DNA. The six-component telosome is shown schematically on the t-loop, with POT1 interacting with the D-loop. (B) During DNA replication, the presence of WRN at the replication fork is postulated to enable the replication complex to efficiently replicate telomeric DNA. (C) The presence of WRN at telomeres may facilitate unwinding of the D-loop, enabling telomerase to extend telomeres. The linear 3' overhang is probably protected by POT1. Model adopted from (Multani and Chang 2007).

2.2 RECQ5 helicase

The RECQ5 gene has 19 exons and by alternate splicing, it gives rise to three isoforms, namely RECQ5α, RECQ5β, and RECQ5γ. These proteins are 46 kDa, 108.9 kDa, and 49 kDa, respectively (Shimamoto et al., 2000). Among the three isoforms only RECQ5β has the nuclear localization signals. Sequence analysis revealed that RECQ5 gene is located onto chromosome 17q25 and mutations in this region leads to ovarian cancers and

breast carcinomas (Sekelsky et al., 1999). RECQ5 isoforms are ubiquitously expressed with relatively high expression in testis and their expression level does not change during any stages of the cell cycle (Ozsoy et al., 2001). There are no reports about genetic diseases associated with RECQ5 gene mutation published to date.

2.3.1 Clinical manifestations of RECQ5 deficiency

A) Murine model

The *Recq5* knockout mice generated by deleting exon4 showed a cancer susceptibility phenotype. Specifically, they developed lymphomas. However, *Recq5* knockout mice had a normal life span suggesting RECQ5 is not essential for normal embryonic development in mice (Hu et al., 2009).

B) Cellular phenotype

Primary mouse embryonic fibroblasts (MEF) cells derived from *Recq5* knockout mice showed an elevated rate of spontaneous double-strand breaks (DSBs), homologous recombination (HR), and showed significant increase in sister chromatid exchange frequency. Recq5-deficient cells also accumulate Rad51 and γ-H2AX foci and are prone to gross chromosomal rearrangements in response to replication stress. Moreover, RECQ5 defecient cells were sensitive to camptothecin treatment and cell survival assays showed proliferation defects upon CPT treatment (Hu et al., 2009). Wang and colleagues deleted the chicken *RECQL5* homologue in DT40 cells. They detected no effect of *RECQL5* deletion on the frequency of spontaneous SCE. However, the same *RECQL5* deletion in a $BLM^{-/-}$ background increased the frequency of SCE (Wang et al., 2003). These

observations suggest that *RECQL5* suppresses SCE but only under *BLM* function-impaired conditions.

2.3.2 Structural aspects of the RECQ5 protein

A) Domain architecture

RECQ5β contains an N-terminal, RQC, HRDC, and C- terminal domain. The large C-terminal region includes a domain homologous to the non-helicase domain of the *E. coli* RecQ DNA helicase, and it is responsible for the strand annealing activity of RECQ5β. This domain is also required for unwinding lagging strand duplex DNA and for DNA strand exchange (Aygun and Svejstrup). Interestingly, the N-terminal is small in contrast to the N-terminal of other RecQ helicases, and the function of this short N-terminal of RECQ5 is not known yet (Kawasaki et al., 2002).

B) Assembly state

Unlike other RecQ helicases, RECQ5 forms monomers both in its free and DNA/ATP bound forms suggesting that RECQ5 unwinds DNA as a monomer (Garcia et al., 2004). However, baculovirus expressed dmRecQ5β was suggested to form higher-order oligomeric structures dmRecQ5β by gel-filtration experiments. Interestingly, GTP binding triggers the helicase activity of dmRecQ5β (Kawasaki et al., 2002).

2.3.3 Biochemical characteristics of the RECQ5 protein

A) Enzymatic properties

The *in-vitro* biochemical characterization of the purified recombinant human RECQ5 protein shows DNA-dependent ATPase and ATP-dependent DNA helicase activities. The

DNA helicase activity of RECQ5 has 3′–5′ polarity, consistent with the general properties of the other RecQ helicases. Interestingly, unlike other RecQ helicases the unwinding activity is not stimulated by RPA and the annealing activity is inhibited by the addition of RPA (Garcia et al., 2004). Like other RecQ helicases, RECQ5 unwinds conventional model replication forks and Garcia *et al*. showed that RECQ5 has the ability to branch migrate Holliday junctions (Garcia et al., 2004).

2.3.4 Functional role of the RECQ5 protein

Available literature suggests that RECQ5 is a tumor suppresser protein. In particular, it minimizes the propensity of oncogenic rearrangements by suppressing the accumulation of DSBs and attenuating HR by disrupting inappropriate RAD51 presynaptic filaments (Hu et al., 2007). Conflicting data regarding the role of RECQ5 in transcription have been reported. RECQ5 association with RNAPII *in vivo* suggests the possible role of RECQ5 in transcription However, latter reports suggested that RECQ5 acts as an inhibitor of transcription (Aygun et al., 2008; Aygun et al., 2009). The exact mechanism of transcription regulation by RECQ5 is not yet clear Thus, RECQ5 has been proposed to promote genome stability through two parallel mechanisms: by participation in homologous recombination-dependent DNA repair and by regulating initiation of Pol II to reduce transcription-associated replication impairment and recombination.

RECQ5 may play an additional role in stabilization and/or restart of stalled replication forks. This was suggested by the findings that mouse *Recq5-/-* embryonic stem (ES) cells and primary embryonic fibroblasts are hypersensitive to camptothecin (CPT), a topoisomerase I inhibitor that blocks DNA replication (Hu et al., 2009).

2.3.5 Regulation of the RECQ5 protein function

A) Protein protein interactions

In line with its proposed functions in transcription and homologus recombination, RECQ5 has been shown to interact with various proteins involved in these pathways. In particular, RECQ5 is the only member of RecQ helicase which directly interacts with RNA polymerase II (RNAPII). This interaction is mediated by the RPB1 subunit of RNAPII (Aygun, Svejstrup et al., 2008) (Figure 2.8). Recent finding's from Weidong Wang's lab, using chicken DT40 cells inactivated for RECQL5, demonstrated that Pol II is a critical partner of RECQL5 in suppression of SCE and resistance of CPT-induced cell death. Moreover, they identified two conserved domains in RECQL5, KIX and SRI, and showed that they are responsible for interacting with Pol II (Islam et al.).

RECQL5β interacts and co-localizes with FEN1 in the nucleus in response to DNA damage and this interaction stimulates the flap endonucleases activity of FEN1 suggesting that the interaction with FEN1 is essential for the DNA repair function of RECQ5 helicase (Speina et al.). In addition, Pavel Janscak's group has purified FLAG-RECQ5 in complex with PCNA, Topoisomerase III α/β, and the MCM complex proteins, but the functional significance of these interactions is not known yet.

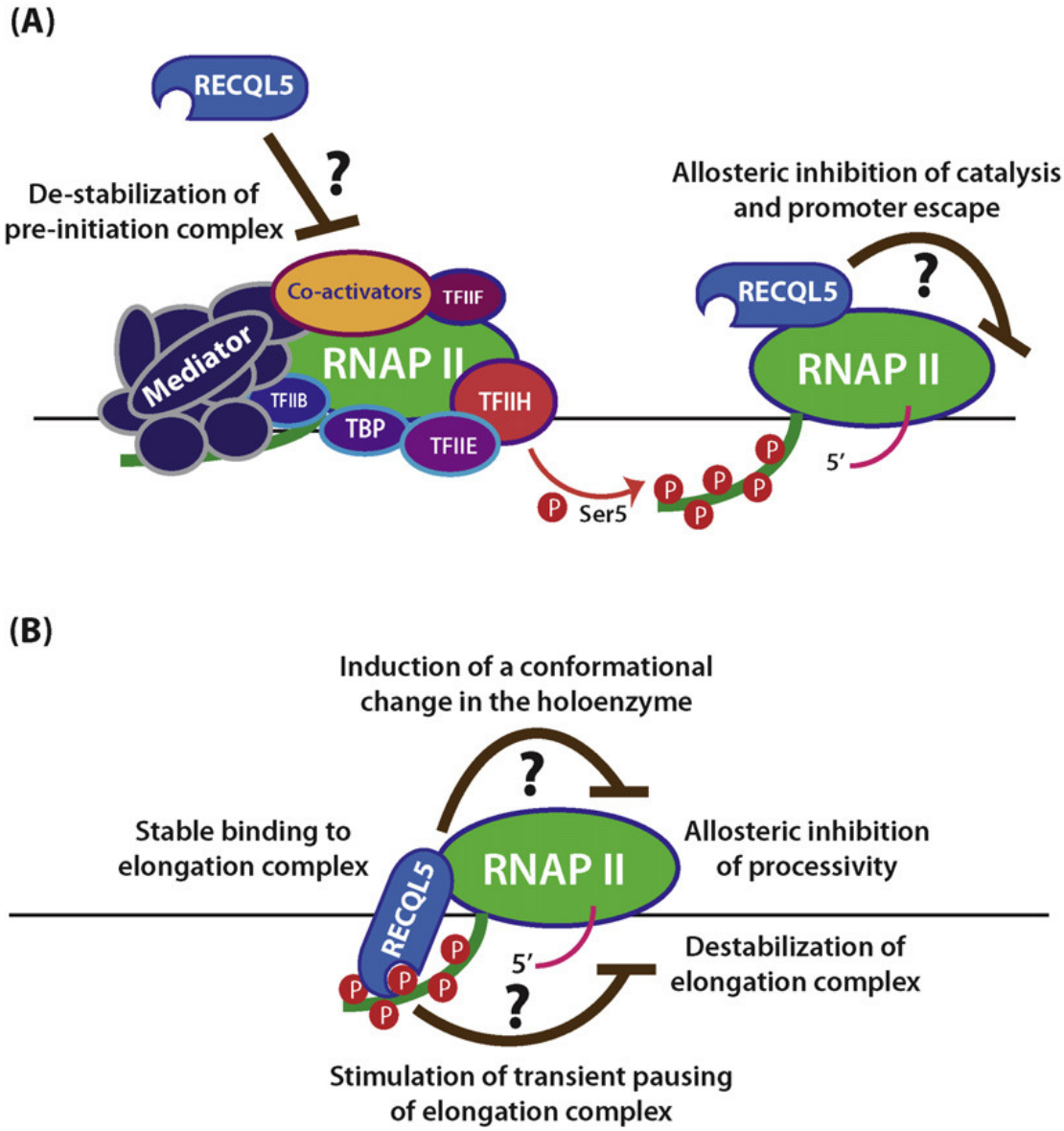


Figure 2.8: Potential role of RECQ5 and its interaction partner RNAP II in human cells. Potential mechanisms by which RECQ5 may affect RNAPII transcription (A). RECQ5 may inhibit at least 2 critical events in the early initiation stages of RNAPII transcription. First, RECQ5 may directly compete with one of the initiation factors and de-stabilize pre initiation complexes. RECQ5 may also exert an allosteric inhibitory effect on the catalysis of first phosphodiester bond formation by RNAPII (B). During elongation RECQ5 may form a stable complex with RNAPII ternary elongation complex and this binding may result in a conformational change in the holoenzyme. Consequently the processivity of the enzyme may be reduced. Finally, RECQ5 may destabilize the associated RNAPII elongation complex or stimulate its transient pausing, which may be relieved by a positive elongation factor such as TFIIS. However, the fact that RECQ5 is capable of inhibiting RNAPII transcription in the absence of other co-factors indicates that the effect is directly targeted at an intrinsic RNAPII function. Adapted from **(Aygun and Svejstrup).**

2.4 RECQ4 helicase

The RECQ4 helicase is a structure specific DNA helicase of 1208 amino acid residues. The Recq4 gene has been identified on chromosome 8q24.3 (Kitao et al., 1998). Mutations in this gene leads to three different genetic disorders namely Rothmund –Thomson (RTS), RAPADILINO, and Baller-Gerold (BGS) syndromes (Islam et al., ; Kitao et al., 1999; Lindor et al., 2000; Lype et al., 2008; Siitonen et al., 2003). RECQ4 is expressed in testis, prostate and thymus. At cellular level, RECQ4 is found both in nucleus and cytoplasm and the expression of RECQ4 is regulated throughout the cell cycle with a peak of expression during the S phase (Sengupta et al., 2005).

2.4.1 Clinical manifestations of RECQ4 deficiency

A) Human syndromes associated with RECQ4 deficiency

Rothmund –Thomson syndrome (RTS): Mutations in helicase domain of Recq4 gene leads to RTS. The patients show poikiloderma, growth deficiency, juvenile cataracts, premature aging and a predisposition to malignant tumours, particularly osteosarcomas (Figure 2.9) (Balraj et al., 2002; Kitao et al., 1999; Lindor et al., 2000).

RAPADILINO: Mutations which leads to in-frame deletion of exon 7 of Recq4 gene are the cause of RAPADILINO syndrome, where the patients show **RA**dial hypo-/aplasia, **PA**tellae hypo-/aplasia and cleft or highly arched palate, **DI**arrhoea and DIslocated joints, LIttle size and LImb malformation and **No**se slender (Siitonen et al., 2003).

Baller-Gerold syndromes (BGS) : Mutations in the exon 9 of Recq4 gene leads to BGS. The clinical features of the patients are radial aplasia/hypoplasia and craniosynostosis (Lype, Henry et al., 2008).

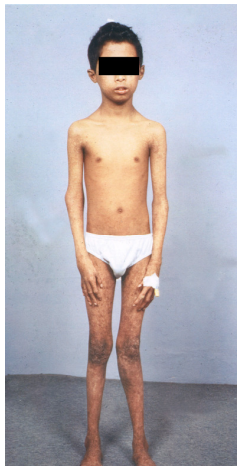


Figure 2.9 : A RTS patient showing bone abnormalities

B) Murine model

Three RECQ4 knockout mice have been reported so far. The first mice with a knockout of exon 5-8, covering the N-terminal, proved to be embryonic lethal (Ichikawa et al., 2002) suggesting a very important function of this N-terminal domain. The second mouse where part of exon 13 which codes for part of the helicase domain was knocked out, showed severe growth retardation and other organismal phenotypic characteristics resembling RTS patients (Hoki et al., 2003). The third mouse where exon 9 to 13 were knocked out showed typical RTS clinical features such as hypo-/hyperpigmented skin, skeletal limb defects and palatal patterning defects (Mann et al., 2005). The existence of two mouse models with similar phenotype of RTS patients confirms that mutation in the helicase domain of RECQ4 is the reason behind the RTS.

C) Cellular phenotype

The cells derived from RTS patients show genomic instability, including trisomy, aneuploidy and chromosomal rearrangements. Likewise, cytogenetic analysis of the embryonic fibroblasts (MEF) derived from Recq4 knockout mice revealed overall aneuploid phenotype and a significant increase in the frequency of premature centromere separation (Hoki et al., 2003). Such wide range of disruptions of the chromosome could likely be the reason for the carcinoma's, RTS, BGS, and RAPADILINO. Additionally, RTS cells are sensitive to ionizing radiation and oxidative stress/damage suggesting a possible role in DNA break repair. Acute depletion of RECQ4 protein in primary murine embryo fibroblasts leads to defects in DNA synthesis and cell proliferation (Sangrithi et al., 2005) which is consistent with embryonic lethality of RECQ4 knockout mice.

2.4.2 Structural aspects of RECQ4 protein

A) Domain architecture

Although RECQ4 has the seven domain that characterize the helicase domain of all the RecQ helicases, it lacks the RQC and HRDC domain that characterize most of the members of the RecQ family. A number of acidic amino acid residues are found at the C-terminal region of RECQ4, while this acidic amino acid residues are generally found at the N-terminus of other RecQ helicases (Xu and Liu, 2009). The N terminal domain of Xenopus RECQ4 shows sequence similarity to yeast Sld2/DRC1 complex which is responsible for the establishment of replication forks (Matsuno et al., 2006; Sangrithi et al., 2005) However, this similarity only limited to the first 70 amino acids in the case of

human RECQ4 and, surprisingly the N-terminal of human RECQ4 was found to have an intrinsic DNA helicase activity (Xu and Liu, 2009). Several mutations in the helicase domain have been identified in RTS patients (Figure 2.10).

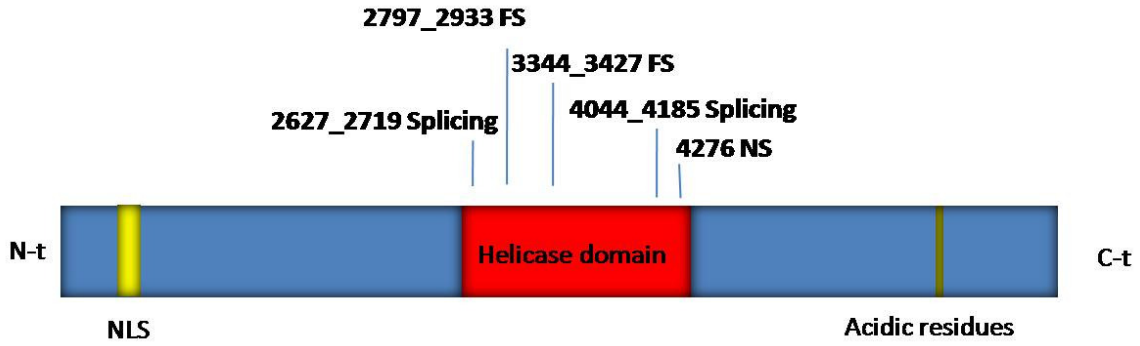


Figure 2.10 :Schematic depiction of wild type RECQ4 with characterized mutations leading to RTS.
The positions of the mutations found in the RTS patients with FS referring frameshift type of mutation (Kitao *et al.,* 1999; Lindor *et al.,* 1999).

2.4.3 Biochemical characteristics of RECQ4 helicase

A) Enzymatic properties

Recombinant RECQ4 purified from *E.Coli* showed ATP dependent DNA unwinding activity with a 3' to 5' polarity (Macris et al., 2006). Interestingly, a recent study suggested that the unwinding activity is derived both from the helicase domain and the Sld2 domain (Xu and Liu 2009). Moreover, RECQ4 has strand annealing activity as demonstrated using the *E.coli* purified recombinant protein and the immuno-purifyed endogenous protein (Macris et al., 2006; Xu and Liu, 2009; Yin et al., 2004).

Apart from usual replication fork like structures, RECQ4 can unwind 61 bp blunt-end duplex DNA containing either a 12-nt bubble, a 4-nt bubble, or even without a bubble (Xu and Liu 2009).

2.4.4 Functional role of the RECQ4 protein

RECQ4 is implicated in different DNA metabolic processes, as suggested by three different genetic diseases associated with *Recq4* mutation. First, RECQ4 has been proposed to function in initiation of DNA replication. Consistently, the N-terminal of xRECQ4 shows sequence similarity to yeast Sld2 which has been shown to participate in DNA replication initiation. Its role in DNA replication is further supported by lethal phenotype of knockout mice, the cellular phenotype showing defect in DNA synthesis, and recent immunoprecipitation experiments showing that human RECQ4 is in association with replication complex proteins (Matsuno et al., 2006; Sangrithi et al., 2005; Xu et al., 2009a). Moreover, a recent study demonstrated that RECQ4 is essential for the assembly of CDC45-MCM-GINS (CMG) complex, thus initiating the DNA replication (Im et al., 2009).

Second, RECQ4 has been shown to participate in various DNA repair pathways. Petkovic *et al* reported that after etoposide treatment RECQ4 nuclear foci coincide with the foci formed by Rad51, a crucial protein functioning in homologous recombination (Petkovic et al., 2005). In addition, fibroblasts from RTS patients (RTS cells) are sensitive to ionizing radiation (Jin et al., 2008) and Kumata *et al* recently provided the evidence that RECQ4 participates in DSB repair in *Xenopus* egg extracts (Kumata et al., 2007). RECQ4's role in DSB repair pathway was further supported by a recent report from Wilhelm Bohr's group where they showed that RECQ4 participates in Nucleotide Excision Repair (NER). The authors of this study demonstrated that the complementation with functional RECQ4 could remove the UV lesions and rescued the UV sensitivity of RTS cells (Park et al., 2006). Third, Woo and Werner reported that RECQ4 plays a role in oxidative stress.

They observed that, upon oxidative stress, RECQ4 shifts to nuclear foci and interacts with PARP1 (Woo et al., 2006). Fourth, RECQ4 has also been reported to control S phase arrest. Cells defective in RECQ4 escaped from the S-phase arrest following UV treatment (Park et al., 2006). These data altogether suggest that RECQ4 is involved in DNA replication, distinct DNA repair processes, and possibly cell cycle arrest.

2.4.5 Regulation of the RECQ4 protein function

A) Post translational modifications

RECQ4 is acetylated both *in-vitro* and *in-vivo* by p300 and the acetylated lysine residues are found in the nuclear localization signal region. Acetylation of RECQ4 regulates the nuclear trafficking as the acetylated form of RECQ4 stays in the cytoplasm (Dietschy et al., 2009) suggesting that acetylation might also regulate the DNA repair function of RECQ4. RECQ4 is a phosphoprotein, CDK phosphorylates Sld2-like domain of RECQ4 and this phosphorylation modulates the interaction of RECQ4 with MCM10, thus suggesting that phosphorylation might also regulate the replication role of RECQ4
(Xu et al., 2009a). RECQ4 was also shown to be poly(ADP-ribosyl)ated *in vitro* (Woo *et al.,* 2006). When cells are treated with oxidative stress, RECQ4 interacts with PARP1 and gets poly(ADP-ribosyl)ated suggesting that this modification might also regulate the RECQ4 activity in DNA repair.

B) Protein - protein interactions

Two independent studies support a role of RECQ4 in DNA replication by showing that RECQ4 co-purifyies with the replication initiation complex proteins. Xu *et al* isolated the

complex containing MCM and GINS complex proteins through immuno-purification of FLAG tagged RECQ4. They also demonstrated that the association of RECQ4 with initiation complex is mediated through MCM10 (Xu, Rochette et al. et al., 2009a). Jun-Sub Im *et al*. used a bimolecular florescence complementation assays to demonstrate the existence of the same complex in HeLa cells. Furthermore, they observed that the stable association of CMG (CDC45,GINS,MCM) complex requires RECQ4 (Im et al., 2009) (Figure 2.10).

RECQ4 also interacts with PARP1 and PARP cn poly(ADP-ribosylate) RECQ4 suggesting that the interaction with PARP1 is essential to withstand the oxidative stress (Woo, Futami et al., 2006)

Furthermore, RECQ4 interacts with RAD51 *invivo* and co-localizes with RAD51 when the cells are treated with the DNA damaging agent etoposide, supporting a role of RECQ4 in DNA replication (Petkovic et al., 2005). RECQ4 interacts with UBR1 and UBR2, however RECQ4 is not ubiquitylated (Yin et al., 2004) and the functional significance of this interaction is not known yet.

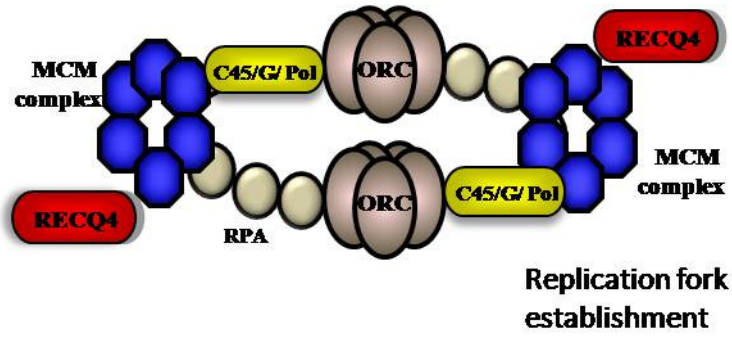


Figure 2.11: Potential role of RECQ4 and its interacting partners. RECQ4 interaction with the MCM complex, GINS, and CDC45 is essential for the establishment of replication forks.

2.5 RECQ1 helicase

RECQ1 helicase is the first RECQ helicase identified to be a human homologue of the *E.coli* RecQ helicase (Puranam and Blackshear, 1994). The cDNA cloning and FISH analysis localized the RECQ1 gene on the short arm of human chromosome 12 at 12p12 (Puranam and Blackshear, 1994). It encodes a 649 amino acid polypeptide with a predicted molecular weight of 73 kDa. The protein shares a high sequence similarity with *E.coli* RecQ and other human RecQ helicases. The expression of RECQ1 is significantly high in testes and ovaries (Olesen, 2007; Wang, 2003). At the cellular level, RECQ1 is found both in the cytoplasm and the nucleus, and it is ubiquitously expressed throughout the cell cycle. Though there is no human syndrome associated with RECQ1 deficiency, mutations in the RECQ1 gene are associated with testicular germ cell tumours (Suijkerbuijk et al., 1993).

2.5.1 Clinical manifestations of RECQ1 deficiency

A) Knockout mice

RECQ1 knockout mice was produced by replacing helicase domain IV and part of helicase domain V with PGKNeo of the targeting vector, Pjn52/RECQL. The resulting RECQ1 deficient mice did not show any apparent phenotypic differences compared with the wild type mice (Sharma et al., 2007).

B) Cellular phenotype

Cytogenetic analysis of the embryonic fibroblasts (MEF) derived from RECQ1 knockout mice revealed aneuploidy, suggesting a role of RECQ1 during segregation of chromosomes during mitosis. M-FISH analysis revealed increase in fragmented

chromosomes, chromatid breaks, translocation events, and sister chromatid exchanges. These cells accumulate DNA damages, evident by the increase in γH2AX and RAD51 foci formation. In addition, RECQ1 deficient cells are hypersensitive to ionizing radiation.

Interestingly, acute depletion of RECQ1 in HeLa cells resulted in reduced cell proliferation. Colony forming assays performed by Sharma et al., showed a significant reduction in both the size and number of colonies, when HeLa cells were inhibited for the expression of RECQ1. Along with the cell proliferative role, a cancer-specific role of RECQ1 was suggested based on two observations. First, RECQ1 silencing in cancer cells resulted in mitotic catastrophe and second, the expression of RECQ1 is upregulated in actively proliferating cells and also upon cellular transformation by EBV or SV40 T antigen (Sharma, Stumpo et al. 2007) .

2.5.2 Structural aspects of the RECQ1 protein

A) Domain architecture

The domain organization of RECQ1 closely resembles that of the bacterial RecQ with high similarity in helicase domain and in RecQ family specific zinc domain. However, the C-terminal domain which folds as Winged Helix (WH) domain is characterized by a β-hairpin loop that is longer than in the bacterial counterpart. The C-terminal domain also possesses nuclear localization signal (NLS), while the N terminal domain has been shown to be involved in the formation of higher order oligomers (Pike et al., 2009).

The truncated RECQ1, consisting of residues 49-616 (of 649) was crystallized with ADPand Mg^{2+} (Figure 2.11). Crystal structure of RECQ1 is similar to that of *E.coli* RecQ. However, the Winged Helix (WH) domain is positioned differently and the β-hairpin loop

of the WH domain is longer when compared to *E.coli* RecQ. The biochemical characterization of a truncated form of RECQ1 revealed that this β-hairpin loop plays an important role in DNA strand separation (Pike et al., 2009).

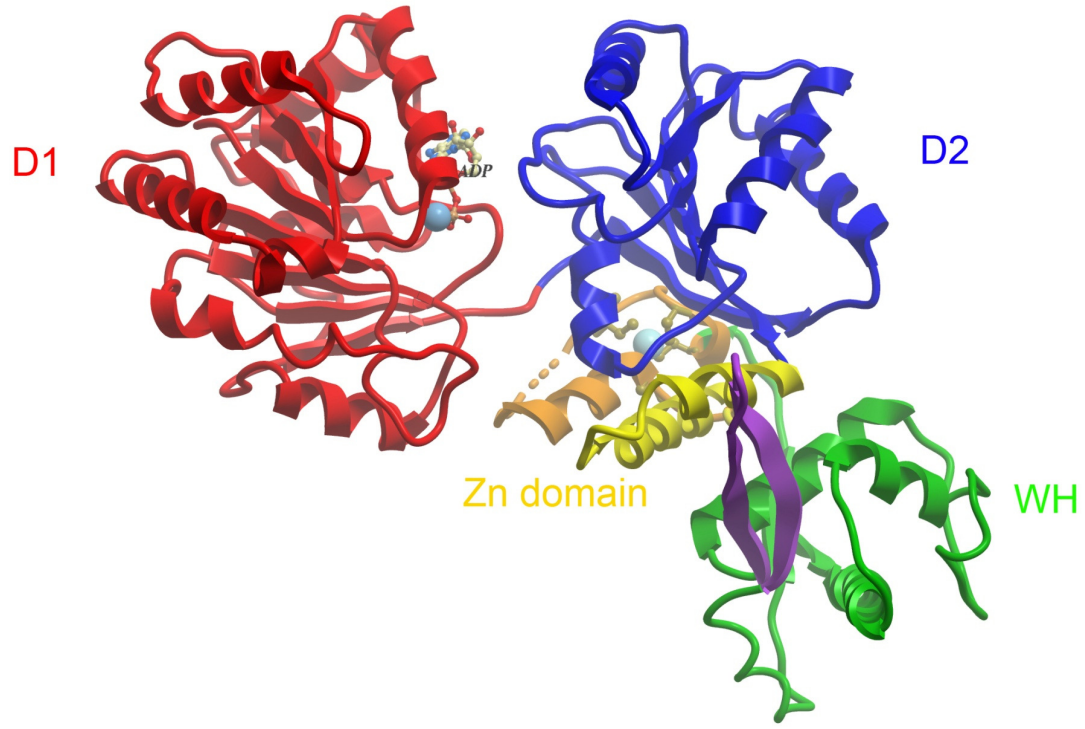


Figure 2.12: Crystal structure of human RECQ1. Ribbon representation of a single RECQ1 molecule, viewed from 3 perpendicular orientations. The subdomains are identified by color: Core helicase domain D1, red;core helicase domain D2, blue; zinc motif (ZnD), yellow;helical hairpin (HH), orange; WH domain, green; and the β-hairpin in purple. ADP is shown in spacefilling form (Pike et al., 2009).

C) Assembly state

ATP binding and ssDNA modulate two different quaternary states of RECQ1. These two different states are associated the unwinding and annealing activity of RECQ1 (Figure 2.12). RECQ1 forms higher-order oligomeric structures and these oligomers promote the

strand annealing acivity. On the other hand, ATP binding promotes the formation of smaller oligomers (dimers or monomers) and favours the unwinding reaction (Muzzolini et al., 2007).

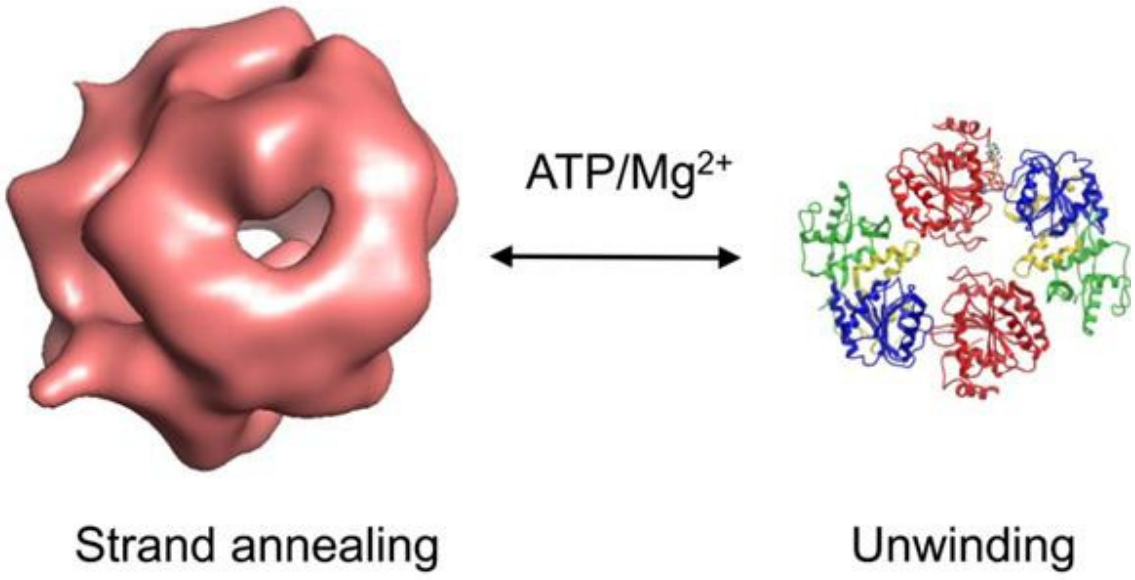


Figure 2.13 : Schematic representation of different quaternary structures of human RECQ1. Higher order oligomers promote strand annealing and smaller oligomers catalyze DNA unwinding.

2.5.3 Biochemical characteristics of the RECQ1 helicase

A) Enzymatic properties

The RECQ1 helicase is an ATP- and Mg^{2+}-dependent enzyme that unwinds DNA with a 3' to 5' polarity. RECQ1 also possesses a DNA strand annealing activity and the assembly state of RECQ1 regulates the unwinding and annealing activity (Muzzolini et al., 2007). RECQ1 can unwind a variety of DNA substrates including the conventional B-form duplexes. The higher order oligomers of RECQ1 are required to resolve synthetic HJ structures in an ATP dependent way. RECQ1 also unwinds D-loops which are the

intermediates of homologous recombination. The unwinding function of RECQ1 is distinct from that of BLM since RECQ1 cannot unwind G-quadruplexes, DNA-RNA hybrids and cannot catalyze fork regression or displace plasmid D loops lacking a 3' tail. However, RECQ1 is able to unwind HJ lacking an homologous core, unlike the BLM (Popuri et al., 2008).

2.5.4 Functional role of the RECQ1 protein

The increased load of DNA damage and the elevated sister chromatid exchanges in the RECQ1 defecient cells suggest that RECQ1 is involved in suppressing chromosomal instability (Sharma, Stumpo et al. 2007). A role of RECQ1 in DNA repair has been suggested by the ionic radiation sensitivity of RECQ1 defecient cells and by the interaction of endogenous RECQ1 with some DNA mismatch repair proteins (Doherty et al., 2005). However, the exact function of RECQ1 in the maintenance of genome stability is still unknown.

2.5.5 Regulation of the RECQ1 protein function

A) Post translational modifications

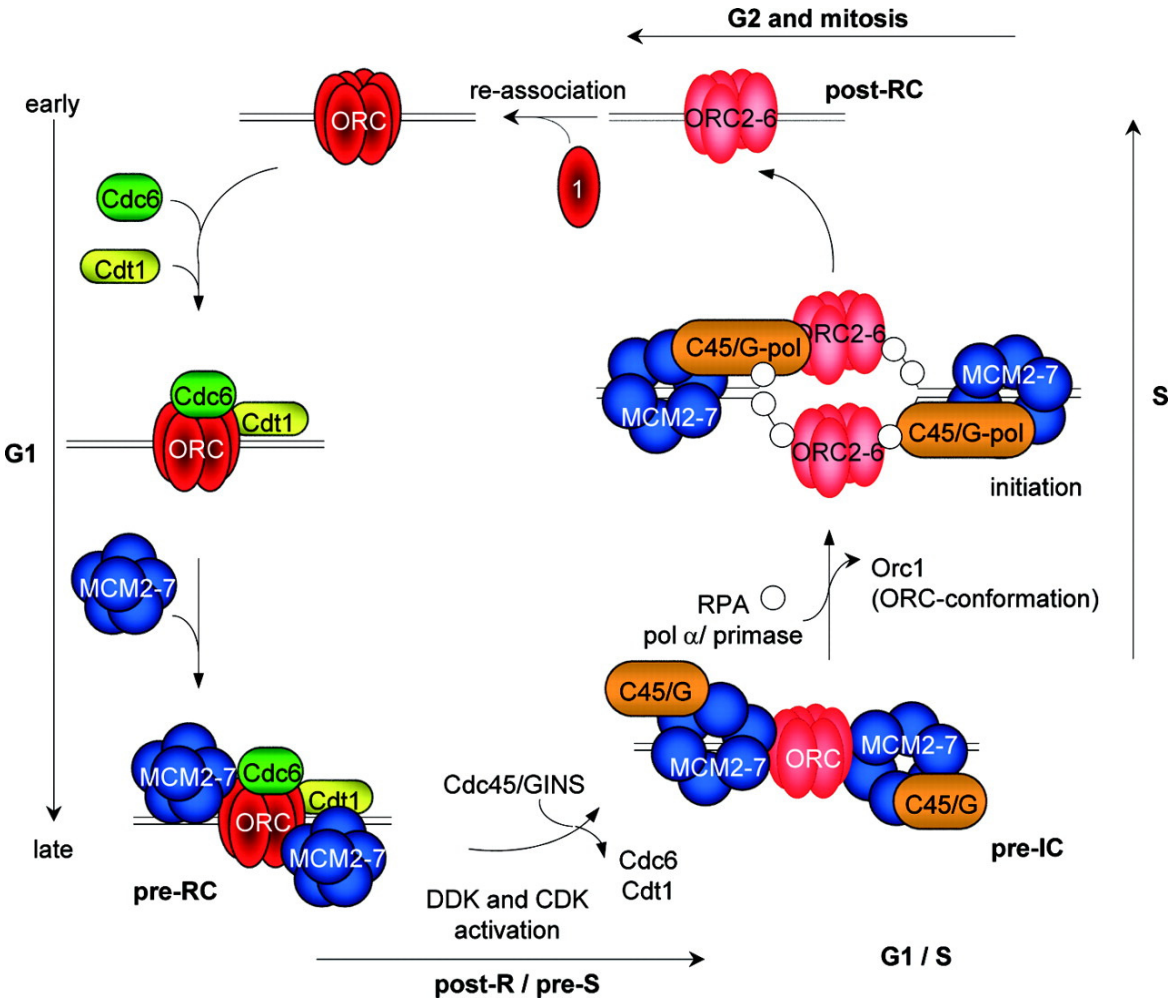
RECQ1 appears to be phosphorylated when the cells are exposed to ionic radiations (IR). This phosphorylation event was suggested to help RECQ1 in associating with chromatin when the cells are exposed to IR (Sharma and Brosh, 2007). However, the phosphorylating kinase and the specific role of phosphorylated RECQ1 remain elusive.

B) Protein - protein interactions

Several DNA repair proteins such as MSH2/6, MLHI-PMS2, EXO1 and RAD51 have been shown to associate with RECQ1 helicase by co-immunoprecipitation experiments. The functional role of these interactions is still unknown (Doherty et al., 2005; Sharma and Brosh, 2007). RPA also interacts with RECQ1 and this interaction is mediated by the RPA70 subunit. Co-immunoprecipitation experiments using human nuclear extracts confirmed that RECQ1 and RPA interact in cells. Importantly, RPA stimulates the ability of RECQ1 to unwind duplex DNA substrates. RPA stimulation of RECQ1 helicase activity increases the processivity of RECQ1. RECQ1 alone in unableto unwind DNA duplexes exceeding 100 bp, while it can unwind much longer duplexes of 500 bp in the presence of RPA (Cui et al., 2004).

3. DNA replication

DNA replication is a highly coordinated process which ensures efficient inheritance of the genetic information and accurate doubling of the cell. In eukaryotic cells, this event is initiated at particular sequences called origins of replication and this region is marked by the binding of the origin recognition complex (ORC). ORC acts as a landing pad and the pre-replicative complex proteins namely ORC, Cdc6p, Cdt1p, and MCM assemble as a multiprotein complex on ORC-defined sequences (Aparicio et al., 1997; Diffley et al., 1994; Tanaka et al., 1997). This loading is permitted by the low activity of cyclin dependent kinases (CDK) during the G1 phase of the cell cycle. An increase in CDK activity at the G1/S transition allows the binding of CDC45 to the MCM complex, followed by the binding of GINS proteins . Thus, pre-RC complex is converted into the initiation complex and starts unwinding. The high CDK activity during S and G2 phases prevents the re-formation of pre-RC until the end of mitosis (Aparicio et al., 1997; Nasmyth, 1996; Zou and Stillman, 1998). The unwound origins are stabilized in the single stranded state by the binding of RPA and the subsequent binding of DNA polymerase α and ε leads to the synthesis of daughter strand (Aparicio et al., 1997). The entire DNA replication process is tightly regulated along cell cycle to ensure the formation pre-RC during G1 phase and initiation complex just before the S phase. This process is monitored by check point mechanisms. In case of any block in DNA replication or DNA damage, a signal transduction pathway is activated resulting in a block of further initiation events in S phase and promoting the entry in mitosis (Nyberg et al., 2002)(Figure 2.13) .



. **Figure 2.14 : Model for eukaryotic DNA replication**.The origin recognition complex is assembled at origins in the early stages of G_1. Cdc6 and Cdt1 are required for the association of the Mcm2-7 protein family, resulting in the formation of the prereplicative complex (pre-RC). After activation of the DBF4-dependent kinase (DDK) and cyclin-dependent kinase (CDK) complexes, the transition from the pre-RC to the preinitiation complex (pre-IC) occurs at G_1/S and is characterized by the formation of the CMG complex (consisting of Cdc45p, Mcm2-7p, and GINS).Adapted from Gerhardt J et al.,2006.

Protein	Function
ORC complex proteins (Orc1, Orc2, Orc3, Orc4, Orc5, Orc6)	ATPase for DNA binding
Cdc6 Cdt1	ATPase helicase clamp loader
Geminin	Inhibits helicase clamp loader
MCM complex proteins (Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, Mcm7, Mcm8, Mcm9, Mcm10)	ATP ase helicase for unwinding/ elongation
CDK-kinase	Inhibits helicase loading to block re-replication.
DDK-kinase	Protein kinase for helicase activation and replisome loading
Cdc45	Helicase activation and replisome loading
GINS complex proteins (Psf1, Psf2, Psf3)	Helicase activation and replisome loading
Polα primase holoenzyme	Polymerase priming; replication of lagging strands
Polδ holoenzyme	Bidirectional replication
Polε holoenzyme	Bidirectional replication
Rfc1-5 complex	PCNA clamp loading
PCNA	Polymerase clamp loading
RPA1-3 complex	Coats ssDNA

Table 3.1: **Proteins involved in DNA replication and the functions associated with them.**

3.1 Human DNA Replication origins

Mammalian cells regulate the rate of DNA replication by activating or deactivating the starting points of DNA replication called “Origins of DNA replication”. This regulation is achieved through the cellular phase-specific assembly and reorganization of multi protein complexes on the DNA sequences marked as origins by the ORC. Nascent DNA analysis and 2-D gel electrophoresis helped initially to identify the replication origins. However, recent studies using origin trapping assay, high throughput assays combining nascent strand abundance analysis with microarray data and genome wide high resolution maps based on hybridization of short nascent strands (SNS) on DNA microarrays covering The Encyclopedia of DNA Elements (ENCODE), helped to identify almost 283 replication origins in 1% of the human genome (Cadoret et al., 2008; Giacca et al., 1997; Giacca et al., 1994; Lucas et al., 2007; Todorovic et al., 2005).

There are several features that decide the particular locus of the DNA to function as origins. Unlike *S. cerevisiae*, there are no consensus sequences in humans that function as origins; however chromatin conformations, chromatin hyperacetylations which recruits ORC complex, presence of CpG islands, presence of chromosomal scaffold attachment sites, presence of transcriptional regulatory elements (ORC is localized to specific sequences that overlap with RNA pol II binding sequences) probably decide the location of DNA replication origins (Falaschi et al., 2007; Paixao et al., 2004). The firing of replication origins are tightly connected with “ S” phase of the cell cycle. Even though several origins have characteristic early S phase firing while others have mid/late S phase firing, the firing occurs only in the S phase of the cell cycle (Gilbert 2001).

3.2 Human Lamin B2 replication origin

Although 283 replication origins are identified so far, very few origins have been characterized in detail. The human lamin B2 replication origin, which is located downstream of the human lamin B2 gene is one of the best characterized so far. Competitive PCR analysis of the nascent DNA library from HL-60 cells was used to localize a genomic segment with "origin activity" in the G-negative p13.3 band of chromosome 19, at the 3' portion of the ORF for lamin B2 and upstream of the promoter of TIMM13 (Biamonti et al., 1992). This origin fires immediately at the onset of S phase and its activity has been reported in various cell lines including myeloid, epithelial, neuronal, fibroblasts, and primary peripheral lymphocytes (Kumar et al., 1996). The *in vivo* foot printing experiments revealed a footprint around replication initiation sites and these protein DNA interactions at the origins underwent changes during the cell cycle. There was no footprint observed in G0 phase cells but there was a footprint covering 100bp during G1/S and this footprint shrinks to 70 bp during S phase and completely disappears during mitosis. Of note, there were no such footprints in non proliferating cells. The footprinting experiments combined with ligation mediated PCR analysis showed that a 1.2-kb fragment of the lamin B2 replicon functions as a replicator even if it is integrated at ectopic positions of the genome.YThis study precisely showed that a 290 bp region containing the OPR (Origin protected Region) decides the replicator activity and is influenced by the nearby CpG island (Paixao, Colaluca et al., 2004). Successively, two precise nucleotides of the lamin B2 origin from which bidirectional DNA synthesis starts have been identified (Abdurashidova et al., 2000). Several proteins have been shown to interact with the lamin B2 replication origin. In particular, the

interaction of the pre-replication complex proteins and initiation complex proteins with the lamin B2 origin have been reported (Abdurashidova et al., 1998; Dimitrova et al., 1996; Stefanovic et al., 2003).

3.3 Replication fork stability

At each fired origin, two sister replication forks (RFs) are established that move away from the origin as the parental DNA duplex is unwound by the action of DNA helicases. The RFs can be hindered by structural and chemical alterations in the DNA. When one RF is terminally blocked or arrested, firing of dormant or nearby origins ensures that replication is complete (Doksani et al., 2009). Checkpoints are cellular surveillance and signalling pathways that detect DNA lesions, such as ssDNA or DSBs, and then boost a DNA damage response that ensures replication fork (RF) stabilization (Branzei and Foiani, 2007). Ataxia telangiectasia mutated (ATM) dependent pathway and ATM- and Rad3-related protein (ATR) dependent pathway are key checkpoint mechanisms that safeguards the replication fork. ATM responds mostly to DSBs (Costanzo et al., 2000) and is recruited to the damaged replication fork through the MRN complex (Uziel et al., 2003) which is composed of MRE11, RAD50, and NBS1. The MRN complex proteins activate ATM (Lee and Paull, 2004) leading to phosphorylation of the downstream cellular targets p53 and Chk2 that inhibita the further firing of origin by degrading CDC25 (Costanzo et al., 2004; Falck et al., 2005; Jazayeri et al., 2008; Lee and Paull, 2005; Uziel et al., 2003). On the other hand, ATRresponds mostly to single-strand DNA gaps coated with RPA at stalled RFs (Costanzo et al., 2003). Similar to ATM activation,

ATR phosphorylates downstream proteins and induces the degradation of CDC25 thereby inhibiting firing of the origins.

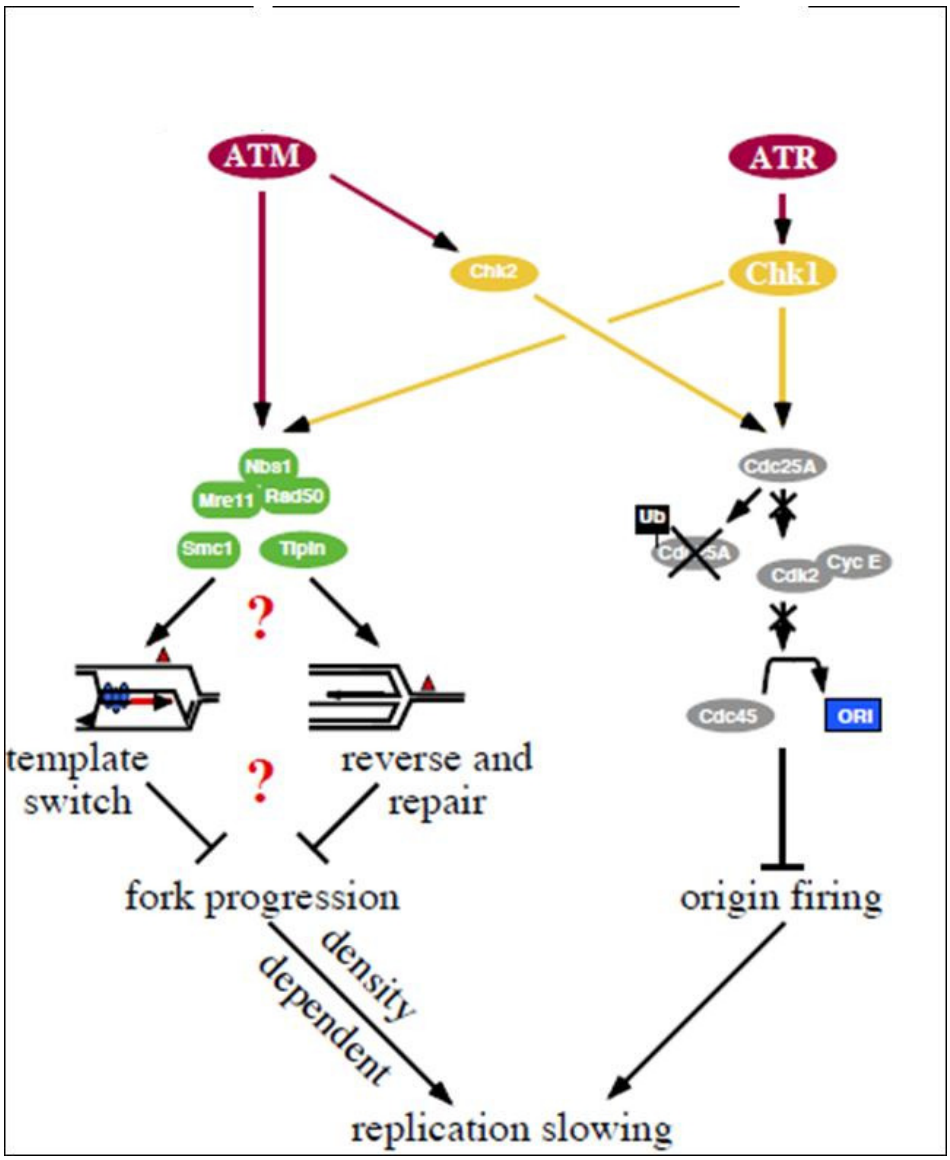


Figure 2.15 : Maintenance of active replication forks by checkpoint system. ATM or ATR pathway is activated to slowdown the progression of damaged replication forks and to activate the dormant or nearby origin.

4. RECQ helicases in DNA replication

DNA replication is one of the key processes where any defect could potentially lead to genome instability. Several lines of evidence suggest that RecQ helicases play an important role in DNA replication control (Bachrati and Hickson, 2008; Chu and Hickson, 2009). In particular, RecQ helicases are thought to facilitate replication by preserving the integrity of stalled replication forks and by remodeling or repairing damaged or collapsed forks to allow the resumption of replication. Consistent with these ideas, several investigators have shown that primary fibroblasts from BS, WS and RTS patients, and RecQ5- deficient mouse embryonic fibroblasts show differential hypersensitivity to agents that perturb DNA replication (Davalos et al., 2004; Dhillon et al., 2007; Hu et al., 2009; Jin et al., 2008). Moreover, BLM and WRN are recruited to DNA replication forks after replicative stress, and DNA fiber track analyses have shown that both BLM and WRN are required for normal fork progression after DNA damage or replication arrest (Constantinou et al., 2000; Davies et al., 2007; Sakamoto et al., 2001; Sidorova et al., 2008). Biochemical and functional data indicate that RecQ helicases may act directly on the replisome or replication fork. For example, several RecQ helicases have been shown to interact physically or functionally with core replication components such as proliferating cellular nuclear antigen (PCNA), replicative and translesion synthesis DNA polymerases, human replication protein A (RPA) and DNA topoisomerases (Brosh et al., 2000; Cui et al., 2004; Kamath-Loeb et al., 2000; Lebel et al., 1999; Rodriguez-Lopez et al., 2003; Selak et al., 2008). The conserved interaction of WRN and BLM with FEN1

nuclease suggests a role of these two helicases in Okazaki fragment maturation during lagging strand replication (Sharma et al., 2004a; Sharma et al., 2004b).

RecQ helicases may also act on replication intermediates to facilitate the completion of replication. For example, BLM, WRN, and RECQ5 have all been shown to promote the regression or remodeling of model stalled forks *in vitro*, a reaction that could facilitate replication restart (Kanagaraj et al., 2006; Machwe et al., 2006; Ralf et al., 2006). In particular, BLM in conjunction with DNA toposiomerase III and other two accessory proteins, RMI-1 and RMI-2, has been shown to catalyze the resolution of double Holliday junction recombination intermediates to generate non-crossover products. This 'dissolution' reaction could play an important role in the error-free recombinational repair of damaged or stalled forks during S-phase (Singh et al., 2008; Xu et al., 2008). WRN also appears to promote error-free repair by contributing to the resolution of gene conversion events to generate non-crossover products (Saintigny, Makienko et al., 2002). In line with the above observations, WRN and BLM can be found associated with replication foci or other DNA damage response proteins in damaged cells. In contrast, in unperturbed cells, a majority of each protein is found in the nucleolus (WRN) or associated with PML bodies (BLM) (Bischof et al., 2001; Marciniak et al., 1998). RECQ4 has also been implicated in DNA replication. Recent studies have shown that hypomorphic mutants of the *D. melanogaster* homolog of human RECQ4, DmRECQ4, have reduced DNA replication-dependent chorion gene amplification (Wu, Capp et al., 2008). These findings are thus consistent with a postulated role for *X. laevis* RECQ4 (XRECQ4) in the initiation of DNA replication (Matsuno et al., 2006; Sangrithi et al., 2005). The N-terminus of XRECQ4 bears homology to the N-termini of the yeast

proteins Sld2 (*S. cerevisiae*) and DRC1 (*S. pombe*), which in association with budding yeast Dpb11 and the fission yeast homolog Cut5/Rad4 play a central role in the establishment of DNA replication forks (Masumoto et al., 2002; Noguchi et al., 2002; Wang and Elledge, 1999). Consistently, the N-terminus of XRECQ4 has been shown to interact with the *X. laevis* variant of Cut5, and XRECQ4 depletion severely perturbs DNA replication initiation in *X. laevis* egg extracts (Matsuno et al., 2006; Sangrithi et al., 2005). The notion that the function of XRECQ4 is evolutionarily conserved in mammals is supported by the observations that the human protein can complement its *Xenopus* counterpart in cell free assays for replication initiation, and that depletion of human RECQ4 inhibits cellular proliferation and DNA synthesis (Matsuno et al., 2006; Sangrithi et al., 2005). Moreover, deletion of the N-terminal region of mouse RECQ4 has been shown to be embryonic lethal (Ichikawa, Noda et al.et al., 2002). These observations suggest that vertebrate RECQ4 might be a functional homolog of Sld2, although its precise function during replication initiation and progression is not known. Recent reports, indicate that human RECQ4 interacts with the MCM replicative complex during replication initiation, and that this interaction is regulated by CDK phosphorylation of RECQ4 (Xu, Rochette et al., 2009a).

RECQ1, the most abundant of the human RecQ helicases, has been demonstrated as an integral component of pre-replication complex required for the Kaposi sarcoma-associated herpes virus replication. However, the role of RECQ1 in human DNA replication has not been investigated in detail..

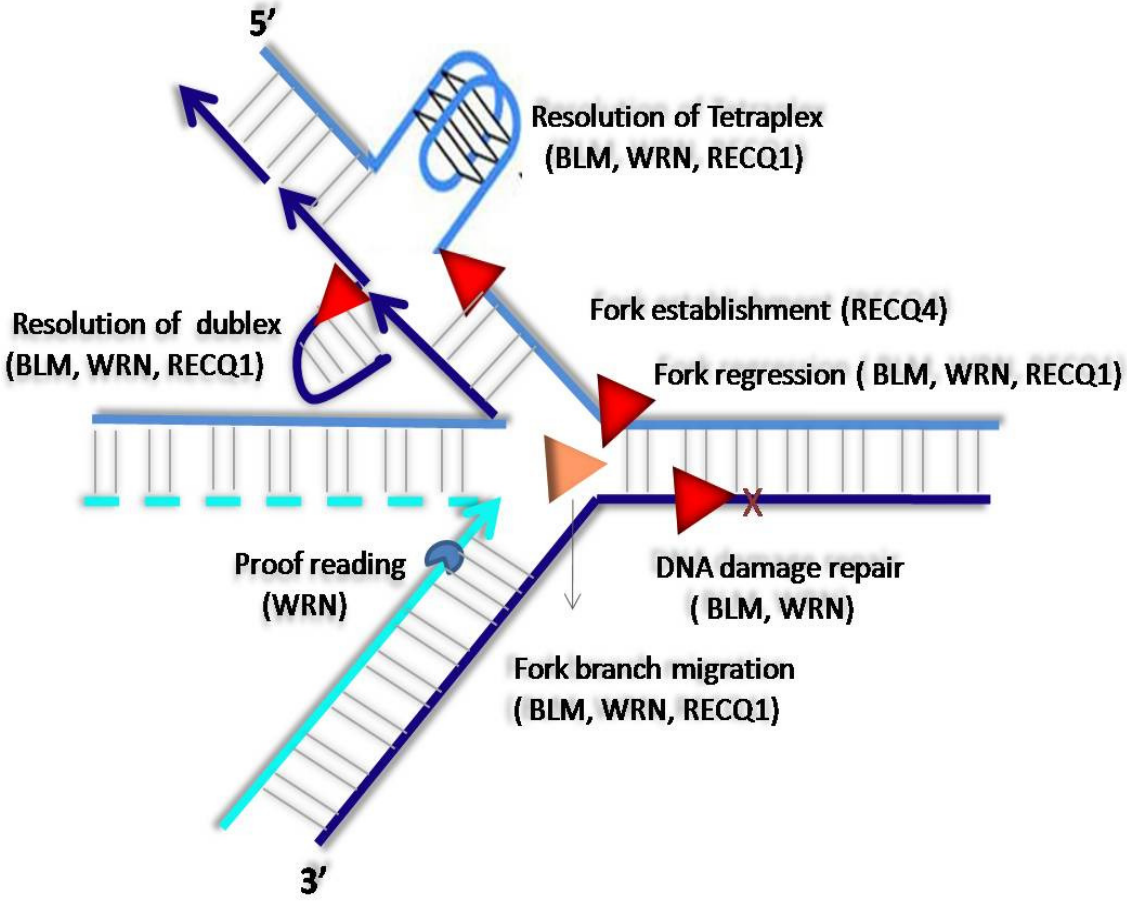


Figure 2.16 : Possible Role of RecQ helicases in DNA replication. RecQ helicases have been proposed to function in several steps of DNA replication such as fork establishment, fork regression, proof reading, resolution of dublex and tetraplex.

Aim of the thesis

Although RecQ helicases have shown to be involved in DNA replication, the exact roles of the five human RecQ enzymes in this process is still unclear. In order to better delineate the roles of human RecQ helicases in DNA replication, I investigated the *in vivo* interactions of all five human RecQ enzymes with three well-characterized human DNA replication origins by quantitative chromatin immunoprecipitation (ChIP) assays. I also determined how cell proliferation, DNA synthesis, nascent-origindependent DNA synthesis, chromatin binding of replication proteins, origin firing frequency, and replication fork rates were altered by depleting specific human RecQ helicase proteins.

3A. MATERIALS AND METHODS

3.1. Antibodies.

Polyclonal antibodies against RECQ1 (BL2074) and WRN (NB 100-471) were purchased from Bethyl Laboratories and Novus Biologicals, respectively. Polyclonal antibody raised against residues 60-111 of human RECQ4 was produced in Weidong Wang's laboratory as described (Yin et al., 2004). Polyclonal antibodies against BLM and RECQ5 were, respectively, generous gifts from Ian Hickson (Wu et al., 2001) and Pavel Janscak (Kanagaraj et al., 2006). Anti-Orc2 (3B7) antibodies were from MBL. Polyclonal antibodies anti-ORC1 were produced and purified by immunization of rabbits with a His-tagged Orc1 250-480 aa polypeptide (Todorovic et al., 2005). Cdc6 (N-19), MCM3 (N-19), MCM4 (H-300), Cyclin E (M-20), Cyclin A1 (H-432), PCNA (F-2), antibodies were from Santa Cruz Biotechnologies. RPA (BL915) antibody was purchased from Bethyl Laboratories. Anti-α-Tubulin (B-5-1-2) antibody was from Sigma. Anti-p84 (3F10) and rat anti-CldU/BrdU (BU1/75(ICR1)) antibodies were from Abcam. Mouse anti-IdU/BrdU (#347580) and anti-BrdU-FITC (#347583) antibodies were from BD Biosciences. Anti-rat Alexa 594-conjugated (A11007) and anti-mouse Alexa 488-conjugated (A11001) were from Molecular Probes.

3.2. Oligonuceoltides.

All oligonucleotides were chemically synthesized and purified by reverse-phase high pressure liquid chromatography (RP-HPLC) (Sigma-Aldrich, Suffolk, UK), and were

resuspended in Tris buffer (10 mM Tris-HCl, pH 7.5). Oligonucleotide sequences are reported in Table 3A.1.

Primer set	Sequence (5'→3')	Position (NCBI sequence)	Annealing temp (°C)
Real Time PCR			
B48 RT For	CTCCACCCCCAAGGAAAAAG	2368081-2368062	60
B48 RT Rev	GGCAGGGTCCCATGCA	2368005-2368020 (NT_011255.14)	
B13 RT For	CCCCAGGGAGTAGGTTGTGA	2363991-2363972	60
B13 RT Rev	TGTTATTTGAGAAAAGCCCAAAGAC	2363892-2363916 (NT_011255.14)	
17 RT For	AGCCAAGGTGCCTCTTTCAG	33833183-33833202	60
17 RT Rev	GTCCGGGAGGAGCAGACA	33833240-33833223 (NT_034772.5)	
21 RT For	TGTACAAATGAAGTGCACAAATCTTAAG	33834440-33834467	60
21 RT Rev	CCTGGGTGCCAGTTATTTATATAGG	33834522-33834498 (NT_034772.5)	
23 RT For	GTCCACCTCACTAATGCAGACAAT	33836142-33836165	60
23 RT Rev	AGAGAAGCAGGAAGGGCTTAGAGA	33836213-33836193 (NT_034772.5)	
BG-9 For	TGTGTTCACGACTGACATCACCG	52790-52812	62
BG-9 Rev	GCTGGGCTTCTGTTGCAGTAGGG	52879-52857 (U01317.1)	
BG-3 For	CTGCCGTTACTGCCCTGTGGG	62215-62235	62
BG-3 Rev	ACCAACCTGCCCAGGGCCTC	62284-62265 (U01317.1)	
Competitive PCR			
B48 F	TAGCTACACTAGCCAGTGACCTTTTTC	2368042-2368068	56
B48 R	GACTGGAAACTTTTTTGTAC	2368210-2368191 (NT_011255.14)	
B13 F	CCTCAGAACCCAGCTGTG	2363655-2363638	56
B13 R	GCCAGCTGGGTGGTGATAGA	2363490-2363509 (NT_011255.14)	

Table 3.1. Oligonucleotides used for real time PCR and competitive PCR analysis of the Lamin B2, GMCSF and beta-globin origins.

3.3. Cell culture, synchronization and cell-cycle analysis.

T98G/HeLa and IMR-90 cell lines were maintained in DMEM and MEM media, respectively, supplemented with Glutamax (Life Tecnologies) and 10% (v/v) FBS (Life Technologies). HEK293 cells containing stably expressing strep-hemagglutinin double-tagged (SH) RecQ helicases were grown in DMEM media with 100µg/ml of hygromycin and 15µg/ml of blasticidine. K562 cell lines were instead maintained in RPMI media

supplemented with Glutamax and 10% (v/v) FBS. T98G cells were synchronized by serum starvation as previously reported (Paolinelli et al., 2009; Takahashi et al., 2000) or by drug arrest with 0.8 mM mimosine (Sigma). HeLa and K562 cells were synchronized with 0.8 mM mimosine. Synchronized cells were then analyzed for their cell cycle profile (DNA content) by propidium iodide staining (Sigma) and flow cytometry on a FACS Calibur (Becton Dickinson). Cell cycle profile distributions were determined with the Modfit LT 3.0 software. BrdU incorporation experiments were performed on transiently siRNA transfected cells 72 h post-transfection. Cells were pulsed for 1 h with BrdU (Sigma, final concentration 10 μM), and BrdU-positive cells were detected by using a mouse anti-BrdU-FITC primary antibody followed by an anti-mouse Alexa 488-conjugated secondary antibody. Cells were collected and analyzed by flow cytometry on a FACSCalibur (Becton Dickinson) to simultaneously determine the cell-cycle profile (DNA content) by incorporation of propidium iodide and the S-phase cell population by incorporation of BrdU. Cell-cycle profile distributions were determined with the CellQuestPro and Modfit LT 3.0 software. DNA replication perturbation was achieved with 2 m M hydroxy urea for 20 hours.

3.4. Chromatin immunoprecipitation assays.

Cells were fixed by adding formaldehyde (Fluka) directly to the cell culture medium at 1% final concentration. Cross-linking was allowed to proceed for 7 min at 37°C and was stopped by the addition of glycine (Sigma) at a final concentration of 125 mM. Cells were washed and digested with 125 U micrococcal nuclease S7 (Roche) for about 1-2 x 10^7 cells, at 37°C for 15 min. Reactions were stopped by adding EDTA (20 mM final). Cells were washed and resuspended in HNNG buffer (20 mM HEPES pH7.5, 250 mM

NaCl, 0.5% NP40, 10% glycerol, 1 mM PMSF) supplemented with protease inhibitors, then chromatin was further sheared by sonication to generate DNA fragments of less than 0.5 kb. The resulting sonicated lysates were then used for immunoprecipitation with anti-RECQ1, -BLM, -WRN, -RECQ4, -RECQ5, -ORC2, -ORC1, -MCM3, -CDC6 antibodies. A rabbit IgG was used as a negative control. Immunocomplexes were collected with protein A beads, washed sequentially with HNNG buffer and HLNG buffer (15 mM HEPES pH7.5, 250 mM LiCl, 0.5% NP-40, 10% glycerol, 1 mM PMSF), resuspended in TE buffer and treated with 100µg /ml RNase A (Roche) for 30 min at 37°C. Samples were then incubated for 1 h at 56°C with 0.5 mg/ml Proteinase K (Sigma) and for 15 h at 65°C to revert the crosslink. DNA was extracted with phenol:chloroform:isoamyl alcohol 25:24:1 (Invitrogen), ethonol precipitated and resuspended in 10mM Tris-HCL, pH 7.5.

3.5. Real-time PCR.

The abundance of specific immunoprecipitated DNA sequences prepared as described above was determined by quantitative real-time PCR and/or competitive PCR. Sequence-specific primers for real-time PCR analysis were designed to amplify and detect origins as well as non-origin control regions near the human lamin B2,GM-CSF1/2, and beta-globin replication origins (Table 1). Real-time PCR experiments were carried out in a total volume of 10 µl with 1 µl of genomic, nascent or immunoprecipitated DNA and 800 nm of primers using IQ SYBR Green Supermix (Bio-Rad) on CFX96 Real-Time System (Bio-Rad). The amplification conditions for all primer sets are shown in Table 2. Genomic DNA (100, 10, 1 ng) from crosslinked cells (input) was used to generate the standard curves needed for quantification of the PCR

products. A negative control without template DNA was included with each set of reactions. PCR products were also resolved on 1% agarose gels, visualized with ethidium bromide, no extraneous bands were generated with any of the primer sets. Enrichments were calculated using the $\Delta\Delta$Ct method**.**

3.6. Competitive PCR.

The abundance of two different sequences was quantified using competitive PCR. The sequences chosen for amplifcation are defined as B48 and B13, the B48 corresponding to the lamin B2 ori, whereas B13 is displaced by 5 kb. A single competitor molecule was used for both markers. The core of the molecule is a 110 bp region derived from the β-globin gene harboring a 20 bp insertion. The core competitor was built directly from the amplification products obtained by the overlap extension method (Higuchi et al., 1988; Ho et al., 1989). A set of four primers was synthesized for a region of the β-globin gene. Two external primers (PCO3 and PCO4) were synthesized together with two internal primers (PCO/+1 and PCO/+2) consisting of a common 5' tail of 20 nucleotides linked to the specific sequences complementary to genomic targets on the 3' end. Pairs of internal and external primers were used in two separate PCR reactions for the construction of two intermediate products. These intermediate products were eluted from polyacrylamide gel, mixed, denatured and annealed. Subsequently, after one round of extension, the hybrid product was amplified using the external primers to obtain the core competitor molecule, which has the same sequence as the β-globin genomic target, except for the addition of 20 nucleotides in the middle. The forward and reverse primers spanning the lamin B2 origin area (SB12, SE10, B13, B48 see Giacca et al., 1994,1997), arranged in a head-to-tail fashion, were joined to the core molecule by

PCR amplifications, using chimerical primers. This approach allowed us to use a single competitor for the quantification of the relative abundance of different PCR markers. The competitor was quantified in competitive PCR experiments against a known amount of DNA molecules harboring the lamin B2 origin area. The PCR cycle profile was as follows: denaturation at 95°C, annealing at 56°C and extension at 72°C, time for each step was 30 s; 35 cycles were performed with 1 U of GO Taq polymerase (Promega). PCR amplified products were resolved on acrylamide gels, stained with ethidium bromide , intensities of the bands were quantified using ImageJ software and the ratio of competitor, genomic DNA were plotted in excel.

3.7. RNA interference.

Cells were transiently transfected for 72 h with a SMART pool siRNA against RECQ1 (NM_032941) or RECQ4 (NM_004260) (Dharmacon) at final concentration of 100 nM by HiPerFect reagent (Qiagen) following the manufacturer's instructions. RNAi control experiments were performed using a duplex siRNA against luciferase (Dharmacon). RECQ1 depletion was also obtained by transfection with the pcDNASup expression vector encoding a short harpin RNA against RECQ1 mRNA (target sequence: 5'-GAGCUUAUGUUACCAGUUA-3') using the calcium-phosphate method. The RECQ1 downregulated clones were selected using G418 (0.5 mg/L) after 48 h of transient transfection followed by 2 weeks of selection.

3.8. Colony forming assays.

Colony forming assays were conducted as previously described (Franken et al., 2006) in six-well plates using glioblastoma T98G cells that had been transfected with RNAi pools

targeting RECQ1, RECQ4 or luciferase as a negative control. Transfected cells were seeded at different dilutions (200, 400, 800 cells/well). Colonies formed after growth for at least 7 days were counted by VersaDoc 4000 imaging system (BioRad). The colony forming capacity was calculated as the average ratio of the number of formed colonies to the number of cells seeded, expressed as a percentage.

3.9. Nascent DNA quantitation.

Total DNA was extracted from RECQ1, RECQ4 or luciferase RNAi- transfected T98G cells by standard procedures (Giacca et al., 1997), were denatured by a 10 min incubation in boiling water, and size-separated (300 mg per gradient) on 35 ml of 5–30% neutral sucrose gradients for 20 h at 26 000 r.p.m. in a Beckman SW28 rotor at 20^{o}C. Fractions of 1 ml each were collected from the gradient and quantification of the abundance of the different origin and control sequences in gradient fractions was performed by quantitative real-time PCR analysis as described above.

3.10. Microfluidic-assisted replication track analysis (maRTA).

Origin firing frequency and replication fork progression rates were determined by using a recently described microfluidic-assisted replication track analysis protocol (maRTA) (Sidorova et al., 2009). In brief, RECQ1-, RECQ4- or mock-depleted T98G glioblastoma cells were labeled for 40 min each with 100 μM IdU followed by 100 μM CldU, then collected by trypsinization and used to prepare agarose plugs as previously described (Sidorova et al., 2009). High molecular weight labeled DNA was isolated from cells embedded in agarose by brief heating to 75°C to melt agarose, followed by agarase digestion. The resulting high molecular weight DNA was then loaded by capillary tension

into microchannels to uniformly stretch and capture DNA on glass coverslips for immunostaining and fluorescence microscopy (Sidorova et al., 2009). Origin firing efficiency was determined by counting the fraction of origin firing events among all active replication events (i.e. ongoing forks and converging forks). Replication elongation efficiency was determined by measuring the mean length of first label replication tracks in double-labelled tracks that were scored in order to unambiguously analyze active/ongoing forks. Track lengths were measured in digital images of tracks using the AxioVision software package (Carl Zeiss). Three replicate samples of RECQ1-, RECQ4- or mock-depleted cells were analyzed for each determination, where from 250 to 450 replication tracks were measured in each sample.

3.11. Biochemical fractionation.

Biochemical fractionation was performed following a previously described procedure (Mendez and Stillman, 2000). To prepare total cell extracts, tissue-cultured cells were harvested by centrifugation, washed in PBS, and resuspended in HNNG buffer followed by sonication for 15 s in a Tekmar CV26 sonicator set at 25% amplitude samples were quantified by bradford reagent (Biorad) and denatured by heating 100^0c for 5 min in Laemmli buffer. To prepare nuclear extracts, the cells were washed once with PBS and lysed by Dounce homogenization in hypotonic buffer (20 mM Hepes-KOH [pH 8.0], 5 mM KCl, 1.5 mM $MgCl_2$, 5 mM Na butyrate, 0.1 mM dithiothreitol [DTT]). To isolate chromatin, cells were resuspended (4×10^7 cells/ml) in buffer A (10 mM HEPES, [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 5 µg of aprotinin per ml, 5 µg of leupeptin per ml, 0.5 µg of pepstatin A per ml 0.1 mM phenylmethylsulfonyl fluoride). Triton X-100 (0.1%) was added, and the cells were

incubated for 5 min on ice. Nuclei were collected in pellet 1 (P1) by low-speed centrifugation (4 min, 1,300 × *g*, 4°C). The supernatant (S1) was further clarified by high-speed centrifugation (15 min, 20,000 × *g*, 4°C) to remove cell debris and insoluble aggregates. Nuclei were washed once in buffer A, and then lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease inhibitors as described above). Insoluble chromatin was collected by centrifugation (4 min, 1,700 × *g*, 4°C), washed once in buffer B, and centrifuged again under the same conditions. The final chromatin pellet (P3) was resuspended in HNNG buffer and sonicated for 15 s in a Tekmar CV26 sonicator using a microtip at 25% amplitude .

3.12. Immunoprecipitation.

Immunoprecipitation experiments were performed as described above for ChIP experiments. After washing with HLNG buffer, the sample was boiled for 30 min in SDS-PAGE sample buffer to reverse formaldehyde cross-links. Protein samples were loaded and run on 8% or 10% SDS–PAGE gels prior to transfer onto PVDF or nitrocelluose membranes (Amersham) for Western analysis and detection by ECL (Amersham).

3.13. Western blot analysis.

Western blot analysis was carried out according to standard protocols. Briefly, the indicated amounts of whole cell extracts, chromatin-enriched fractions, cross-linked immunoprecipitated samples were resuspended in SDS loading buffer (50mM Tris–HCl, pH 6.8, 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 10 min and loaded onto a 5% stacking/8% separating SDS-PAGE gel. Following electrophoresis and transfer onto a PVDF membrane, the membrane was immunoblotted with the

indicated primary and corresponding HRP-conjugated secondary antibodies. Proteins were visualized using the enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham Biosciences, Arlington Heights,IL, USA).

4. RESULTS

Chapter 1 : Association of RecQ helicases with human DNA replication origins.

4.1.1 Chromatin immunoprecipitation of RecQ helicases

To investigate the *in vivo* association of different human RecQ helicases with replication origins, I performed Chromatin Immunoprecipitation (ChIP) experiments. First, the efficiency of antibodies to immunoprecipitate the crosslinked proteins was tested by Western blot analysis after immunoprecipitation. Briefly, anti-RecQ helicases antibodies were incubated with the formaldehyde treated cell extracts and the immuno-complex was collected with protein A agarose beads and subjected to repeated washes, DNase treatment and de-crosslinking. The de-crosslinked immuno complexes were run on SDS page and probed with specific anti-RecQ antibodies. Western blot analysis confirmed the successful immunoprecipitation of all human RecQ helicases and the positive control ORC2 (fig.4.1.1). No specific pull down was observed with anti-IgG antibodies.

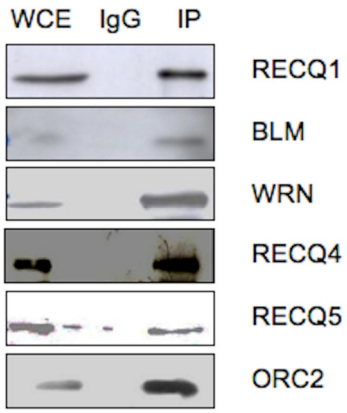


FIG 4.1.1: Efficiency of anti RecQ antibodies. Western analysis after immunoprecipitation of the cross-linked material with specific antibodies against the five human RecQ proteins. ORC2-specific immunoprecipitation served as a positive control, and rabbit IgG immunoprecipitation served as a negative control. Whole T98G cell lysates (WCL) were used to confirm the endogenous expression of the five RecQ helicases.

4.1.2 Interaction of RecQ helicases with the human lamin B2 replication origin

Following the confirmation that all anti-RecQ helicases antibodies could successfully immunoprecipitate their target proteins, I investigated the *in vivo* association of different RecQ helicases with the well-characterized human lamin B2 replication origin by ChIP and quantitative real-time PCR analysis. The lamin B2 replication origin is located on chromosome 19q, in the 3'- end of the lamin B2 gene and upstream of the promoter of the TIMM13 gene (Fig.4.1.2A). The ChIP experiments were initially done using asynchronous T98G human glioblastoma cells and then repeated with a normal human lung fibroblast cell line (IMR-90) to demonstrate that my results were not cell type specific. Asynchronously growing T98G/ IMR-90 cell line were treated with formaldehyde and the crosslinking reaction was quenched with glycine. Cross-linked cells were digested with with MNase to limit the size of DNA to ≤ 500bps, thus reducing unspecific background. The cells were lysed and sonicated. Anti-RecQ antibodies were added to the whole cell lysates to immunoprecipitate respective proteins. The efficiency of the immunorecipitation reactions was confirmed using the ORC2 antibody as positive control and IgG as negative control. After reversal of cross-links, the immuno-complex associated DNA was used for quantitative real-time PCR to determine the amount of origin-containing DNA bound by the immunoprecipitated protein (region B48 of the lamin B2 origin) compared with an adjacent region that does not contain the origin sequence (region B13 for the lamin B2 origin) (Fig. 4.1.2A). Genomic DNA prepared directly from crosslinked cells was used to construct the standards for real time PCR calibration. DNA purified from negative control IgG was used to fix the threshold. Analysis of the real time data was performed through $\Delta\Delta$Ct method where the

amplification of both the samples (B48 and B13) are normalized, then the given difference (delta-ct) is subtracted from the calibrator sample resulting in the delta-delta-ct value**.** Results obtained from three independent analyses were shown as a fold enrichment of the B48 origin sequence over the B13 non-origin sequence. ChIP analysis showed that the origin-specific DNA was enriched three to six fold in untreated T98G cells relative to control sequences after immunoprecipitation with anti-RECQ1 and anti-RECQ4 antibodies (Fig. 4.1.2B). On the other hand, I didn't observe any enrichment for origin-containing regions above control levels when immunoprecipitations were performed with anti-BLM, anti-WRN, and anti- RECQ5 antibodies. We used the ORC2 replication protein as a positive control in all experiments, since ORC2 has already been shown to bind to the lamin B2 origin (Abdurashidova et al., 2003). Consistent origin specific DNA enrichment was observed also in diploid fibroblast cell line established from lung tissue of female fetus.

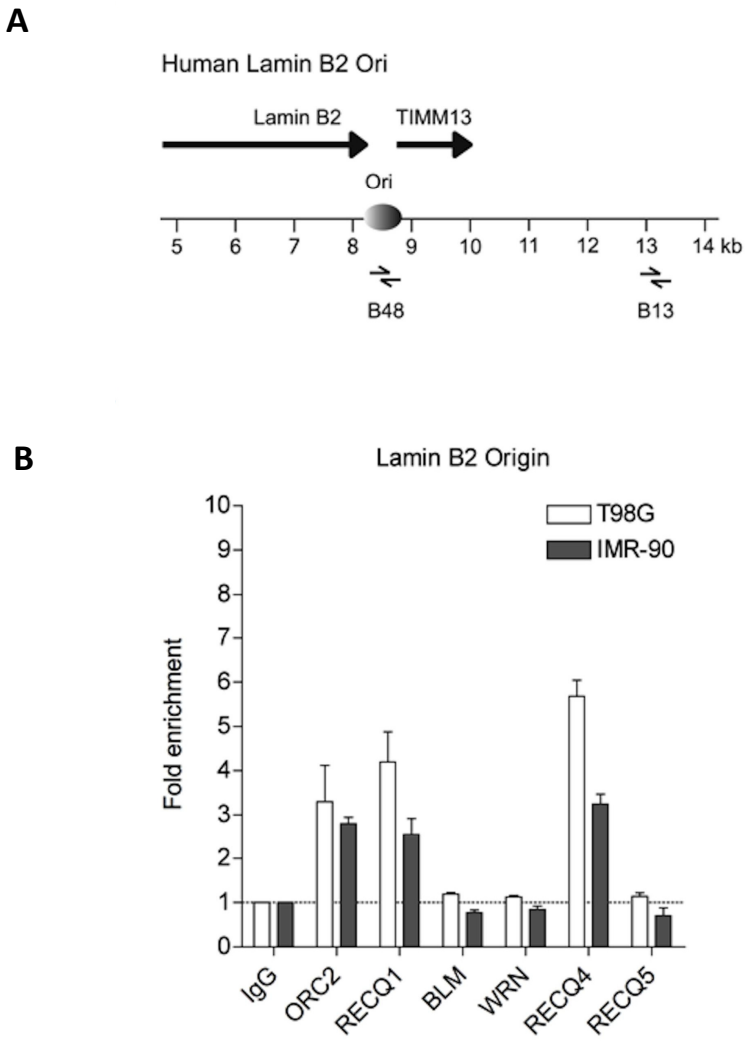


FIG.4.1.2 : RECQ1 and RECQ4 helicases are recruited to human lamin B2 origins of DNA replication. (A and B) Genomic regions containing the laminB2 origin (A) are shown together with the locations of sets of primers (converging arrow pairs) used for quantitative real-time PCR analysis. (B) Quantification of cross-linked lamin B2 origin DNA immunoprecipitated by ChIP from T98G and IMR-90 cells, using the antibodies indicated at the bottom. Fold enrichments of origin sequences were determined versus nonorigin control sequences, and the dashed line indicates the threshold enrichment level obtained by using a negative-control antibody (normal rabbit IgG). Results are reported as means ± standard errors of the means (SEM) (indicated by error bars) for at least three independent experiments.

4.1.3. Interaction of RecQ helicases with the lamin B2 replication origin by competitive PCR analysis

The results of the real-time PCR analysis were also confirmed by competitive PCR experiments in which the amount of origin sequence was estimated by comparing the intensities of corresponding amplified bands to those of bands generated from known amounts of competitor present in the same reaction tubes. Quantification was done by adding scalar quantities of competitor and a fixed quantity of immuno purified DNA (Fig. 4.1.3A). The competition samples after PCR amplification were run on TBE acrylamide gels, stained with ethidium bromide and the intensities of the bands were measured with the image J software. The values obtained were then plotted as a function of the log10 of the known competitor DNA copy number. The point of equivalence corresponds to the point where the amounts of the competitor and target were equal, and represented the number of copies of B48 origin sequence and B13 non-origin sequence in the starting sample. This procedure provides an additional level of specificity and gives more sensitive quantification.

Competitive PCR quantification confirmed the enrichment of human lamin B2 origin sequences in the immunoprecipitations with anti-RECQ1 and anti-RECQ4 antibodies (Fig. 4.1.3A). Origin-specific DNA was enriched four- to five fold in untreated T98G cells relative to control sequences after immunoprecipitation with anti-RECQ1 and anti-RECQ4 antibodies. Conversely no such enrichments was observed with anti-BLM, anti-WRN and anti-RECQ5 antibodies (Fig. 4.1.3B).

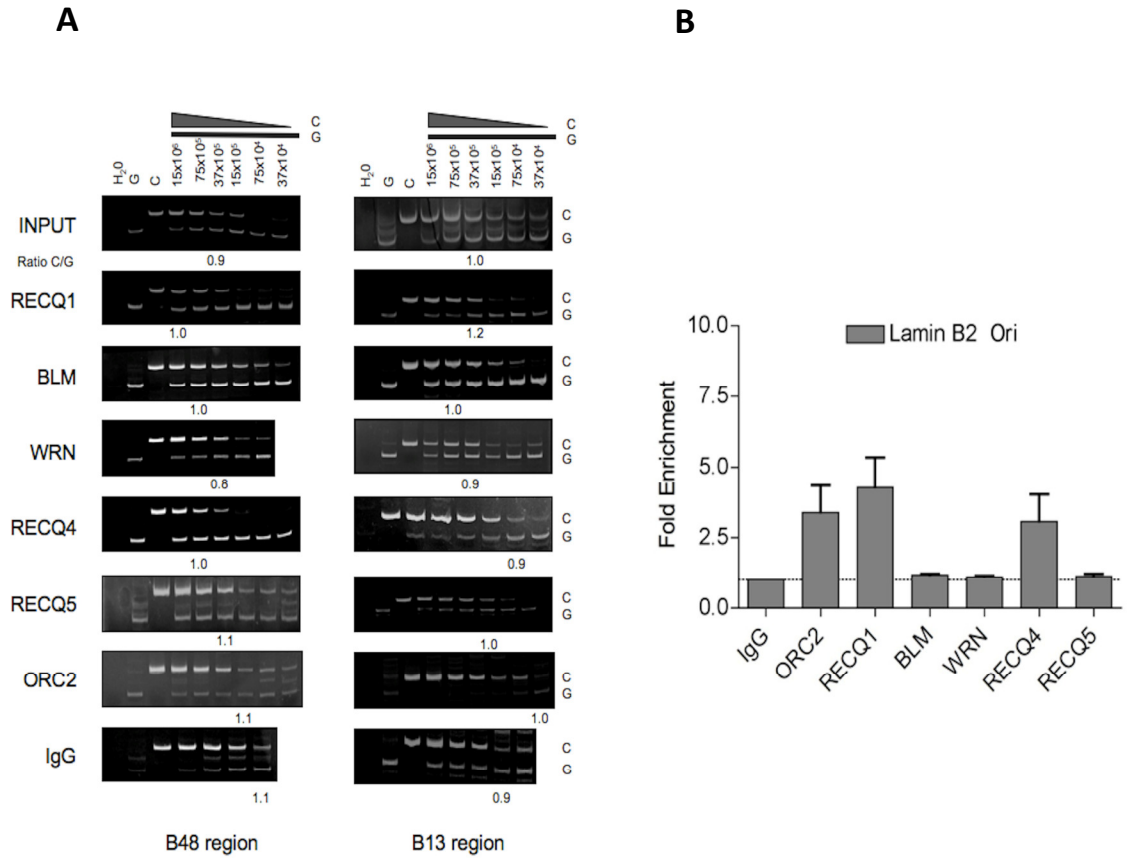


FIG.4.1.3 : Analysis of the association of RECQ1 and RECQ4 with the human lamin B2 replication origin by competitive PCR. A) Gel photos show the results of quantitative competitive PCR analysis performed using ChIP samples prepared by immunoprecipitation with antibodies against the five human RecQ enzymes or with ORC2 protein or normal rabbit IgG as positive and negative controls, respectively. A typical competitive PCR experiment is performed by addition of scalar amounts of competitor (C) of known concentration to a fixed amount of the genomic DNA (G) to be quantified. The amplification products are resolved by gel electrophoresis, stained with ethidium bromide, and quantified as previously described (Abdurashidova et al., Embo J.2003). B) The bar graph quantifies cross-linked lamin B2 DNA immunoprecipitated by ChIP using antibodies specific to the proteins shown across the bottom. Fold enrichments of origin sequences were determined versus non-origin control sequences, and the dashed line indicates the threshold enrichment level obtained by using a negative control antibody (normal rabbit IgG). Results are reported as means ± SEM (standard error of the mean, indicated by error bars) of at least three independent experiments.

4.1.4 Interaction of RecQ helicases with human GM-CSF replication origins

The interaction of the human RECQ1 and RECQ4 helicases with the lamin B2 replication origin was confirmed using two addition origin sequences. These two origins (GM-CSF Ori1 and Ori2) are located 5 kb apart and approximately 5 kb downstream of the 3' end of the GM-CSF gene, on chromosome 5q, and were shown to interact with different members of the DNA pre-replication complex (Fig. 4.1.5A). These two origins are fully competent to direct the assembly of a pre-RC and to drive initiation of DNA replication. They lie in an intergenic region and are neither associated with a promoter, nor linked to a CpG island, and do not share sequence similarity to other known origins (Todorovic et al., 2005). ChIP experiments were done using asynchronous T98G human glioblastoma cells. Origin specific DNA (17 for GM-CSF Ori1 and 23 for GM-CSF Ori2) was enriched approximately four- to five fold in comparison with nonorigin sequence (region 21) in the immuno-purified DNA obtained with RECQ1 and RECQ4, respectively. Consistent with my lamin B2 origin observations, RECQ1 and RECQ4 were the only two RecQ helicases associating with GM-CSF Origins. Immuno-purified DNA from BLM, WRN and RECQ5 showed no specific enrichment of GM-CSF1 and GM-CSF2 origin sequences in relation to nonorigin sequences (Fig. 4.1.5B).

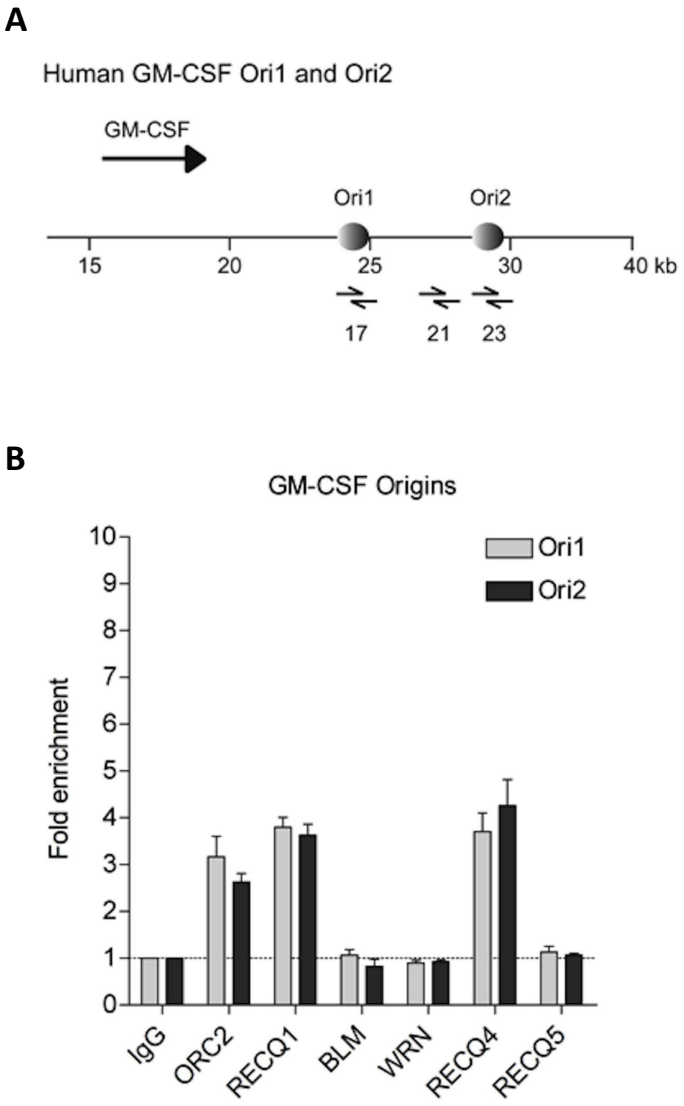


FIG. 4.1.4: RECQ1 and RECQ4 helicases are recruited to human GM-CSF1 and GM-CSF2 origins of DNA replication. (A) Genomic regions containing the two GM-CSF replication origins are shown with the locations of sets of primers (converging arrow pairs) used for quantitative real-time PCR analysis.
(B) Quantification of cross-linked GM-CSF1 and GM-CSF2 origin DNAs immunoprecipitated by ChIP from T98G cells, using the antibodies indicated at the bottom. Fold enrichments of origin sequences were determined versus nonorigin control sequences, and the dashed line indicates the threshold enrichment level obtained by using a negative-control antibody (normal rabbit IgG). Results are reported as means ± standard errors of the means (SEM) (indicated by error bars) for at least three independent experiments.

4.1.5. Interaction of RecQ helicases with human Lamin B2 replication origin in replication perturbed cells

The origin specific interaction of only two human RecQ helicases in unperturbed cells, raised the question whether the same behavior would be observed upon replication stress induciton. Thus, the same ChIP experiments were repeated in the presence of the replication inhibitor hydroxyurea (HU). HU induces DNA damage leading to stalled or reduction in the speed of replication fork movements. Briefly, T98G cells were treated with 2mM HU overnight and ChIP experiments were repeated with anti RecQ antibodies.

Treatment with HU showed that all five human RecQ helicases, including RECQ1 and RECQ4, interacted with the lamin B2 origin upon replication stress (figure 4.1.6). It has been shown that BLM and WRN relocalizes from PML bodies to replication sites upon treatment with replication inhibitors and they accumulate at replication foci either to restart or to repair the stalled forks. My observation that BLM, WRN and RECQ5 interacts with the replication origins in HU treated cells provides further support for the proposed roles of these helicases in promoting fork recovery or repair (Fig. 4.1.6). However, my results indicate that of the five human RecQ helicases, only RECQ1 and RECQ4 specifically bind replication origin regions in unperturbed cells, and that origin binding is not origin or cell type specific, suggesting that these two RecQ helicases are required during normal replication, whereas BLM, WRN, RECQ5 might be needed under conditions of replication stress.

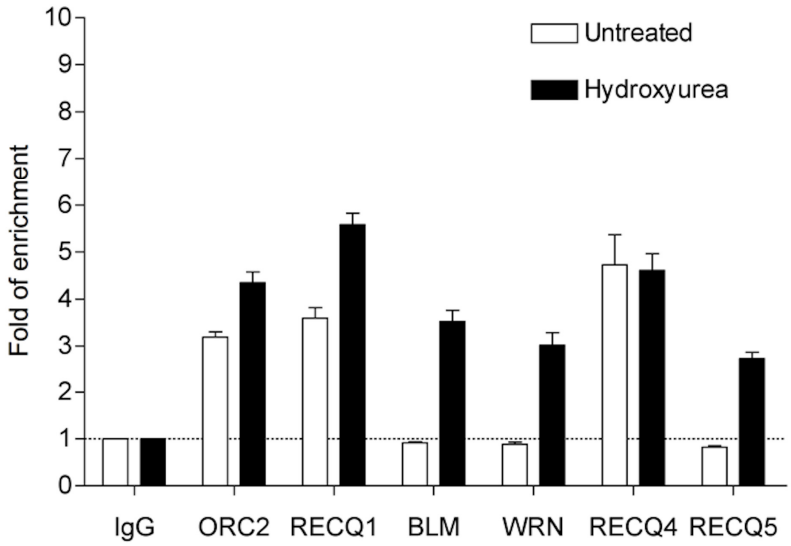


FIG.4.1.5: Comparison of association of RECQ1 and RECQ4 helicases with the lamin B2 origin in untreated versus HU-treated cells.T98G cells were treated with 2 mM HU for 24 h. The bar graph shows the quantification of cross-linked lamin B2 origin DNA immunoprecipitated from T98G glioblastoma cells, using antibodies specific to the proteins shown across the bottom. Fold enrichments of origin sequences were determined versus nonorigin control sequences, and the dashed line indicates the threshold enrichment level obtained by using a negative-control antibody (normal rabbit IgG). Results are reported as means ± SEM for at least three independent experiments.

4.1.6. Interaction of BLM complex proteins with human lamin B2 replication origins

The interaction of BLM, WRN and RECQ5 with the human lamin B2 replication origin in replication perturbed cells indicates that these helicases may regulate origin activity when cells are under replication stress. Protein partners of RecQ helicases may also be required to regulate the origin activity. Consistent with this hypothesis, cells lacking RMI1 or Rif1, two recently discovered protein partners of BLM, are sensitive to hydroxyurea treatment. To check the hypothesis that these BLM complex components participate in modulating origin activity we performed ChIP with Rif1 and RMI1 in unperturbed and hydroxyurea treated cells (Fig.4.1.7A). Both Rif1 and RMI1 immuno-purified DNA showed no specific enrichment of origin sequences in unperturbed cell lines. However, Rif1 and RMI1 showed approximately five-to six fold enrichments, respectively, when the cells were treated with hydroxyurea (Fig.4.1.7B). Rif1 and RMI1 followed the same behaviour of BLM in both unperturbed and perturbed cell lines. TBE-PAGE analysis of real time PCR end product revealed a clear enrichment of origin sequences in immuno-purified DNA of BLM, Rif1 and RMI1, obtained from hydroxyurea treated cell lines (Fig.4.1.7C).

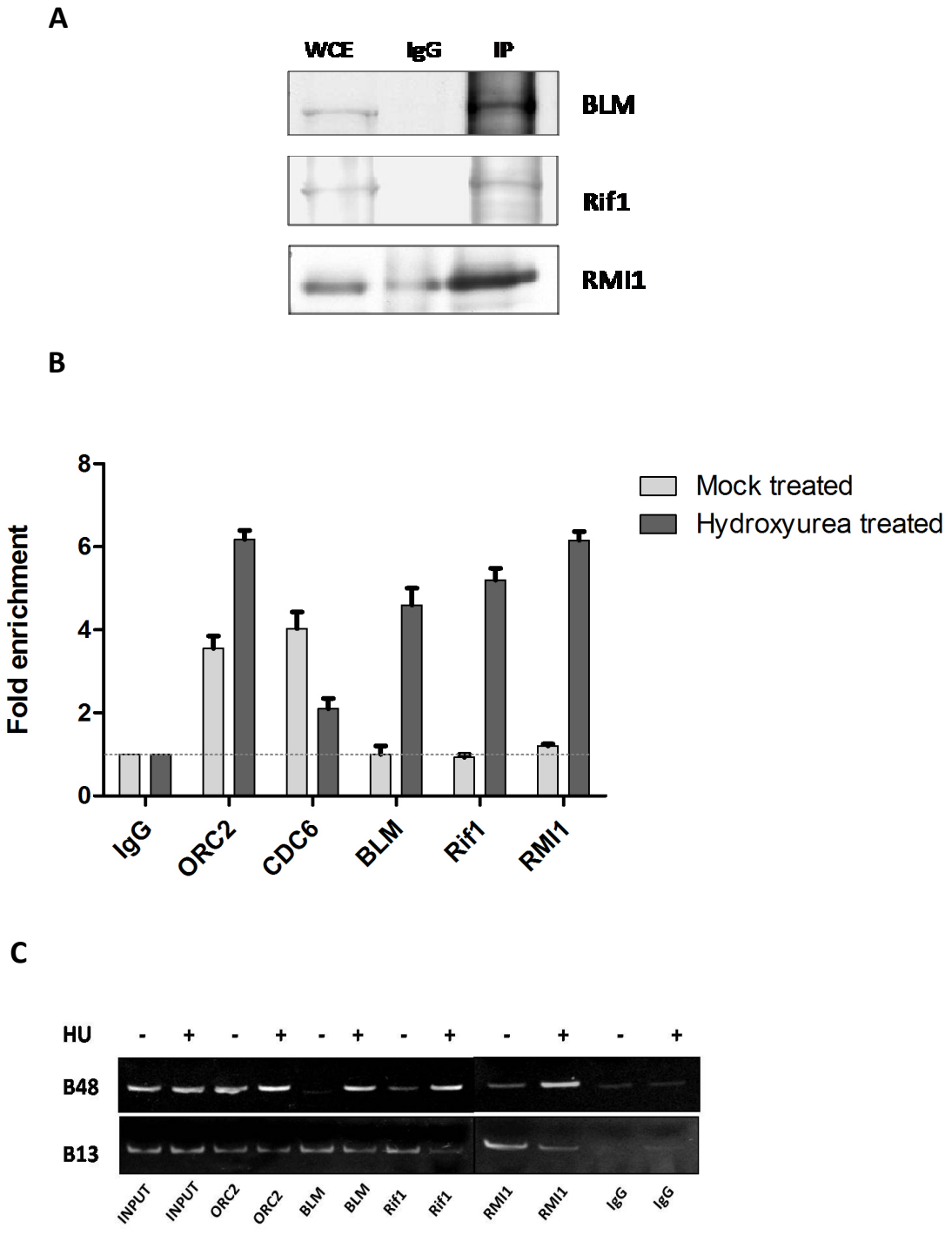


FIG.4.1.6: **Comparison of association of BLM comples proteins with the lamin B2 origin in untreated versus HU-treated cells**. A) Western blot analysis after immunoprecipitation of cross-linked material with anti-BLM, anti-Rif1, anti-RMI1 antibodies. Whole T98G cell lysates (WCL) used to confirm the exogenous expression, and mouse IgG immunoprecipitation served as negative control. B) T98G cells were treated with or without 2 mM HU for 24 h. The bar graph shows the quantification of cross-linked lamin B2 origin DNA immunoprecipitated from T98G glioblastoma cells, using antibodies specific to the proteins shown across the bottom. Fold enrichments of origin sequences were determined versus nonorigin control sequences, and the dashed line indicates the threshold enrichment level obtained by using a negative-control antibody (normal rabbit IgG). Results are reported as means ± SEM for at least three independent experiments. C) TBE-PAGE analysis of PCR products obtained hydroxyurea treated (+) and mock treated (-) cell lines using antibodies specific to the proteins shown across the bottom.

Chapter 2: Regulation of RECQ1 and RECQ4 association with human DNA replication origins.

4.2.1 Cell cycle regulated expression of RECQ1 and RECQ4 helicases

My finding that RECQ1 and RECQ4 associate with DNA replication origins raises the possibility that the interaction of these two helicases may be cell cycle regulated. In order to address this possibility, I tested the expression of RECQ1 and RECQ4 during cell cycle. I used the T98G cell line, because T98G cells could be efficiently synchronized by serum deprivation (Takahashi et al., 2000). Although it is a continuous cell line, T98G has retained growth arrest mechanisms characteristic of normal cells, including density mediated growth inhibition and induction of quiescence in response to serum deprivation (Stein, 1979). Serum starvation resulted in the accumulation of a quiescent population G_0 that synchronously progressed through G1 and S phase after serum stimulation (Fig. 4.2.1A), allowing for the isolation of populations in early G1 (8 h), mid-G1 (12 h), late G1 (18 h), at the G1/S transition (20 h), early S (22 h), mid S (24 h), and in G2/M phase (28 h) (henceforth, all cell cycle phase designations refer to these time points). Synchronization and cell cycle progression were monitored by fluorescence-activated cell sorter (FACS) analysis and by western analysis of cyclin E and cyclin A1 expression (Fig. 4.2.1A and 4.2.1B). In agreement with previous findings, cyclin E and cyclin A1 protein levels started to rise in middle G1 (12 h) and early S (22 h), respectively, while both cyclins were present at the G1/S boundary (20 h) (Mailand and Diffley, 2005). RECQ1 was present throughout synchronization and release, with a small but reproducible increase at the G1/S border (20 h), a pattern similar to, though not as

pronounced as, that observed for cyclin A1. In contrast, RECQ4 was almost undetectable in G0 or resting cells but was readily detectable in late G1 and early S (18 h) (Fig. 4.2.1B).

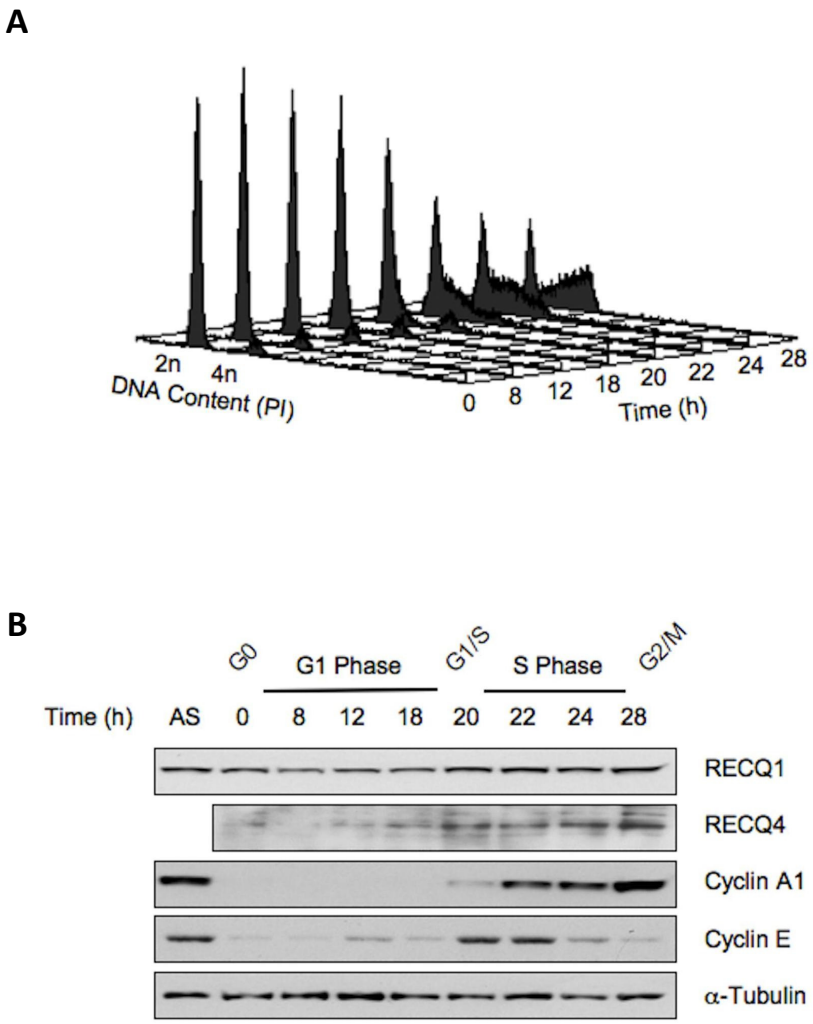


FIG.4.2.1: Cell cycle-dependent expression of RECQ1 and RECQ4 helicases. (A) Cell cycle phase timing was determined by flow cytometry profiling of synchronized T98G cells that had been cultured without serum for 72 h and then sampled over a 28-h time course after the addition of serum. PI, propidium iodide. (B) Western blot analysis of whole-cell extracts of asynchronous T98G cells (AS) or cells from different times after synchronization and release.

4.2.2 Cell cycle regulated interaction of RECQ1 and RECQ4 helicases with replication origins

Following my observation that RECQ1 and RECQ4 expressions are cell cycle regulated, I tested if the association of these RecQ helicases with replication origins is also changing as a function of the cell cycle. In order to address this possibility, I used ChIP analyses to determine whether RECQ1 or RECQ4 was recruited to origins in a cell cycle phase-dependent fashion. ChIP experiments from synchronized T98G cells showed that neither RECQ1 nor RECQ4 was associated with replication origins in G0 or early G1. A threefold enrichment of RECQ4 binding to the lamin B2 origin region could be detected at the G1/S boundary and increased to approximately six fold above background with the onset of S phase (Fig.4.2.2). Conversely, RECQ1 was found enriched at origin sequences only in early S. For comparison, ORC2, a well-characterized component of the pre-replication complex (pre-RC), was found on origin regions in early G1 and remained bound through early S (Abdurashidova et al., 2003).

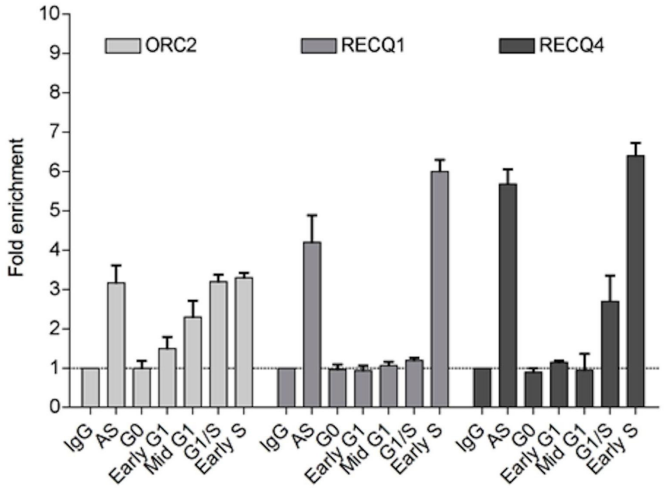


FIG.4.2.2 : Cell cycle-dependent association of RECQ1 and RECQ4 with the human lamin B2 replication origin. Quantification of cross-linked lamin B2 origin DNA immunoprecipitated by ChIP from synchronized cells. The key on top indicates the antibodies used for ChIP analyses. Fold enrichments of lamin B2 origin region (B48) DNA over control B13 region DNA are reported for each antibody, where the dashed line indicates the threshold enrichment obtained using a negative-control normal rabbit IgG antibody. Histogram bars report the mean ± SEM for at least three independent experiments for each antibody and cell cycle fraction.

4.2.3. Analysis of RECQ1 and RECQ4 association with replication origins in mimosine blocked cells.

My analysis of RECQ1 and RECQ4 loading to the replication origins in the synchronized cells revealed RECQ4 loads to the origins before RECQ1. To confirm the loading of RECQ4 to replication origins precedes RECQ1 loading, asynchronously growing T98G cells were treated with mimosine. The plant amino acid mimosine has been demonstrated to arrest cell cycle progression in late G1 (Tsang et al., 2007). FACS analysis confirmed the accumulation of late G1 cells by mimosine treatment (Fig. 4.2.3A). ChIP of mimosine treated T98G cells showed two fold enrichment of origin specific sequences from RECQ4 immunoprecipitated DNA but not with RECQ1

immunoprecipitated DNA. ORC2, the positive control, showed consistent enrichment of origin sequences in the immunoprecipitated DNA (Fig. 4.2.3B). My observation that RECQ4 is loaded onto the origins in late G1 suggests that RECQ4 could be a new member of human pre-initiation complex.

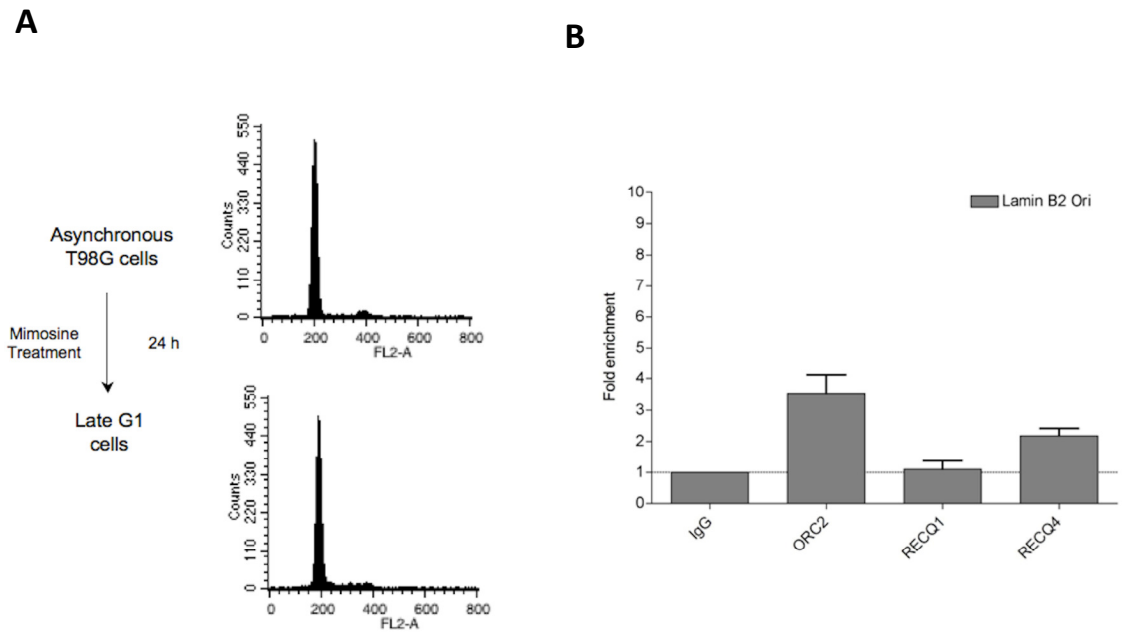


FIG 4.2.3: Recruitment of RECQ4 to replication origins precedes RECQ1 loading. A) FACS profile showing T98G cells were synchronized in late G1 by mimosine treatment for 24 h. B) Synchronized cells were used to quantify proteins cross-linked to the lamin B2 origin DNA that could be immuno-precipitated by antibodies specific to the proteins indicated at the bottom. The bargraph shows fold enrichments of origin sequences versus non-origin controlsequences, where the dashed line again indicates the threshold enrichment level obtained byusing a negative control antibody (normal rabbit IgG). Results are reported as means ± SEM (standard error of the mean, indicated by error bars) of at least three independent experiments.

4.2.4. Sequential loading of RecQ helicases on replication origins: a detailed analysis.

To better define the temporal loading of the human RECQ1 and RECQ4 helicases on the replication origin, a detailed analysis of the cell cycle-dependent association of RECQ4, RECQ1, and other known replication factors with the lamin B2 origin was tested by ChIP experiments. T98G cells were synchronized by serum starvation and the cells representing late G1, G1/S, early S, mid S and late S were collected. Synchronization was confirmed by FACS profile. Western blot analysis of cyclin expression pattern in the synchronized cells was consistent with prior literature (Mailand and Diffley, 2005), confirming again the effective synchronization (Fig.4A.2.4B). Through my ChIP experiments I found, consistent with published results, that CDC6, ORC1, ORC2, and MCM4 assembled on the origin in G1 to complete the prereplication complex (Abdurashidova et al., 2003; Ghosh et al., 2006; Schaarschmidt et al., 2002). RECQ4 loading was detected in late G1 as part of the prereplication complex and was most abundant at the G1/S border as CDC6 was lost. In early S phase, ORC1 was also lost from origins, as expected (Abdurashidova et al., 2003), whereas RECQ1 and additional RECQ4 could now be detected on the lamin B2 origin. RECQ4, RECQ1, and MCM4 binding to the origin region of lamin B2 was no longer detectable after mid-S phase (Fig.4.2.4). In agreement with my findings, a recent study showed that a Flag-tagged version of RECQ4 specifically interacted with the lamin B2 origin during the G1 and S phases of the cell cycle in human 293T cells (Xu et al., 2009a). In this analysis, the lamin B2 origin appeared to be enriched five-fold at G1 compared with S phase. These results are difficult to assess in the absence of detailed ChIP analysis time course data. The apparent enrichment in origin binding at G1 versus S may reflect the fact that the

sample used for S-phase experiments corresponded to mid or late S, as suggested from the flow cytometry data, where I showed that RECQ4 was almost undetectable on the lamin B2 origin. Collectively, my results and those reported by Xu et al (Xu et al., 2009a), indicate that RECQ4 is part of the prereplication complex and thus might play a role in the transition from the prereplication to the preinitiation complex. RECQ1, in contrast, may be assembled on origins only at the start of bidirectional DNA synthesis. The subsequent loss of both RECQ1 and RECQ4 from origin regions could indicate either disassembly or the tracking of one or both RecQ helicases with the newly formed replisome after origin firing.

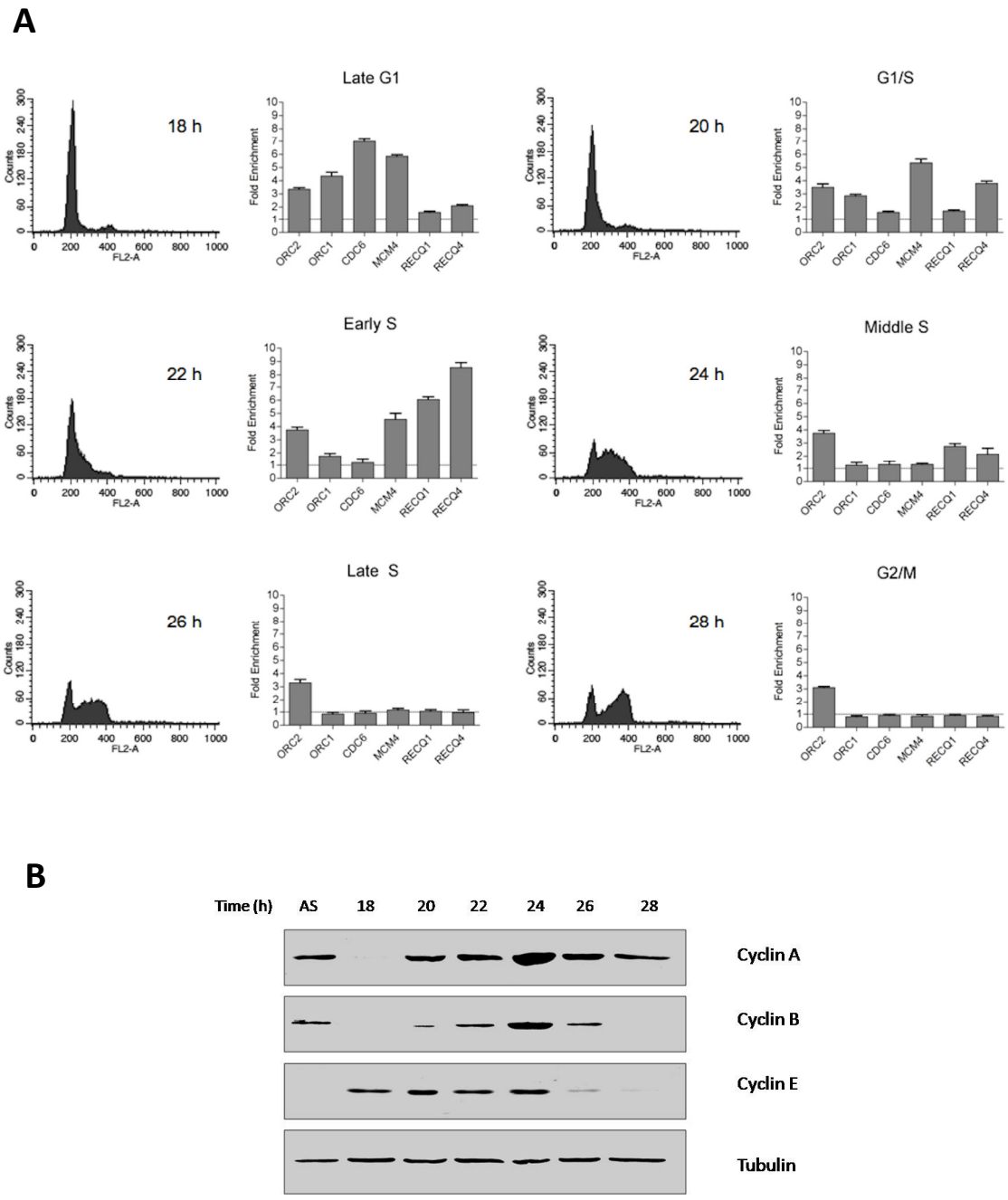


FIG.4.2.4 : G1- and S-phase-specific loading of RECQ4 and RECQ1 on the lamin B2 origin. A) Paired columns show flow cytometry profiles of synchronized T98G cells at different times after release from serum starvation, together with bar graphs of cross-linked lamin B2 origin DNA immunoprecipitated by ChIP with antibodies to each of the proteins shown across the bottom of the bar graph at that time point. Histograms report the mean ± SEM for at least three independent experiments at each time point, where fold enrichments of lamin B2 origin region (B48) DNA over control B13 region DNA are reported for each antibody, where the dashed line indicates the threshold enrichment obtained using anegative-control normal rabbit IgG antibody. B) Western blot analysis of whole-cell extracts of T98G cells (AS) or cells from different times after synchronization and release.

4.2.5 Cell cycle regulated chromatin association of RECQ1 and RECQ4 helicases

Since the early S phase specific cellular upregulation of RECQ1 and the late G1 expression of RECQ4 are in accordance with the cell cycle dependent origin loading of these proteins, I investigated whether such a cell cycle specific origin association is also regulated at subcellular level. To examine this, I synchronized and collected cells representing various phases of the cell cycle. These cells were then fractioned into cytoplasmic (S2), nucleoplasmic (S3) and chromatin enriched (P3) fractions. Western analysis was performed with the biochemically fractioned, synchronized cells. Tubulin served as a control for cytoplasmic fractionation and also as a loading control of cytoplasmic fractions, whereas p84 served as a control for chromatin fractionation. In agreement with the ChIP results, biochemical fractionation experiments showed that RECQ4 was enriched in the chromatin fraction during the late G1 and early S phases (Fig 4.2.5). The continued presence of RECQ4 bound to chromatin in late S is consistent with the idea that RECQ4 might leave origin regions to travel with the replisome after replication initiation. Although most of the RECQ1 protein was bound to chromatin throughout all phases of the cell cycle, ChIP experiments showed that RECQ1 interacted with replication origins only at the onset of S phase, when origins are licensed for firing. Interestingly, the partitioning of RECQ1 and RECQ4 between cytosolic and nucleoplasmic fractions changed over the cell cycle. This suggests that RECQ1 and RECQ4 function might be regulated by a combination of cell cycle-dependent synthesis and subcellular localization (Fig 4.2.5). This cell cycle dependent sub-cellular localization correlated well with the origin loading and thus appear to support the notion that the function of RECQ1 and RECQ4 at origins is also regulated at subcellular level.

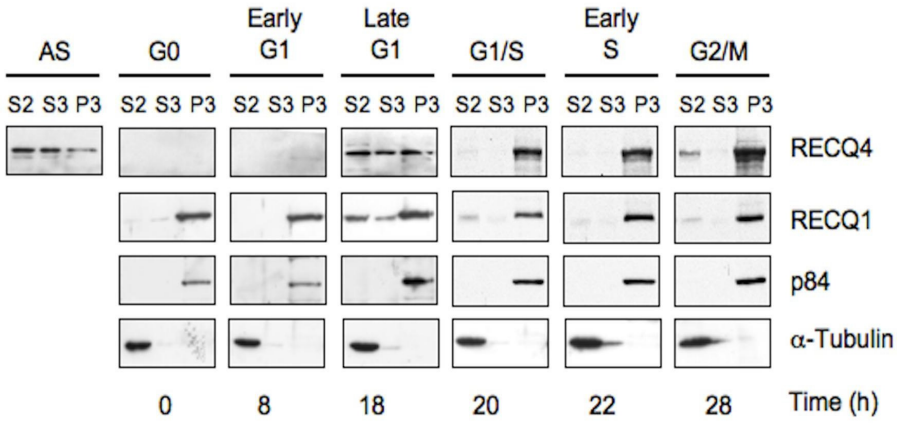


FIG 4.2.5: Cell cycle-dependent subcellular distribution of RECQ1 and RECQ4. Whole-cell lysates were fractionated to generate cytosolic (S2), soluble nuclear (S3), and chromatin-enriched (P3) fractions, in which protein levels were assessed by Western blotting. Antibodies against p84 and α-tubulin were used as controls for nuclear and cytoplasmic localization, respectively.

4.2.6. Analysis of the replication timing-regulated association of RECQ1 and RECQ4 with replication origins.

The lamin B2 and GM-CSF origins are early firing DNA replication origins (Abdurashidova et al., 2000; Todorovic et al., 2005). To test if the temporal loading of RECQ1 and RECQ4 to replication origins might change as a function of replication timing, I compared the loading of these two helicases on the human beta-globin replication origin in HeLa versus K562 cell lines in ChIP experiments (Fig.4.2.6A). The beta-globin origin is located on chromosome 11, in the region encompassing the human beta-globin gene, and it is known to replicate in early S phase in hematopoietic cells (K562 cells) and later in S in non hematopoietic cells (HeLa cells) (Buzina et al., 2005; Epner et al., 1988; Kitsberg et al., 1993). The K562 and HeLa cell lines were synchronized by mimosine treatment in late G1 and then released from the drug and harvested at different time points during S phase. Cellular synchronization and progression were monitored by FACS analysis (Fig.4A.3B and Fig.4A.3C). Control experiments confirmed that RECQ1 and RECQ4 were enriched at the lamin B2 origin sequence in early S and were lost from the lamin B2 origin in late S in both cell lines. These results support my previous conclusion that the interaction of RECQ1 and RECQ4 with the early-firing lamin B2 origin is not cell type specific (Fig.4.2.6B and Fig.4.2.6C). My results using the beta-globin origin in K562 cells showed that the timing of the interaction of RECQ1 and RECQ4 was identical to what I observed for the lamin B2 origin (Fig.4.2.6B). Conversely, in HeLa cells, where beta-globin origin firing occurs later during S, RECQ1, RECQ4, and ORC2 were already detected on the origin in early S and remained bound in late S (Fig.4.2.6C). The presence of origin-bound RECQ1, RECQ4, and ORC2 already in early S phase indicates that the

temporal loading of these factors is likely to be independent of origin replication timing. These results suggest a model in which replication factors assemble on most or all origins during G1/early S, when cyclin-dependent kinase levels are permissive, but that further signals are necessary to start replicating at a specific point in S phase. Further analyses will be required to test this model.

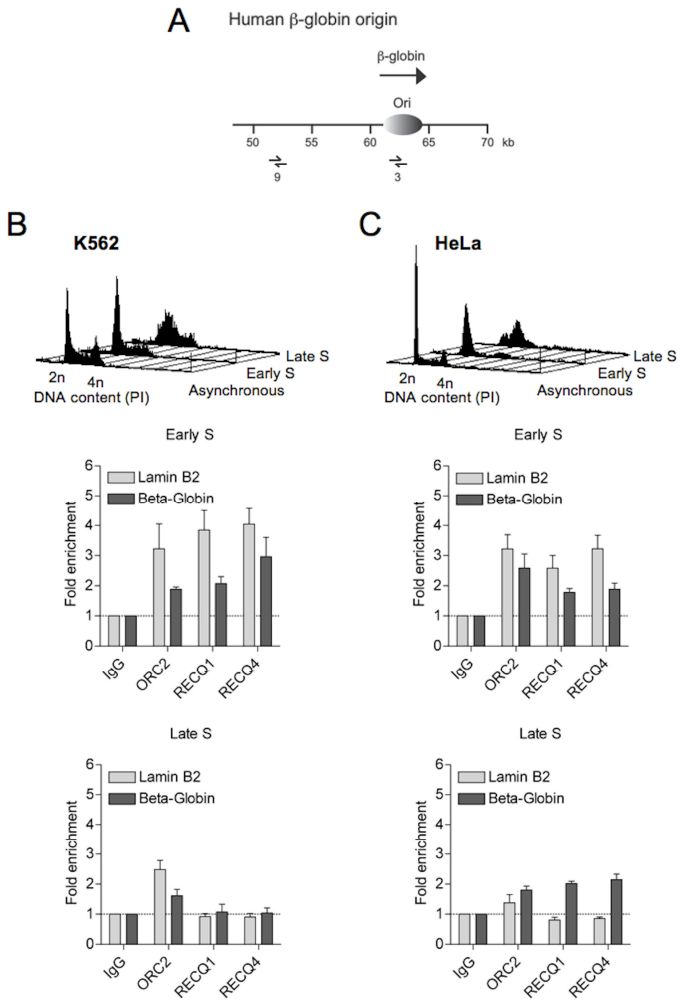


FIG.4.2.6: Association of RECQ1 and RECQ4 with beta-globin replication origin as a function of origin timing. (A) Genomic region containing the beta-globin replication origin, together with the locations of sets of primers (converging arrow pairs) used for quantitative real-time PCR analysis. (B) Flow cytometry profiling of synchronized K562 cells that had been cultured with mimosine for 24 h and then sampled 3 (early S) and 9 (late S) h after the removal of the drug is shown at the top. The histograms below quantify cross-linked lamin B2 and beta-globin origin DNAs immunoprecipitated by ChIP from early- and late-S-phase-synchronized K562 cells. (C) Flow cytometry profiling of synchronized HeLa cells that had been cultured with mimosine for 24 h and then sampled 3 (early S) and 9 (late S) h after the removal of the drug is shown at the top. The histograms below quantify cross-linked lamin B2 and beta-globin origin DNAs immunoprecipitated by ChIP from early- and late-S-phase-synchronized HeLa cells. Histograms report the means ± SEM for at least three independent experiments.

Chapter 3: Physiological role of RECQ1 and RECQ4

4.3.1. Analysis of proliferation capacity of RECQ1- and RECQ4-depleted cells.

To elucidate the physiological role of RECQ1 and RECQ4, I analysed the replication phenotype of cells where RECQ1 and RECQ4 were depleted. T98G glioblastoma cells were transfected with RECQ1, RECQ4, or luciferase (mock-depleted control cells) siRNA. Western blot verification of the extent of depletion indicated marked or near-complete depletion of RECQ1 and RECQ4, respectively (Fig. 4.3.1B). Consistent with the idea that RECQ1 and RECQ4 may play a role in DNA replication initiation, I observed a significant reduction in cell proliferation following the siRNA-mediated depletion of RECQ1 or RECQ4 from T98G cells In particular, I compared the colony forming properties of T98G glioblastoma cells transfected with RECQ1 or RECQ4 siRNA versus a T98G cell line transfected with a control siRNA against luciferase (mock-depleted control cells). RECQ1-and RECQ4-depleted T98G cells showed, respectively, a 5-fold and 6-fold reduction in proliferation as measured by colony formation versus mock-depleted control cells (Fig.4.3.1A). My observation that RECQ1 and RECQ4 were important for cell proliferation was consistent with the recent reports (Futami et al., 2008; Sangrithi et al., 2005; Sharma and Brosh, 2007). The same experiment repeated with the human GM00637 SV40 fibroblast cell line indicated that RECQ1 and RECQ4 depletion were growth suppressive, and that the effect of RECQ1 or RECQ4 depletion was not cell type-specific (data not shown).

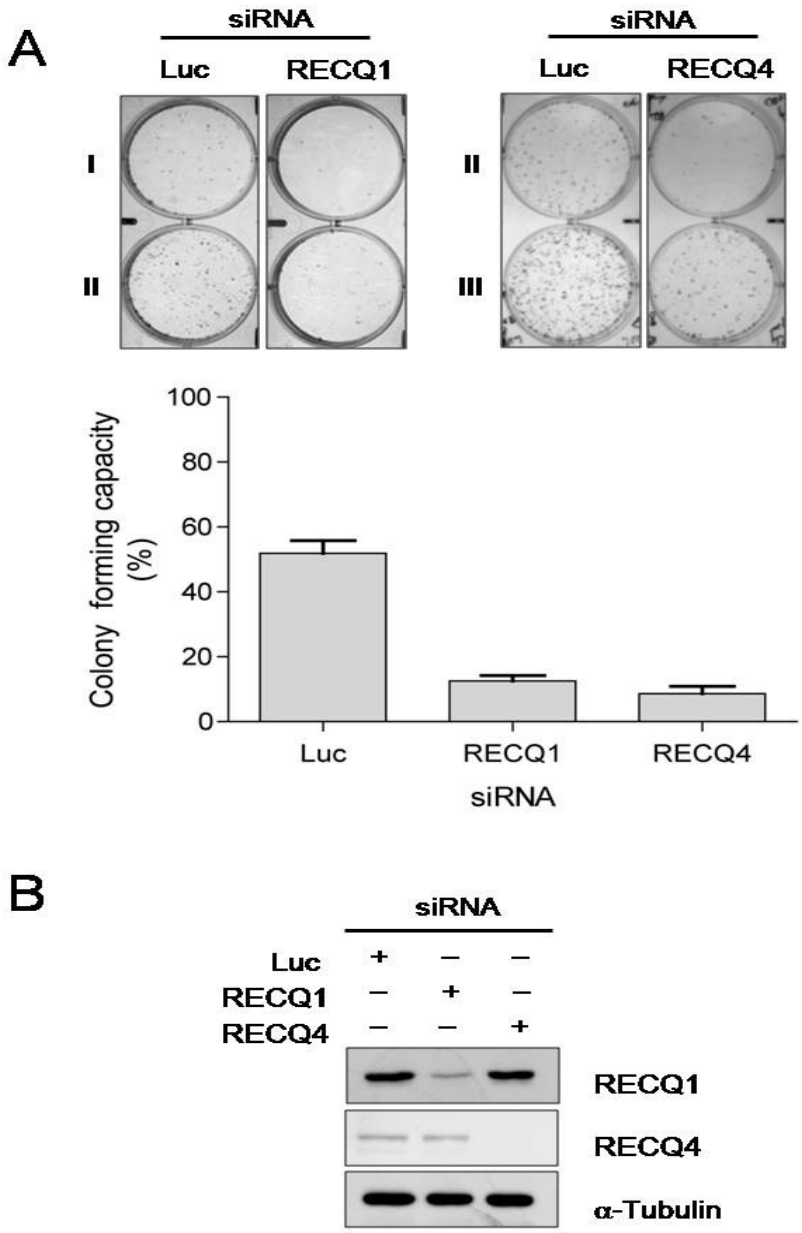


Figure 4.3.1: **Down-regulation of RECQ1 and RECQ4 inhibits cellular proliferation.** A) Colony forming efficiency of T98G cells after siRNA-mediated transfection. Plate photos show representative colony formation after plating cells transfected with siRNA pools directed against the gene named across the top. Cell number plated is indicated by the code along the left: I: 200 cells/well; II: 400 cells/ well; III: 800 cells/well. Colonies were stained and counted after 7 days of growth and counted to determine colony forming efficiencies that are summarized in the bar graph that summarizes results (mean ± SEM) of three independent experiments. B) Western blot analysis of RNAi-mediated depletion of RECQ1 or RECQ4 from T98G cells transfected with siRNA pools against RECQ1, RECQ4 or luciferase (Luc) at 72 h post-transfection. α-tubulin was used as a blot control.

4.3.2. Analysis of cell cycle progression and DNA synthesis in RECQ1- and RECQ4-depleted cells.

To further address the physiological role of RECQ1 and RECQ4's and futher investigate the possible reason of the cell proliferation defects of RECQ1 and RECQ4 depleted cells, I studies tha cell cycle kinetics of RECQ1, RECQ4, and luciferase downregulated cells. Thus, I found a greater fraction of cells in late G1 both in RECQ1 and RECQ4 depleted cells, suggestive of delayed S phase progression. To obtain more detailed information of potential replication defects, I quantitatively monitored the degree of bromodeoxyuridine (BrdU) incorporation. FACS analysis of RECQ1- or RECQ4-depleted cells that had been BrdU -labelled, demonstrated a >50% reduction in both BrdU labelling and S phase fraction together with an increased G1 fraction versus controls (Fig.4.3.2). These results were consistent with previous reports of an increased G1 fraction and decreased BrdU incorporation in RECQ4-depleted cells (Sangrithi et al., 2005). Similar results were again obtained using RECQ1- or RECQ4-depleted GM00637 fibroblasts (data not shown). These results demonstrate that RECQ1 or RECQ4 depletion suppresses cell proliferation, and may do so by interfering with DNA replication.

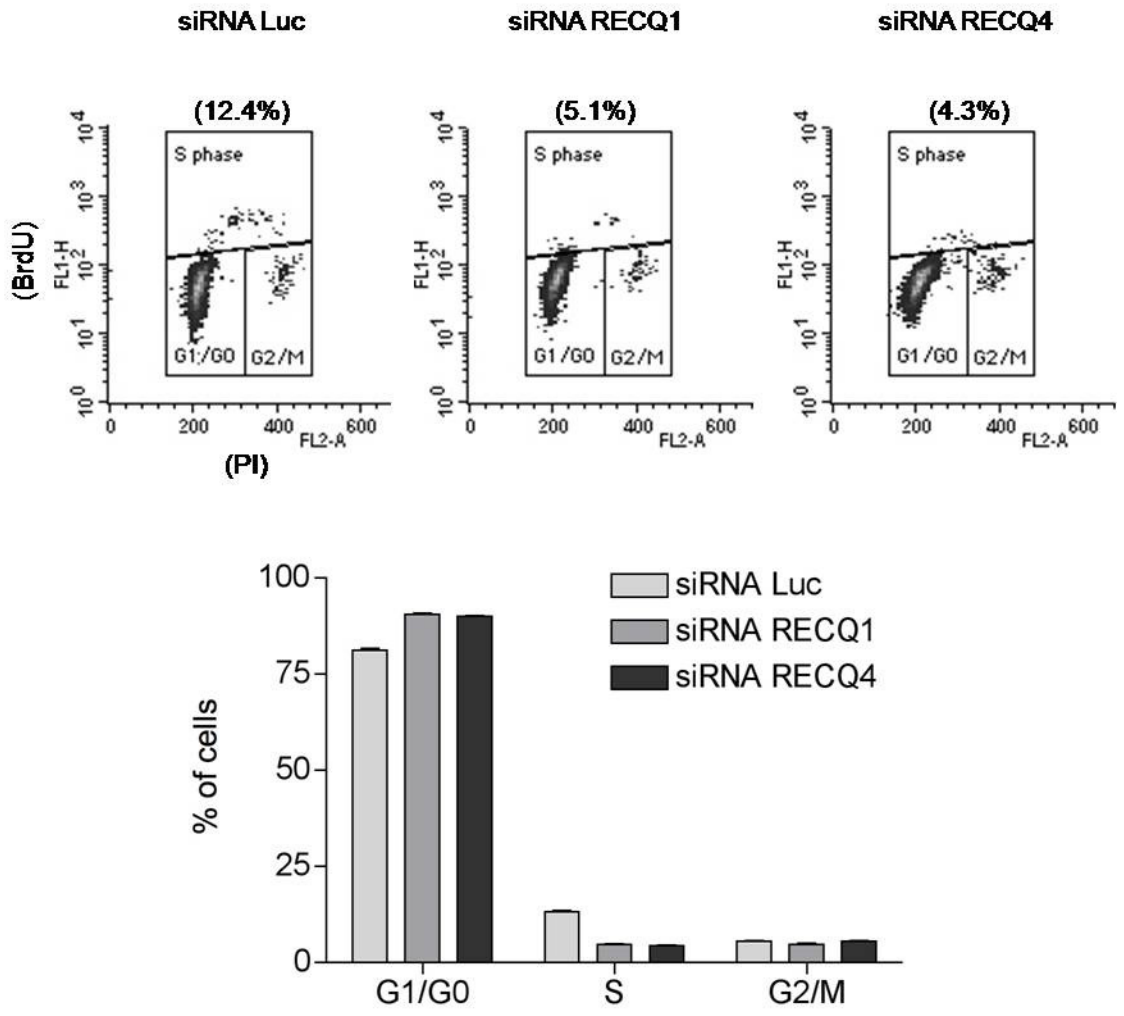


Figure 4.3.2: **Down-regulation of RECQ1 and RECQ4 inhibits DNA synthesis**. Flow cytometry profiles plotting DNA content on the x-axis (propidium iodide/PI staining) versus BrdU incorporation on the y-axis as detected by anti-BrdU immunostaining at 72 h after siRNA transfection. Boxes are labeled to indicate cell cycle phases where the mean percent S-phase cells is shown in parenthesis across the top of each histogram. The bar graph at bottom reports the percentage of G0/G1, S-phase/BrdU positive and G2/M cells in cultures that had been transfected with RECQ1, RECQ4 or luciferase/control siRNA pools (indicated in the key). Results shown are the mean ± SEM from three independent experiments.

Chapter 4: Functional characterisation of the role of RECQ1 and RECQ4 in DNA replication.

4.1. Nascent DNA analysis

In order to functionally characterise the role of RECQ1 and RECQ4 helicases in DNA replication and to determine whether reduced BrdU incorporation and proliferation of RECQ1- or RECQ4-depleted cells reflected impaired DNA replication initiation, I quantified nascent DNA production from origins in RECQ1- and RECQ4-depleted cells. The nascent DNA assay used quantitative real-time PCR to measure newly synthesized DNA corresponding to the lamin B2 origin and the GM-CSF Ori1 and Ori2 regions in neutral sucrose gradient centrifugation fractions from RECQ1- and RECQ4-depleted cells (Giacca et al., 1994). Thus, I found that origin sequence DNA was reduced by approximately 50% in RECQ1-depleted cells, and by >90% in RECQ4-depleted cells 72 h after siRNA transfection (Fig. 4.4.1). The reduced nascent DNA in RECQ1 and RECQ4 downregulated cells suggests that both RECQ1 and RECQ4 are involved in the initiation process of DNA replication.

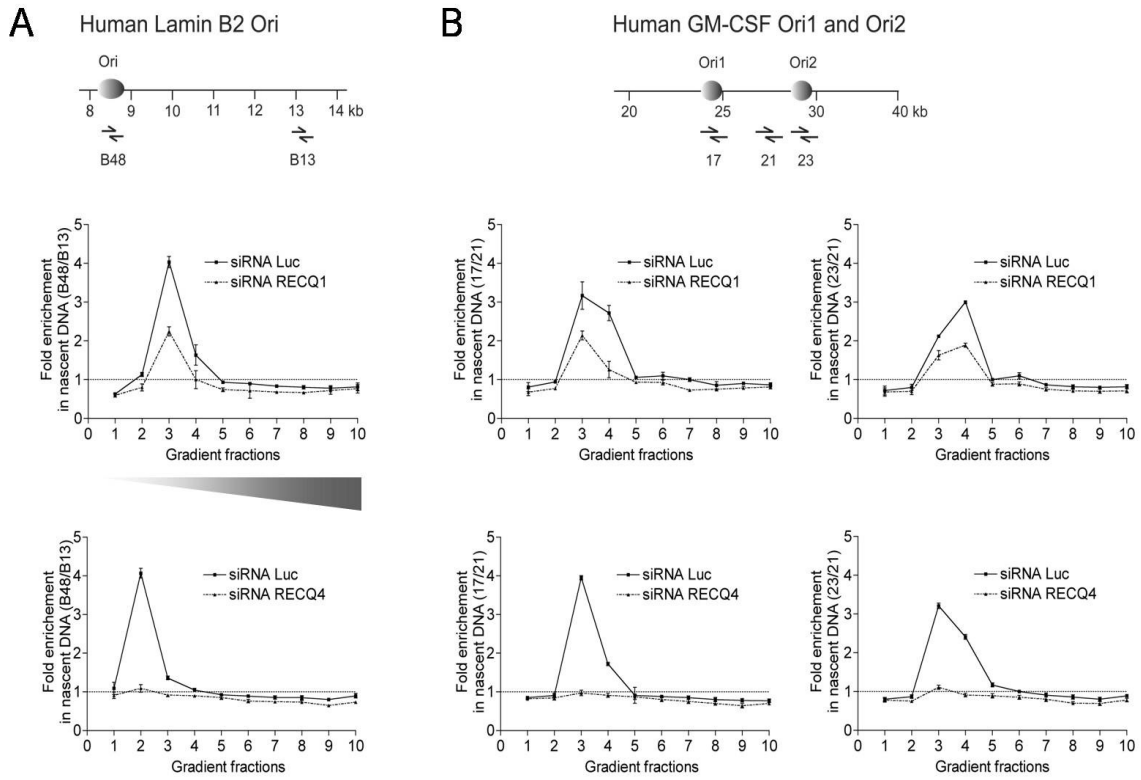


Figure 4.4.1: RECQ1 and RECQ4 depletion reduces newly synthesized nascent DNA from early firing human replication origins. A,B) Figures across the top of each column depict the human lamin B2 (A) and two GM-CSF (B) replication origin regions and the locations of primer pairs used to monitor origin- and adjacent non-origin sequences. Graphs below each figure show the fold enrichment in nascent DNA from origin regions versus adjacent non-origin regions in neutral sucrose gradient fractions prepared from T98G cells depleted of RECQ1 (middle row) or RECQ4 (bottom row) by RNAi transfection compared with control (luciferase RNAi-transfected) cells. Graphs report the mean ± SEM of three independent experiments.

4.2. Analysis of replication origin use and fork progression rate.

The sequential loading of RECQ4 and RECQ1 on origins at different times after pre-replicative complex (pre-RC) formation suggests these two proteins may play distinct roles during or after replication initiation. In order to gain more insight into these roles of RECQ1 and RECQ4, I performed replication track analyses on DNA from T98G cells transfected with RECQ1 or RECQ4 siRNA. These experiments were peformed in collaboration with the group of Prof. Raymond Monnat from the Universirt of Seattle (USA). In these experiments, cells were labelled sequentially for 40 min each with IdU and with CldU, and genomic high molecular weight DNA was isolated and stretched with the aid of microfluidic channels for replication track analysis (Fig.4.4.2) (Sidorova et al., 2008; Sidorova et al., 2009).

To evaluate origin firing efficiency, I estimated the fraction of origin firing events among all replication events during the 80 min labeling period. The origin firing events corresponded to tracks containing CldU only or a central IdU segment flanked by two CldU segments. Other ongoing replication events were elongating forks (IdU-CldU tracks) and converging forks (IdU-CldU-IdU tracks). This analysis revealed that RECQ1 or RECQ4 depletion reduced the probability of origin firing during the labeling period from 20% (in luciferase controls) to approximately 14 and 12% respectively (Fig. 7B). These results were consistent with my nascent DNA assay results, and indicate that RECQ1—and to a greater extent RECQ4—are important for efficient replication initiation. I also determined whether replication elongation was affected in RECQ1- or RECQ4-depleted cells. I measured the lengths of IdU segments in ongoing forks represented by two-segment (IdU-CldU) tracks. Interestingly, this analysis showed that in RECQ1—but

not RECQ4—depleted cells, first label segments were significantly ($p<0.001$) shorter than in control cells (Fig.4A.7C). Lengths of second label CldU segments in these same tracks were also shorter, as were CldU-only tracks in RECQ1-depleted cells (data not shown). These data suggest that RECQ1 may play an additional role in maintaining replication fork progression after initiation.

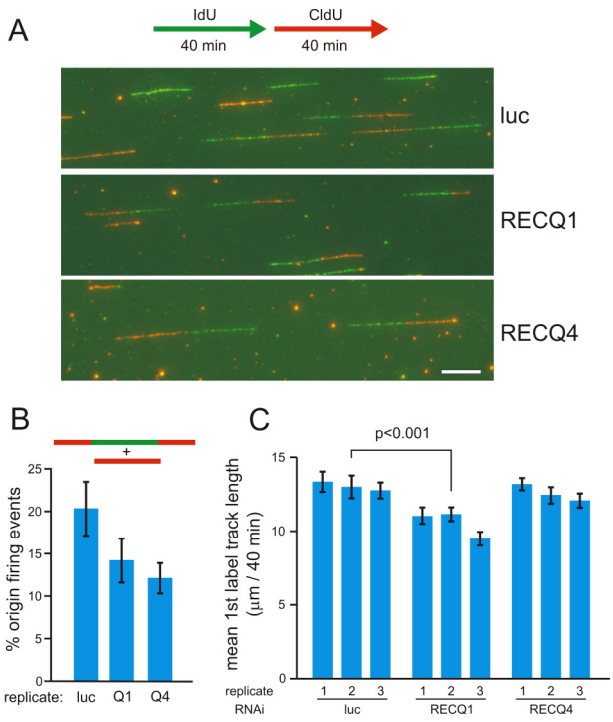


Figure 4.4.2: **Depletion of human RECQ1 or RECQ4 affects DNA replication dynamics.** A) An outline of the experimental protocol is shown on top, in which asynchronous cells were labeled consecutively with IdU (green), then with CldU (red) for 40 min each prior to isolating and stretching DNA for immunostaining as described in Methods. Shown below this are representative images of replication tracks in control (luciferase-), RECQ1- or RECQ4-depleted cells. All track photos are shown at identical magnification, where the white scale bar (lower left) is 10 um long representing approximately 30 kb of DNA or 20 minutes of replication fork progression. B) RECQ1 or RECQ4 depletion reduces the probability of origin firing in stretched DNA samples. Origin firing events among all tracks labeled during the protocol shown in A) were identified as CldU-only (red only) or CldU-IdU-CldU (red-green-red) triple-segment tracks (see diagram at top). The mean percentage of new origin firing events defined by these two track types among all labeled tracks is shown for three independent experiments in which 200 - 450 tracks/experiment were typed for control-, RECQ1- or RECQ4-depleted cells. Error bars are standard deviations. The p values calculated using Student's t-test are: p = 0.074 between luciferase and RECQ1, and p = 0.021 between luciferase and RECQ4. C) RECQ1 depletion, but not RECQ4 depletion, slows ongoing replication forks. The bar graph summarizes mean lengths of first-label IdU (green) segments labeled for 40 min in two-segment (green-red) tracks to ensure fork rate measurements were made from active replication forks (see A diagram above). Track lengths in um were measured using AxioVision Software (Carl Zeiss) for three independent experiments, where 150 - 370 first segment track lengths were measured for each sample. Error bars show 95% confidence intervals for sample means. The statistical significance of differences in mean track lengths was determined by a 2-sample Kolmogorov-Smirnov test. A representative p value is shown for a control (luciferase) vs. RECQ1-depleted sample pair.

4.3. Analysis of chromatin loading of replication factors

In order to provide additional mechanistic insight into the replication role of RecQ helicases, I also examined the order of chromatin loading of replication factors in RECQ1- or RECQ4-depleted T98G cells at the onset of S phase. Cells were synchronized by serum starvation in G0 prior to release by the addition of serum and concurrent transfection with RECQ1 or RECQ4 siRNA (Fig. 4.4.3A). Cells were harvested 24 h after serum stimulation and transfection in order to isolate cell populations highly enriched in S phase. The S phase synchronized luciferase siRNA transfected, RECQ1 siRNA transfected and RECQ4 siRNA transfected cells were biochemically fractioned to cytoplasmic (S2), nucleoplasmic (S3) and chromatin enriched (P3) fractions as described previously (Mendez and Stillman, 2000). Using this protocol I could achieve >90% RECQ4 depletion, and marked though less efficient depletion of RECQ1 consistent with our previous observation that RECQ1 is already expressed in G0 (Fig.4.4.3B). My results showed RECQ1 and RECQ4 depletion had no effect on the chromatin recruitment of MCM3 and ORC1. This suggests that RECQ1 and RECQ4 are loaded on origins after assembly of the pre-replication complex (Fig.4.4.3B). In contrast, the recruitment of PCNA to chromatin was decreased in RECQ1-depleted—and more markedly in RECQ4-depleted—cells, while RPA loading was suppressed only in RECQ4-depleted cells (Fig. 4.4.3B). These results suggest that RECQ4 is loaded onto chromatin and origins before origin firing, followed by RECQ1 loading with or immediately after RPA recruitment.

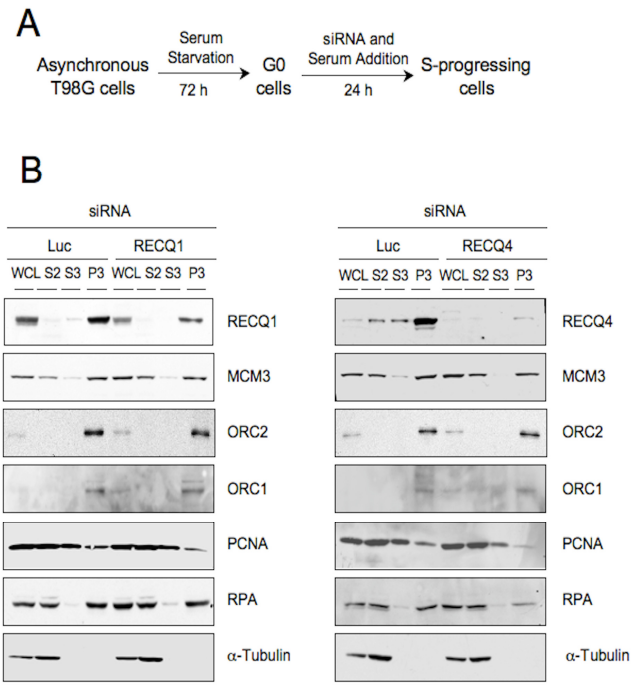


Figure 4.4.3: **RECQ1 and RECQ4 depletion impairs replication factor loading onto chromatin at the start of S phase.** A) Experimental outline for synchronization and release of human T98G cells to generate highly enriched S-phase progressing fractions. Asynchronous cells were grown for 72 h without serum, then refed, RNAi-transfected, and harvested after 24 h for blot analysis. B) Western blot analysis of whole and fractionated cell extracts from synchronized T98G cells. Left and right panels show the subcellular distribution of RECQ1 (left column top), RECQ4 (right column top), and four additional replication proteins together with an α-tubulin control (both columns) in whole cell lysates (WCL) and in cytosolic (S2), soluble nuclear (S3) and chromatin-enriched (P3) fractions prepared from RECQ1-, RECQ4- or control (luciferase)-depleted cells.

4.4. Model of cell-cycle dependent loading of RECQ1 and RECQ4 proteins onto DNA replication origins.

Based on the results of the ChIP analysis (4.2.4), I drew a model of DNA replication including two new proteins, RECQ1 and RECQ4. In this model, RECQ4 associates with replication origins during the late G1 phase of the cell cycle, when pre-RC complex formation takes place, and it remains bound to origins until mid S phase. RECQ1 loads to the origin after RECQ4 during early S phase of the cell cycle and remains at origins until mid S phase (Fig.4.4.4). The possible roles of these two RecQ helicases in DNA replication are discussed in the following paragraph.

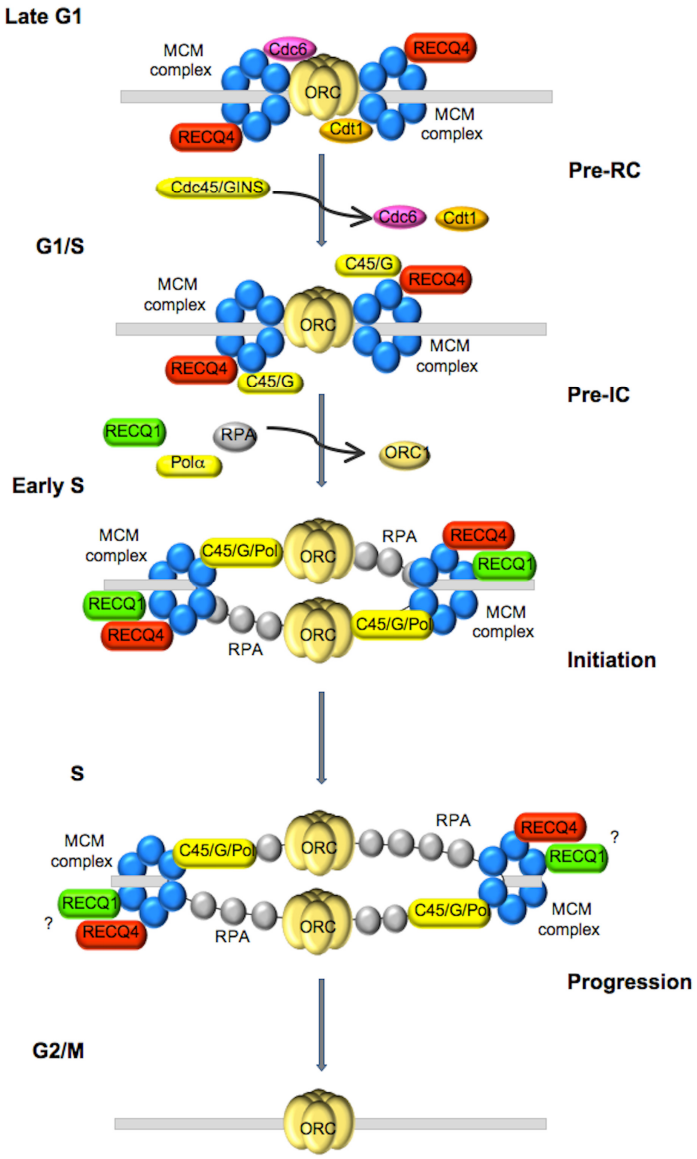


Figure 4.4.4: **Model of cell-cycle dependent loading of RECQ1 and RECQ4 proteins onto DNA replication origins.** RECQ4 is recruited to origins in late G1 as part of pre-replicative complex (Pre-RC) assembly. At the G1/S transition CDC6 release signals pre-initiation complex (Pre-IC) formation. RECQ1, as well as additional RECQ4, are recruited in early S-phase after the release of ORC1. Both RECQ1 and RECQ4 are no longer detectable on the lamin B2 origin by mid-S-phase, when either or both may be associated with active replisomes. This cell cycle phase dependent loading and subsequent loss of RECQ1 and RECQ4 for origins of replication suggest specific roles for each protein in replication initiation and, potentially, other specific aspects of DNA replication such as fork progression.

DISCUSSION

DNA replication is a tightly regulated process essential for the faithful transmission of genetic information in all living organisms (Blow and Ge, 2009; Diffley, 2004; Friedel et al., 2009). Given the centrality of replication, it is not surprising that cells have evolved complex mechanisms to regulate DNA replication origin firing and fork progression, as well as a variety of pathways to prevent replication defects, repair damaged replication forks, and enable fork reactivation. The different cellular and DNA metabolic defects associated with loss of RecQ helicase function in many organisms suggest that the five human RecQ helicases play important and probably distinct roles in DNA replication. However, the nature and mechanistic details of these roles remain controversial.

My work extends existing knowledge of the role of specific human RecQ helicase proteins in DNA replication. I found that only two of the five human RecQ helicases, RECQ1 and RECQ4, bind specifically *in vivo* to three well-defined human replication origins in unperturbed cells (Fig 4.1.2). RECQ4 is bound to origins in late G1, when the pre-replication complex (pre-RC) is assembled (Fig.4.2.4). Additional RECQ4 is found at origins at the G1/S border when CDC6 leaves origins. This suggests that RECQ4 may be an integral component of the human pre-initiation (pre-IC) complex. RECQ1, despite its presence throughout the cell cycle, is first reliably detected at origins at the onset of the S phase when ORC1 is lost and origins are licensed for firing. The amount of origin-bound RECQ1 and RECQ4 are maximal in early S phase, and by mid-S phase both are lost from the early firing human lamin B2 and GM-CSF replication origins. An intriguing mechanism for the loss of RECQ1 and RECQ4 from origins is that one or both proteins

become associated with active replisomes. Similar ChIP experiments performed in budding yeast showed that Sgs1, the RecQ homologue in *Saccharomyces cerevisiae*, interacts with the ARS305 yeast replication origin (Cobb et al., 2003). Interestingly, Sgs1 associate with ARS sequences at the beginning of the S phase similarly to RECQ1. Previous studies suggested that Sgs1 is the functional homologue of BLM and WRN in yeast on the basis of the observation that the cellular phenotype of Sgs1 mutant resembles the hyper-recombination phenotype of Bloom's syndrome patient cells and telomere defective Werner syndrome patient cells (Myung et al., 2001). However, I did not detect WRN and BLM at early replication origins. Thus, it is likely that Sgs1 in yeast plays distinct functions that are shared by the five RecQ helicases in humans. In this regard, role of Sgs1 in replication may be shared by RECQ1 or RECQ4 in humans as they are the only two RecQ helicases able to associate with DNA replication origins in unperturbed cells.

I did not detect the RECQ5 along with the two other human RecQ helicases, BLM and WRN, at early replication origins. However, I found that they interact with DNA replication origin upon treatment with the replication inhibitor HU (Fig.4.1.6) consistent with the idea that BLM, WRN, and RECQ5 function in S phase in response to replication stress to promote fork recovery or repair (Bachrati and Hickson, 2008). Previous reports showed both BLM and WRN translocate from PML bodies to the DNA replication sites when the cells are stressed with replication insults and DNA fiber assays revealed both BLM and WRN are necessary to restart the replication fork after replication stress. In line with these experiments, my finding that BLM, WRN, RECQ5 interact with replication origins under replication stress indicates that RecQ helicases are essential for the

replication machinery to survive against the replication stress generated endogenously or exogenously.

In addition to BLM, I also found that Rif1 and RMI1 interact with DNA replication origins under hydroxyurea induced replication stress conditions (Fig.4.1.7). Biochemical and genetic evidence demonstrated that Rif1 and RMI are integral component of the BLM complex and are essential for the maintenance of genome stability. Importantly, genetic analysis in chicken DT40 cells demonstrated that vertebrate cells lacking Rif1 and RMI1 have a higher level of SCE (Meetei et al., 2003; Xu et al., 2008; Yin et al., 2005). More importantly, *Rif*$^{-/-}$ DT40 cells display cellular sensitivity to replication stress and increased accumulation of stalled replication forks (Xu et al., unpublished results). These phenotypes are similar to those observed in *BLM*$^{-/-}$ cells. My data, along with the observations of Xu et al., favour a model that BLM in complex with Rif1 and RMI1 play an importat role in restarting the stalled replication forks.

I also investigated the origin-association kinetics of ORC2, RECQ1, and RECQ4 in early firing *versus* late firing origins (Fig.4.2.6). Thus, I found that RECQ1 and RECQ4's association with replication origin is not regulated by replication origin timing. The loading of RECQ1 and RECQ4 on late firing β-globin origin in HeLa cell lines follows the same trend observed for the early firing lamin B2 origin of HeLa cell lines or for the β-globin origin of K562 cell lines. However, the interaction of RECQ1 and RECQ4 with the β-globin origin in HeLa cells persists until late S when the origin fires suggesting that RECQ1 and RECQ4 are required also for late firing origins. Our studies indicate for the first time that there are indeed origin-associated loading time differences that may control early versus late firing. Similar analyses have been performed for MCM, CDC45p

and DNA polymerase α in *Saccharomyces cerevisiae.* The ChIP experiments performed by Aparicio et al., revealed a delay in origin association of CDC45p and DNA polymerase α proteins with the late firing ARS 501 origin when compared with the early firing ARS 305 origin (Aparicio et al., 1999). This appears to contradict with my observation that the loading time of RECQ1 and RECQ4 does not change between the early and late replication origins. This discrepancy might be explained considering the differences in the regulatory processes that control DNA replication in mammals and yeast. My experiments suggest a model for the mammalian system according to which replication factors assemble on most or all origins during G1/early S, when cyclin-dependent kinase levels are permissive, but further signals are necessary to start replicating at a specific point in S phase. Although theres is difference in the temporal loading of replication proteins between yeast and mammals, the time RECQ1 and RECQ4 remain associated with late firing origins is interestingly similar to the time that CDC45p and DNA polymerase α remain associated with late ARS501 origins (Aparicio et al., 1999). This supports our idea that RECQ1 and RECQ4 are important also for the firing of late replication origins.

My results suggest that RECQ1 and RECQ4 are integral components of the replication complex and play an important role in DNA replication initiation. Consistent with this idea, nascent DNA experiments showed that RECQ1-depleted, and even more strikingly, RECQ4-depleted, cells have reduced amounts of nascent, newly synthesized DNA containing the lamin B2 and GM-CSF Ori1 and Ori2 origin sequences (Fig 4.4.1). This replication defect phenotype was also supported by colony forming assays and BrdU incorporation experiments (Fig 4.3.1). RECQ1- and RECQ4-depleted cells show reduced

proliferation and an elevated G1 fraction. Similar analyses, in which levels of other known replication initiation proteins such as ORC2 have been reduced, also led to a decrease in proliferation and an elevated G1 fraction (Machida et al., 2005). These responses may protect cells from premature S phase entry without the proper number of activated replication origins.

Replication track analyses confirmed these results by showing that RECQ1, and especially RECQ4, are required for efficient replication initiation (Fig 4.4.2). In addition, RECQ1-depleted cells display shorter replication tracks than control cells, indicating that RECQ1, though not RECQ4, might play an additional role in replication fork progression in unperturbed cells. Similar DNA fiber analyses previously identified a role for BLM in efficient restart of replication forks, and in the suppression of new origin firing after DNA damage (Davies et al., 2007; Rao et al., 2007). WRN, in contrast, is not required for efficient restart but is required to ensure normal fork progression after recovery from HU-mediated arrest (Sidorova et al., 2008). Thus, RECQ1, BLM and WRN may play distinct functions in modulating fork activity and fork rate during S-phase.

My work also establishes new parallels and provides new mechanistic insight into the comparative roles of human RECQ4 and *X. leavis* RECQ4 proteins in replication. *X. leavis* RECQ4 is required to establish active replication forks, and appears to act by facilitating the loading of replication factors at origins (Matsuno et al., 2006; Sangrithi et al., 2005). The recent demonstration that RECQ4, as well as RECQ1, possess intrinsic ATP-dependent helicase activity (Xu and Liu, 2009) suggests that both proteins might facilitate replication initiation by promoting origin unwinding with the MCM helicase complex. Consistently, a recent study reported that RECQ4 physically interacts with the

MCM2-7 replicative complex. This interaction is mediated by MCM10, which regulates the helicase activity of RECQ4 possibly to prevent unlicensed replication initiation (Xu et al., 2009b). Interestingly, the same authors showed that the MCM10-RECQ4 interaction is not shared by other human RecQ helicases, including RECQ1, suggesting that RECQ1 might interact with a different component of the replication complex. Thus, human RECQ4 and RECQ1 recruitment following assembly of the pre-RC might facilitate replication initiation in two ways: by origin unwinding, and by the recruitment of proteins required to assemble the replisome. Consistent with this idea, Xu and collaborators reported that RECQ4 down-regulation does not affect the chromatin binding of MCM and CDC6 supporting the notion that RECQ4 loads on the origin downstream of these pre-RC complex factors, but affects the chromatin recruitment of the GINS complex (Xu et al., 2009b). This finding was also confirmed by a recent study showing that the assembly of the Cdc45-MCM2-7-GINS complex requires RECQ4 and MCM10 (Im et al., 2009). Moreover, we found that RECQ4 and RECQ1 are required for efficient PCNA loading that precedes and is required for polymerase loading onto the replication fork. RECQ4 also facilitates the loading of the ssDNA binding protein RPA, as already shown in *X.leavis* (Sangrithi et al., 2005). Since RPA is likely to act in concert with helicases to stabilize unwound replication origins, a lack of RECQ4 helicase activity could hamper origin unwinding with the generation of single-stranded DNA as well as RPA and PCNA loading onto nascent replication forks.

Previous studies demonstrated that recombinant human RECQ4 can replace *X. leavis* RECQ4 in DNA replication in egg extracts (Sangrithi et al., 2005), and that deletion of the N-terminus of mouse RECQ4 leads to early embryonic lethality (Ichikawa et al.,

2002). Of note, the N-terminus of *X. leavis* RECQ4 shares homology with the N-termini of the yeast proteins Sld2. These proteins are essential for DNA replication in yeast, but do not appear to have readily identifiable, conserved vertebrate homologs (Matsuno et al., 2006).

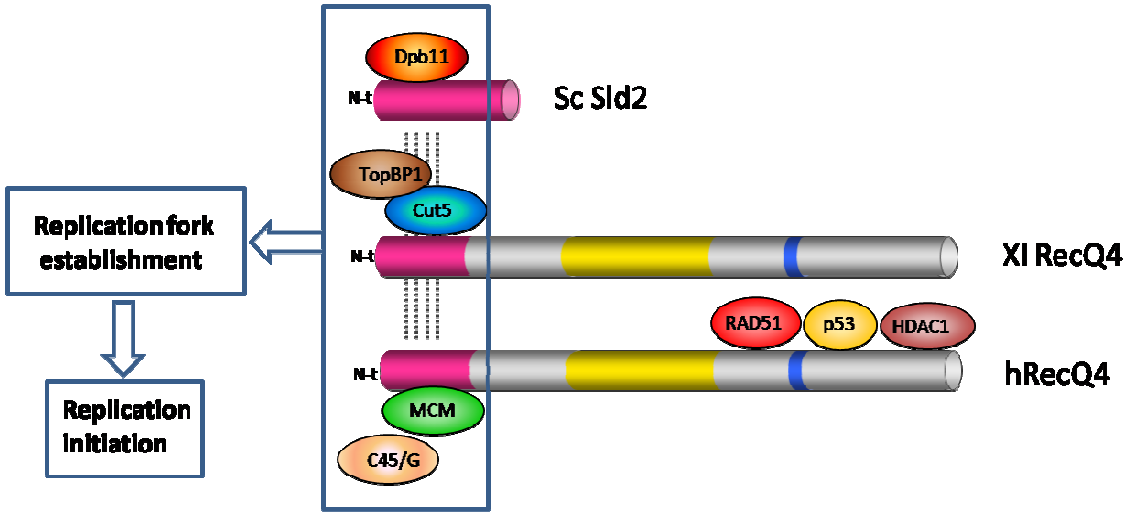


Figure 5.1. **Schematic structures of Sld2 of *Saccharomyces cerevisiae* (Sc), *Xenopus laevis* (Xl) RecQ4, human RecQ4**. The N-termini shown in pink and the dashed lines indicate the location of the Sld2 domains. Proteins interacting with the N-termini are also indicated.

My results support the idea that there may be functional conservation between human and *Xenopus* RECQ4s and these essential yeast proteins during DNA replication initiation. Moreover, my finding that RECQ4 has a role in replication initiation, though probably not in elongation, provides new insight into the function of this helicase during the replication process. The replication function of RECQ4 is likely to be preserved in RTS patients since most of the mutations associated with RTS are located in at the centre of RECQ4 leaving intact the N-terminal domain of the protein.

Cellular functions of RECQ1, in contrast to RECQ4, are not as well-defined. Previous studies have shown that acute depletion of RECQ1 affects cellular proliferation (Futami et al., 2008; Sharma and Brosh, 2007, 2008). Moreover, RECQ1 depletion renders cells sensitive to DNA damage, and leads to spontaneous γ-H2AX foci formation as well as elevated levels of sister chromatid exchanges. These results indicate that RECQ1 plays an important role in the maintenance of genome stability (Sharma and Brosh, 2007). Surprisingly, RECQ1-deficient mice do not show any apparent phenotypic difference when compared to wild-type mice, although embryonic fibroblasts derived from the RECQ1-deficient mice show hypersensitivity to ionizing radiation and spontaneous chromosomal breakage (Sharma et al., 2007). This discrepancy between cellular and organismal phenotypes could be explained by functional compensation for the loss of RECQ1 during development, as has been reported for proteins such as the tumor suppressor pRB (Sage et al., 2003). Alternatively, loss of RECQ1 alone may not reveal a phenotype in the absence of additional defects. This has been the case for Wrn-deficient mice that develop accelerated tumorigenesis only on a p53-minus background (Lebel et al., 2001), or an organismal phenotype resembling Werner syndrome with features of premature aging only when combined with later generation telomerase deficiency (Chang et al., 2004; Du et al., 2004). An additional explanation for the lack of an organismal phenotype is that the RECQ1 deficient mice do not carry a complete knockout of the *RECQ1* gene, but only a deletion of a portion of the gene that includes the helicase domain IV and part of helicase domain V. Thus, the RECQ1 region important for its replication function might be still expressed in these mice. Likewise, RECQ4 mouse models carrying deletions or nonsense mutations of the helicase domain are still

viable, and only the deletion of the N-terminal domain of the *RECQ4* gene leads to embryonic lethality (Dietschy et al., 2007; Ichikawa et al., 2002). One potential role for RECQ1 in genomic stability assurance was suggested by the recent identification of RECQ1 as part of a piRNA complex implicated in transcriptional gene silencing (Lau et al., 2006). The size, single-stranded nature and strand-specificity of short noncoding piRNAs suggest that piRNA generation could occur during DNA replication and thus be influenced by RECQ1 or other replication proteins (Bateman and Wu, 2007). Moreover, a recent study demonstarted that RECQ1 is an integral component of the replication complex required for the Kaposi's sarcoma-associated herpesvirus replication (Wang et al., 2008).

My results are consistent with these findings, and provide new evidence that RECQ1 plays an important role in replication initiation and replication fork progression. An intriguing possibility that cannot be excluded at this stage is that RECQ1 might be required for the firing of a subset of origins that lack RECQ4. This function could be particularly relevant for the firing of dormant origins after DNA replication stress that either stalls or slows normal replication forks. RECQ1, together with RECQ4, may also play a role in early S phase to stabilize or repair replication forks. This role would be consistent with the DNA damage sensitivity of both RECQ1 deficient HeLa cells and RECQ4 deficient primary fibroblasts (Jin et al., 2008; Sharma et al., 2007).

In summary, my work identifies important and distinct roles for two human RecQ helicase proteins, RECQ1 and RECQ4, in DNA replication. RECQ4 appears to function early, in replication initiation, when pre-replication complex assembly takes place and active replisomes are assembled. RECQ1 is also required for efficient replication

initiation and might play an additional role during replication elongation. The mechanisms by which RECQ1 and RECQ4 participate in replication initiation, and whether helicase activities are required in this process, demand further investigation. My studies also begin to suggest how the loss of specific human RecQ helicases may promote genomic instability and promote tumorigenesis as part of characteristic, heritable human disease phenotypes.

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VI. Abbreviations

Abbreviation		Explanation
ATP	-	Adenosine Triphosphate
ADP	-	Adenosine diphosphate
NTP	-	Nucleoside triphosphate
SF	-	SuperFamily
ss DNA	-	Single stranded DNA
dsDNA	-	Double stranded DNA
AAA^+	-	ATPases associated with various cellular activities
RQC	-	RecQ C-terminal domain (RQC)
HRDC	-	Helicase RNase D C-terminus
WH	-	Winged helix
NLS	-	Nuclear localization signals
WS	-	Werner Syndrome
BS	-	Bloom Syndrome
RTS	-	Rothmund- Thomson Syndrome
BGS	-	Baller- Gerold syndrome
BLM	-	Bloom syndrome protein
WRN	-	Werner syndrome protein
RMI	-	RecQ mediated genome instability
FA	-	Fanconi anaemia
PML	-	Promyelocytic leukemia
RNAPII	-	RNA polymerase
MLH	-	mutL homologue

FEN1	-	Flap structure-specific endonuclease
SCE	-	Sister chromatid exchanges
MEF	-	Mouse embryonic fibroblast
ES cells	-	Embryonic stem cells
DSB	-	DNA double-strand break
SSB	-	Single strand binding protein
BER	-	Base excision repair
HU	-	Hydroxyurea
MMS	-	Methyl methanesulphonate
CPT	-	Camptothecin
4NQO	-	4-nitroquinoline-1-oxide
HR	-	Homologous recombination
HJ	-	Holliday junction
SUMO-1	-	Small ubiquitin-related modifier-1;
TRF	-	Telomeric repeat binding factor
PCNA	-	Proliferating-cell nuclear antigen
RPA	-	Replication protein A
MRN	-	The MRE11/RAD50/NBS1 complex
ATM	-	Ataxia telangiectasia mutated
RNAi	-	RNA interference
siRNA	-	Small interfering RNA
IgG	-	Immunoglobulin G
bp	-	Base pairs
nt	-	Nucleotides
UV	-	Ultraviolet
ChIP	-	Chromatin immunoprecipitation

BrdU	-	Bromodeoxyuridine
CldU	-	Chlorodeoxyuridine
IdU	-	Iododeoxyuridine
MNase	-	Micrococal Nuclease
RF	-	Replication Fork
ICL	-	Interstrand CrossLinks
CMG	-	CDC45, MCM, GINS complex

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