Characterization of RTEL/PCNA interaction in the maintenance of genomic stability

By

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To my parents who I owe my genes

&

To my wife with whom I have merged my genes

Abstract

Previously, we have demonstrated that a DNA helicase-like protein, termed RTEL (regulator of telomere length) is essential for the maintenance of genomic stability. RTEL deficiency induced telomere loss and genomic instability, leading to embryonic lethality. However, the role of RTEL in these biological pathways is largely unknown. To uncover RTEL's function(s), we applied several approaches to identify the proteins that could interact with RTEL. Proliferating Cell Nuclear Antigen (PCNA), the key regulator of the replication fork, was found to be a strong candidate. In this study, we have demonstrated the interaction between RTEL and PCNA. Further characterization of the interaction between RTEL and PCNA revealed that the interaction is important for maintaining genomic stability. Due to the essential role of PCNA in nucleic acid metabolism as a component of the replication and repair machinery, its interaction with RTEL could be the key to the role of RTEL in the maintenance of genomic stability and mouse development.

Along with a bioinformatics approach, we have employed several biochemical approaches to identify the interaction of PCNA with RTEL. Using co-immunoprecipitation, we have demonstrated that RTEL can specifically interact with PCNA. A PCR-based mutagenesis method was used to mutate the PCNA-interacting motif (PIP) in RTEL. Further we have demonstrated that several key amino acids in the PIP motif are responsible for mediating RTEL/PCNA interaction by using co-immunoprecipitation and immunofluorescence studies. Using a gene-targeting approach, we have specifically knocked-in a mutant RTEL with a mutation in PIP motif into mouse

genome. Thus we have developed a transgenic mouse model to study the significance of the interaction between RTEL/PCNA *in vivo*.

This study not only validated the interaction of RTEL with PCNA, via the PIP box, but also generated the RTEL PIP mutant alleles for further functional analysis by transgenic approaches. We employed biochemical and cytogenetic studies to characterize the phenotypes in $Rtel^{I1169A/I1169A}$ mouse. This is the first direct genetic approach to address whether PCNA is an important downstream mediator of RTEL's function in the regulation of genomic integrity.

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List of abbreviations

3-AT	3-Amino-1,2,4-triazole
ATP	Adenosine-5'-triphosphate
BLAST	Basic local alignment tool
BLM	Bloom’s syndrome gene
BSA	Bovine serum albumin
DNA	Deoxy-ribonucleic acid
DAPI	4,6-diamidino-2-phenylindole
DSB	Double strand break
DSBR	Double strand break repair
ES	Embryonic stem
FANCJ	Fanconi anemia complementation group J
FISH	Fluorescence *in situ* hybridization
GWAS	Genome-wide association study
HR	Homologous recombination
IF	Immunofluorescence
IP	Immunoprecipitation
MBD6	Methyl-CpG-binding domain 6
MEF	Mouse embryonic fibroblast
MLH1	MutL homolog1
MMR	Mismatch repair
PBS	Phosphate buffered saline

PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEG	Poly ethylene glycol
PIP	PCNA-interacting protein
RNA	Ribonucleic acid
RTEL	Regulator of telomere length
SDS	Sodium dodicyl sulphate
SDSA	Synthesis dependent strand annealing
SLE	Systemic lupus erythematosus
SRS2	Suppressor of RAD6 mutant 2
SUMO	Small ubiquitin like-modifier
TBS	Tris buffered saline
TLS	Trans-lesion synthesis
TMM	Telomere length maintenance mechanism
Q-FISH	Quantitative-fluorescence in situ hybridization
WRN	Werner’s syndrome gene
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
Y2H	Yeast two-hybrid

Chapter 1: Introduction

1.1 Genomic instability

In living organisms, the integrity of the genome is maintained in order to preserve replicative potential and genetic stability. Multiple processes in DNA metabolism including replication, repair, recombination, telomere maintenance and damage signalling are maintained in cells in a highly regulated and coordinated manner protecting the genome from alterations (Anderson, 2001). However, genetic alterations, such as mutations, DNA rearrangements, changes in chromosome number and structure occur in cells that can lead to so called 'genomic instability'. Although such events have detrimental effects in cells, they are also the basis of genetic variation and evolution (Aguilera and Gomez-Gonzalez, 2008). In certain instances, genomic instability forms the basis of generation of variability, such as immunoglobulin (IgG) diversification (Maizels, 2005). It, however, is frequently associated with inherited diseases, premature ageing and predisposition to cancer (van Brabant *et al*., 2000). Parallel to the observation that a strong correlation exists between age and cancer in human, aging is associated with increased genomic instability in yeast (McMurray and Gottschling, 2003).

Genomic instability refers to a broad range of genetic alterations. Depending on the type of pathway and cellular process associated with the alteration, the outcome varies from single point mutations to gross chromosomal changes (Aguilera and Gomez-Gonzalez, 2008). Table 1 lists the major genomic instability mechanisms and the pathways involved. A comparatively large number of genes are associated with these pathways. More than 230 genes have been identified for high fidelity DNA replication

and another 250 genes could be involved in DNA damage repair (Amberger *et al*., 2009; Anderson, 2001).

Table 1: Major pathways involved in the maintenance of genomic integrity

Mechanism	Genetic alteration	Pathways involved	References
Chromosomal Instability (CIN)	Gain or loss of chromosome, resulting in change in chromosome number	Failure in mitotic chromosome transmission and checkpoints	(Draviam *et al*., 2004)
Micro-satellite or Mini-satellite instability (MIN)	Change in copy number or composition of repetitive DNA sequences	Slippage of replication machinery Mismatch repair(MMR) pathway Homologous recombination	(Gorgoulis *et al*., 2005)
Mutations	Changes in base pair, micro-insertions, micro-deletions	Replication errors, failure in DNA repair, specifically, base excision repair, Mismatch repair, error prone synthesis of DNA	(Friedberg, 2003)
Chromosomal rearrangements	Translocation, duplication, inversions, deletions in chromosomes	Homologous or non-homologous recombination pathway	(Friedberg, 2003)

It is not surprising that many of these genes are redundant in function to ensure the high fidelity of the processes and tight regulation at temporal and spatial level (van Brabant *et al*., 2000). Unless redundant safeguards exist, loss of function of a vital gene results in DNA damage or disruption of control mechanisms. However, in cases where a genetic defect is more subtle, genomic instability results in a dynamic process. Mutations accumulate in the genome over long time, most of which have no detrimental effect. Eventually the cells lose their control mechanisms as a result of mutations in key genes

and gene regulatory elements. Examples of such cells are cancer cells, where genomic instability is a common feature (Aguilera and Gomez-Gonzalez, 2008).

1.2 The role of DNA helicases in the maintenance of genomic stability

DNA helicases are defined as a group of enzymes that possess the capacity to unwind duplex nucleotide structures (Matson *et al*., 1994). They can act as molecular motors utilizing the energy from nucleotide 5´ triphosphates, such as ATP to translocate or move and break the hydrogen bonds to facilitate unwinding. In many instances such unwinding capacity is also associated with regulatory functions (Lohman and Bjornson, 1996).

About 1% of the eukaryotic genome is assumed to code for enzymes with a helicase activity (Eisen *et al*., 1995). Despite their diverse function and structure, many of them share common conserved motifs. So far, seven highly conserved signature motifs have been identified in DNA helicases –I, IA, II, III, IV, V and VI. These motifs have been characterized functionally, and serve as a very useful template for identifying new helicases (Gharibyan and Youssoufian, 1999). It has been demonstrated that mutations in motif I or motif II result in loss of ATPase activity, leading to loss of the capability to unwind DNA. Motif I consists of a highly conserved motif GxGKS/T that interacts with the carbon 6 in the sugar atom and phosphate groups of the tri-nucleotide. A conserved aspartate residue in motif II binds to the Mg^{2+} ion required for the ATPase reaction (Gorbalenya *et al*., 1989). In addition, motif II consists of an additional conserved glutamate residue that plays role in ATP hydrolysis. These two residues form the basis of for naming two different class of helicases- DExx-box and DEAD- box helicases (Gross

and Shuman, 1995; Gross and Shuman, 1996). Based on the presence of these conserved helicase motifs, all helicases are categorized into super-families SFI to SFVI (Eisen *et al*., 1995).

Almost all pathways involving DNA metabolism require unwinding of either a duplex DNA or DNA-RNA hybrid. Although DNA helicases share similar DNA-unwinding catalytic activities, they have evolved to function in different biochemical pathways. The functional importance of the activity of DNA helicases is to provide accessibility to the duplex DNA so that other factors can act. Thus, helicases can be involved in co-ordinating important events in DNA replication, recombination, transcription, DNA repair, chromosomal segregation etc. Several DNA helicases have been demonstrated to play vital roles in the maintenance of genomic stability (Eisen *et al*., 1995). Table 2 lists some of them and their demonstrated functions.

Table 2: A selection of eukaryotic DNA helicases with the roles involved in the maintenance of genomic integrity

Gene		Function	References
Yeast	Mammals		
Mcm4	MCM4	Replication	((Friedberg, 2003)
Dna2	DNA2	Replication	(Friedberg, 2003)
Pif1	PIF1	Replication, RNA-DNA helicase	(Klein and Petes, 1981)
Sgs1	BLM	DSB repair, RecQhelicase	(Zieg *et al*., 1978)
	WRN	DSB repair, RecQ helicase	(Raveendranathan *et al*., 2006)
Srs2	RTEL1	DSB repair, homologous recombination	(Barber *et al*., 2008)

1.3 The Regulator of telomere length (RTEL)

Regulator of telomere length (RTEL) is a newly identified DNA helicases that has been shown to be required for the maintenance of genomic instability (Ding *et al*., 2004)(Barber *et al*., 2008). This gene was identified in an effort to find the essential factors that govern the telomere length diversity in mammalian species. RTEL was found to be located at a region that is associated with species specific telomere length regulation in mice (Zhu *et al*., 1998). Genetic inactivation of RTEL demonstrated that RTEL plays an important role in the maintenance of telomeres and genomic stability (see the following sections).

1.3.1 Genomic organization and conserved domains of RTEL

RTEL gene encodes a protein of 1209 amino acids. Based on the highly conserved helicase motifs and other protein features, RTEL is categorized as a member of the helicase super family I (Ding *et al*. 2004). The organization of conserved domains in RTEL is described in figure 1.

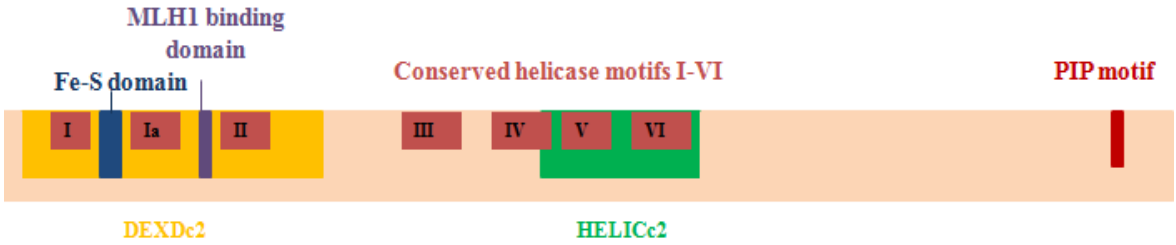


Figure1. Conserved domains present in RTEL. In addition to seven conserved helicase motifs, RTEL possesses Fe-S domain, PIP motif and MLH1 binding domain.

RTEL is also classified as a member of DinG, Rad3 related DNA helicases based on the presence of DEXDc2 and HELICc2 domains (Gorbalenya *et al*., 1989). Interestingly RTEL possesses a Fe-S motif at its N terminus which is absent in most DNA helicase proteins. So far, only five DNA helicases have been found to have a Fe-S motif, which has been shown to be necessary for helicase activity as well (Rudolf *et al*., 2006). Besides the conserved helicase motifs, RTEL also contains two conserved protein interacting motifs- MLH1 (Mut L homolog 1) binding motif at the N terminus and PCNA Interacting Protein (PIP) motif at the C terminus.

1.3.2 Expression of RTEL in cells and tissues

RTEL is widely expressed in proliferating cells (Ding *et al*., 2004). In the developing mouse embryos, its expression pattern coincided with areas of active proliferation. In adult mice, northern blot analysis detected RTEL expression in spleen, testis, thymus, Peyer's patches, kidney and intestine. However, fully differentiated tissues, such as brain, heart, lung, skeletal muscles, skin and white fat tissue did not express RTEL. In addition, RTEL is induced in regenerating muscle or liver cells as well as in the proliferating lymphocytes, implying its role in proliferation. Taken together, these data indicate RTEL expression is associated with cell proliferation.

1.3.3 The phenotypes of RTEL knock out mouse (*Rtel*$^{-/-}$)

In order to determine the *in vivo* function of RTEL, a transgenic approach was employed to knock out RTEL in mice (Ding *et al*., 2004). *Rtel*$^{-/-}$ mice were embryonic lethal, died between E8.5 to E10.5. In addition, these embryos exhibited abnormalities in

tissues that are actively proliferating, such as the nervous system, the heart, the vasculature, and extra-embryonic tissues.

Cytogenetic analyses on *Rtel*$^{+/+ \text{ and }}$ *Rtel*$^{-/-}$ ES cells demonstrated the importance of RTEL at cellular level, specifically for maintaining telomere length. While the average length of telomeres was found to be ~54kb in *Rtel*$^{+/+}$ ES cells with flow FISH analysis, the telomeres in *Rtel*$^{-/-}$ ES cells were only ~37kb. Q-FISH analysis further revealed large increase in the number of the cells without telomeric signals in *Rtel*$^{-/-}$ ES cells as compared to the controls. In addition, differentiating *Rtel*$^{-/-}$ ES cells showed chromosomal abnormalities, including end-to-end fusion, chromosome breaks and fragments (Figure 2) (Ding *et al*., 2004). Taken together, these data indicated that RTEL is essential for maintaining genomic stability and telomere homeostasis. However, how RTEL is involved in this biological pathway is largely unknown.

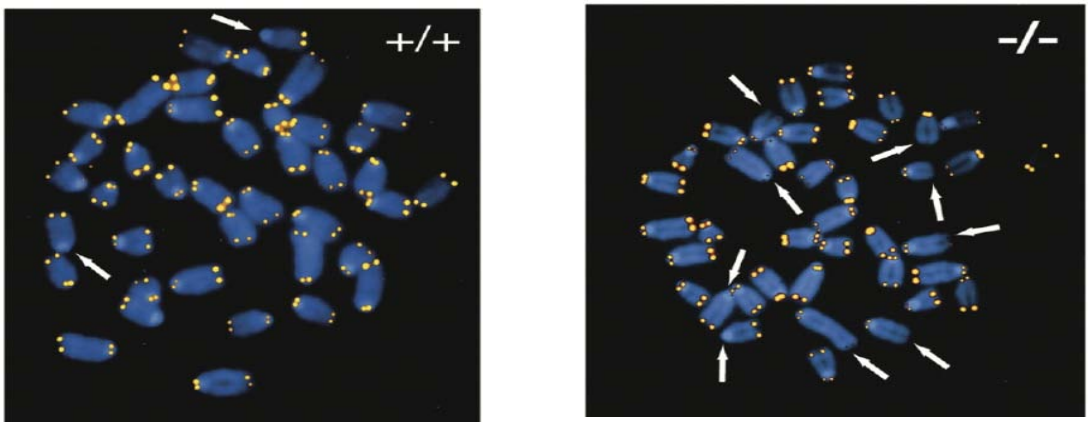
1.4 Telomere dysfunction and Genomic Instability

1.4.1 Telomeres

Telomeres are the specialized DNA-protein structures that protect eukaryotic chromosomal ends (Hodes, 2001). Telomeres of higher eukaryotes consist of long, repetitive TTAGGG subunits, which terminate in a 3′ single-strand G-rich overhang. This 3′ overhang has been shown to form a lariat structure, known as the t-loop, by invading the double-stranded region of the telomeric DNA. The t-loop hides the overhang and protects it from degradation (Griffith *et al*., 1999). Telomeres protect the chromosomal ends from end-to-end fusion, recombination, and DNA degradation that can lead to genomic instability (Greider and Blackburn, 1996).

A. Wild type

Rtel$^{-/-}$



B. 4 hr

8 hr

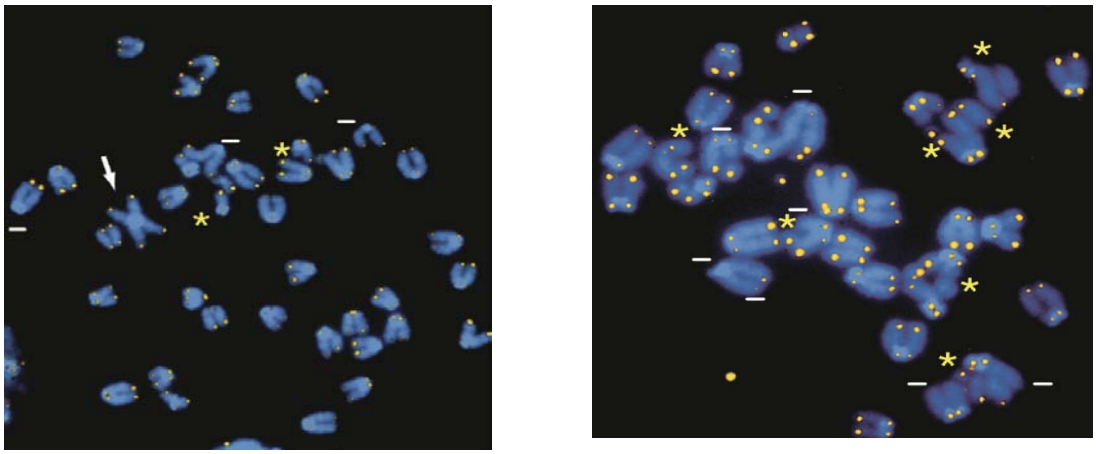


Figure 2. Telomere dysfunction in *Rtel*$^{-/-}$ ES cells. A. Q-FISH analysis of *Rtel*$^{+/+}$ (denoted by +/+) and *Rtel*$^{-/-}$(denoted by -/-) ES cells with fluorescent PNA probes (yellow) on metaphase spreads. Compared to *Rtel*$^{-/-}$ ES cells, a large portion of chromosomes in *Rtel*$^{-/-}$ ES cells lack telomeric signals. **B**. Chromosomal abnormalities in differentiating *Rtel*$^{-/-}$ ES cells. Metaphase spreads were stained with DAPI and probed for telomeric PNA probes (yellow). When the cells were allowed to differentiate without LIF, *Rtel*$^{-/-}$ ES cells showed gross chromosomal abnormalities, such as fusion (arrow), loss of telomeric signals (bar) and chromosomal fragmentation (asterisk). Adapted from (Ding *et al*., 2004).

1.4.2 Mechanisms for the maintenance of telomeres

A minimum telomere repeat length is required for the protection of telomeric structure at chromosomal ends. Somatic cells that lack telomere length maintenance mechanism (TMM) face gradual shortening of telomeres due to an end replication problem, C-strand degradation etc (de Lange, 2005). To counteract the telomere shortening problems, stem cells, cancer cells and other immortalized cells acquire TMM usually by increasing telomerase activity, a reverse transcriptase that adds telomeric repeats *de novo* after each cell division (Shay and Bacchetti, 1997). Alternative ways to maintain telomere length has also been described, *i.e.* alternative lengthening of telomeres (ALT) which uses homologous recombination between telomeric repeat sequences to extend telomere length (Dunham *et al*., 2000).

1.4.3 Telomere dysfunction as a cause of genomic instability

Integrity of the telomeres is essential for cells to maintain replicative potential and genetic stability. Studies have shown that telomere integrity and function require at least three factors: (1) regulation of telomere binding proteins; (2) the integrity of the overhang; and (3) a minimal length in the TTAGGG repeating sequence (Blasco, 2003). The telomere-specific binding proteins form a complex, the so called shelterin or telosome (Figure 3). In this complex, TRF1 and TRF2 bind to duplex TTAGGG repeats and POT1 binds to single strand repeat sequences. While TRF1 is a negative regulator of telomere length, TRF2, in addition to controlling length, is involved in formation and maintenance of the t-loop. Other proteins in this complex, such as TIN2, TPP1 and

RAP1, are required for preventing inappropriate homologous recombination (HR) or non-homologous end joining (NHEJ) recombination at telomeres (de Lange, 2005).

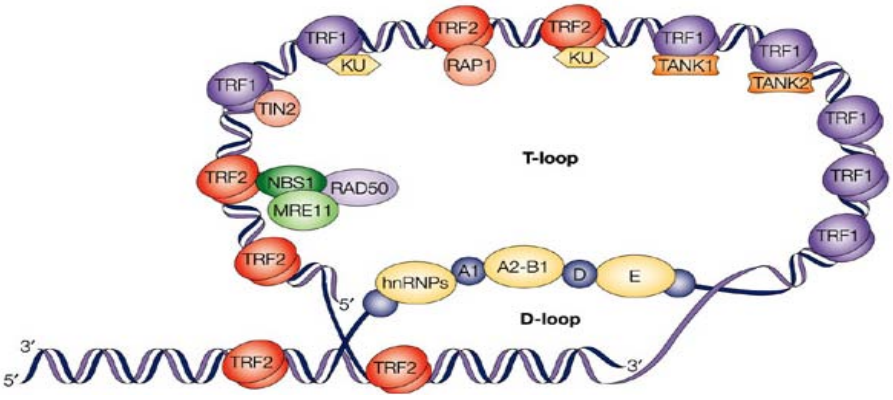


Figure 3: Structure of the shelterin complex. A current model of telomeric structure suggests that the shelterin complex consists of several proteins including TRF1, TRF2, tankyrase and TIN2. The 3' G-overhang of the telomere invades a duplex telomeric region to form t-loop to protect the chromosomal ends. Adapted from (Neumann and Reddel, 2002).

However, the average length of telomere repeats is set by equilibrium between the mechanisms that lengthen and shorten telomere tracts. Progressive loss of telomeric repeat sequences and/or alteration of telomere capping function can lead to loss of telomere function (Harley *et al*., 1990), eventually genomic instability (Figure 4). It has been demonstrated that in absence of telomere capping, DNA damage response pathways are activated. The DNA damage is sensed by the MRE11/Nbs1/Rad50 complex. This event is followed by the activation of pathways, such as p53, p21, Rb/$p16^{INK4A}$, which arrest the proliferation of the genetically damaged cells. In addition, progressive loss of telomeres results in extremely short telomeres that can be recognized as double strand breaks activating other signalling pathways such as ATM/ATR, Chk1 and Chk2

(Allsopp *et al*., 1992; Wright and Shay, 1995). Inappropriate repair or escape from the check point mechanisms can lead to genomic instability by breakage/fusion/bridge (B/F/B) cycle or other mechanisms (Figure 4) (Campisi, 2005). The B/F/B cycle can be initiated by fusion of sister chromatids at the ends after the replication of chromosomes without telomeres. The fused sister chromatids form a bridge during anaphase which is subsequently broken as the two centromeres are pulled apart at later stage of mitosis. However, the breakage point usually lies at a different point than the fusion point, leading to chromosomes with terminal duplication or deletion (Murnane, 2006). The B/F/B cycle can continue for several generations leading to genomic instability (Gisselsson, 2003).

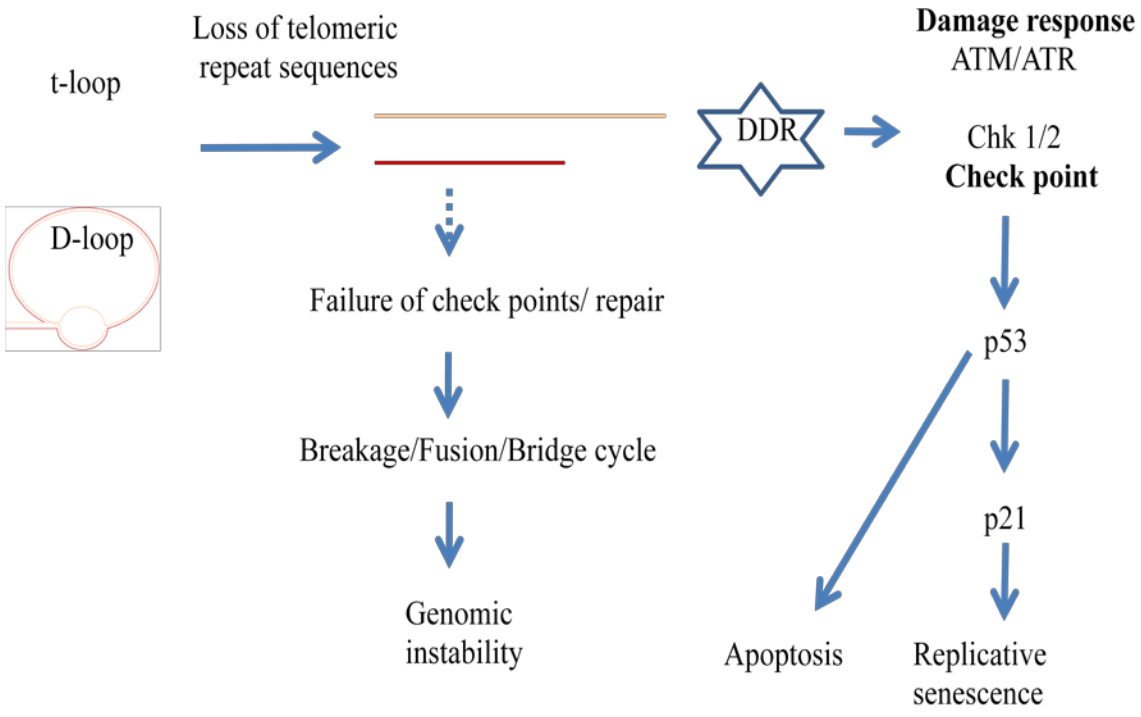


Figure 4: Telomere dysfunction can lead to genomic instability. Progressive loss of telomeric repeat sequences can disrupt the telomeric structure. Further cellular proliferation or unsuccessful repair leads to genomic instability. Adapted from (Deng and Chang, 2007).

1.5 DNA homologous recombination (HR)

Homologous recombination (HR) plays important roles in DNA damage-repair as well as in the maintenance of telomere length. During the process of HR, exchange of DNA between two homologous DNA occurs. It is an essential process for crossing over and segregation during meiosis. This process is also involved in repair of damaged DNA, preventing the blockage of damaged replication forks, and several other aspects of DNA metabolism that are critical for maintenance of genome integrity (Borg *et al*., 2009; Shrivastav *et al*., 2008). It is also important for generation of variation in epitopes, which is important for proper immune response (McEachern and Haber, 2006).

1.5.1 Mechanism of HR

Though the mechanism of HR is little understood in higher eukaryotes, it has been well characterized in yeast. Several lines of evidence suggest that the principle steps of HR are conserved from yeast to human (Sung and Klein, 2006). According to the current model, HR could occur in several steps (Figure 5). A double strand break in DNA molecule is required for the initiation of HR. At the first step of HR, a single stranded overhang at the 3′ end is produced at the double strand break by 5′ to 3′ degradation. The 3′ overhang is first bound by replication protein A (RPA), which is subsequently replaced by RAD51 to form a nucleoprotein filament (San Filippo *et al*., 2008). The nucleoprotein filament searches for homologous DNA molecule and forms Rad51–ssDNA complex to a complementary ssDNA region within the homologous duplex, resulting in D-loop structure. The invading 3′ end is used to prime a second round of leading strand DNA synthesis and D-loop extension (Krogh and Symington, 2004).

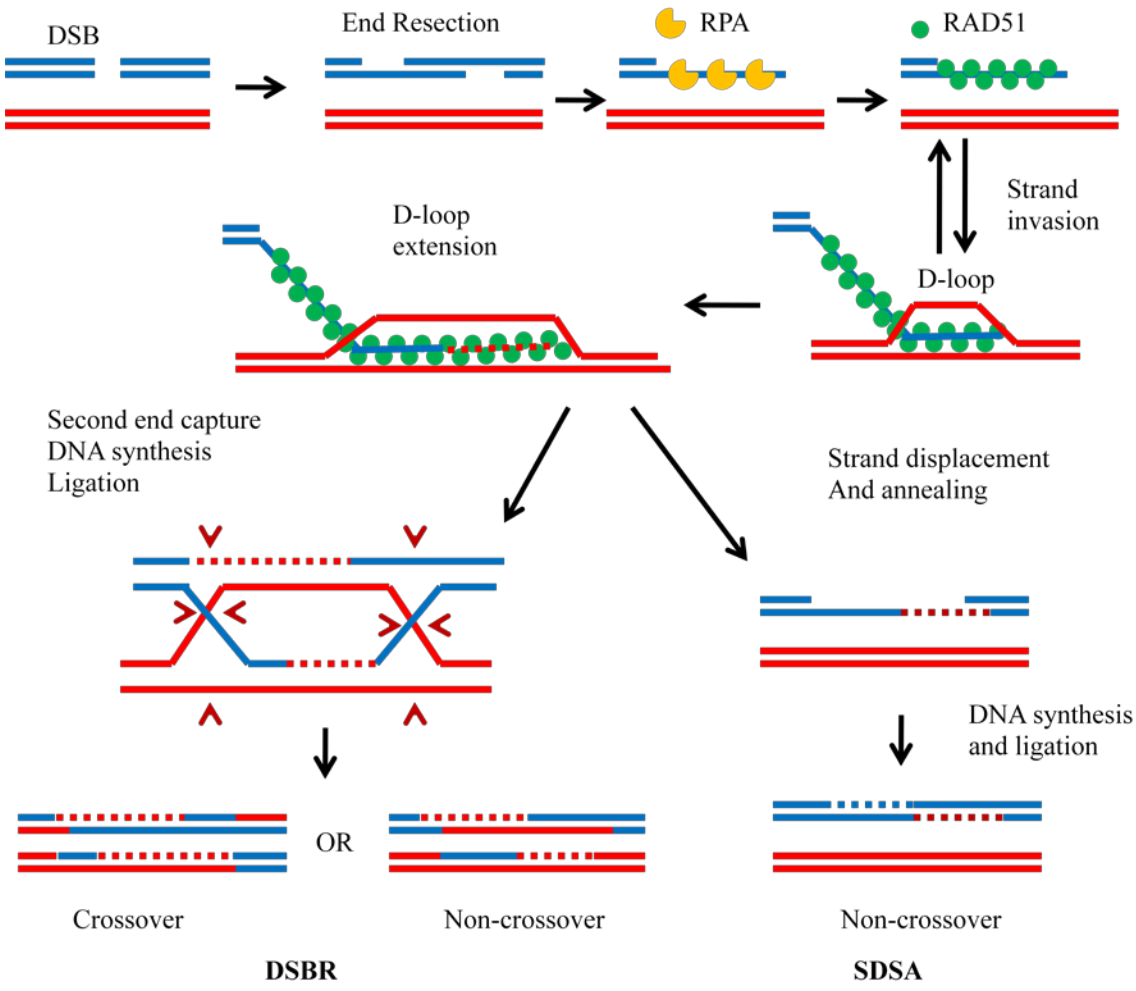


Figure 5: Homologous recombination and double strand break (DSB) repair. Repair is initiated by resection at the DSB to produce 3´ overhangs. RAD51 forms nucleoprotein filaments at the overhang and facilitates invasion at the homologous sites. DNA synthesis is carried out at the invading strand end. In DSBR pathway, the products can be either crossover of non-crossover. Alternatively, SDSA pathway leads to non-crossover products only. Adapted from (Sung and Klein, 2006).

The double strand breaks can be repaired in several ways, including double strand break repair (DSBR) and synthesis dependent strand annealing (SDSA). In DSBR, a double Holliday junction (HJ) intermediate is formed when the second DSB strand is

captured after strand invasion. Depending on the mode of resolution, the product might be non-crossover or crossover. On the other hand, in SDSA, the invading strand is displaced from the intermediate and annealed to the other ssDNA end, followed by gap-filling DNA synthesis and ligation. The result of this process is always a non-crossover product (Sung and Klein, 2006).

1.5.2 Regulation of homologous recombination (HR)

Due to its essential roles, HR is controlled in co-ordination with other cellular processes such as cell cycle progression, replication and meiosis. Unregulated HR has detrimental effects on the cells. For example, loss of heterozygosity (LOH) can occur due to formation of crossovers (via the DSBR pathway) during HR. This can lead to genome instability when it involves genes regulating cellular growth and proliferation. Furthermore, chromosomal translocation can occur when crossover recombination is allowed to occur among repetitive DNA sequences dispersed within the genome (Krogh and Symington, 2004). HR can also interfere with post-replication repair and cause the formation of intermediates that are difficult to resolve resulting in cell-cycle arrest as well as chromosome rearrangements (Gangloff *et al*., 2000).

In addition to regulation at different steps of HR process, it can also be suppressed. Mechanisms exist in cells that can displace the single stranded DNA from the recombination complexes or D-loops (Nicolas, 2009). In cellular contexts during replication or proliferation, HR based repair SDSA pathway plays a predominant role, where a temporarily engaged homologous DNA duplex serves as a template for correct information. This allows minimization of the likelihood of unregulated crossover events

thus preventing DNA rearrangements or complication in meiotic segregation. In addition, dissolving the invading strand during the initiation of HR, can also suppress the pathway allowing a fine control mechanism at cellular level (Paques and Haber, 1999). Several helicases can play role in the displacement step by unwinding of the D-loop intermediate, such as yeast Srs2 (suppressor of rad6), Flm1, human FANCM (Fanconi anaemia group M) and human BLM (Dupaigne *et al*., 2008; Gari *et al*., 2008; Sun *et al*., 2008). It has been recently demonstrated that the *C. elegans* homolog of RTEL, RTEL-1 can unwind the D-loops (Barber *et al*., 2008). RTEL-1 could be a functional homolog of Srs2 which functions as an anti-recombinase. The anti-recombinase function of helicases allows fine regulation of HR both in mitosis and meiosis. In addition, HR is suppressed at telomeres to maintain protective t-loops (Verdun and Karlseder, 2006). Such regulation is indispensible for maintaining genomic stability.

1.6 Role of proliferating-cell nuclear antigen (PCNA)

Proliferating cell nuclear antigen (PCNA) was first described as an antigen in the autoimmune disease systemic lupus erythematosus (SLE) patients (Miyachi *et al*., 1978). While initial studies demonstrated that it is associated with cell proliferation or transformation (Celis *et al*., 1984), it is now known that PCNA is a critical factor involved in DNA replication and repair, chromatin remodelling and cell cycle regulation (Maga and Hubscher, 2003).

1.6.1 The structure and function of PCNA

PCNA is a member of DNA sliding clamp family. The proteins of this family are found in almost all species. Though the amino acid sequence varies among the members,

their ring-like three-dimensional structure is highly conserved (Gulbis *et al*., 1996a). The PCNA monomer consists of two similar domains connected by an interconnection loop. Three identical monomers interact head -to-tail to form a homotrimer with a ring-like shape (Figure 6).

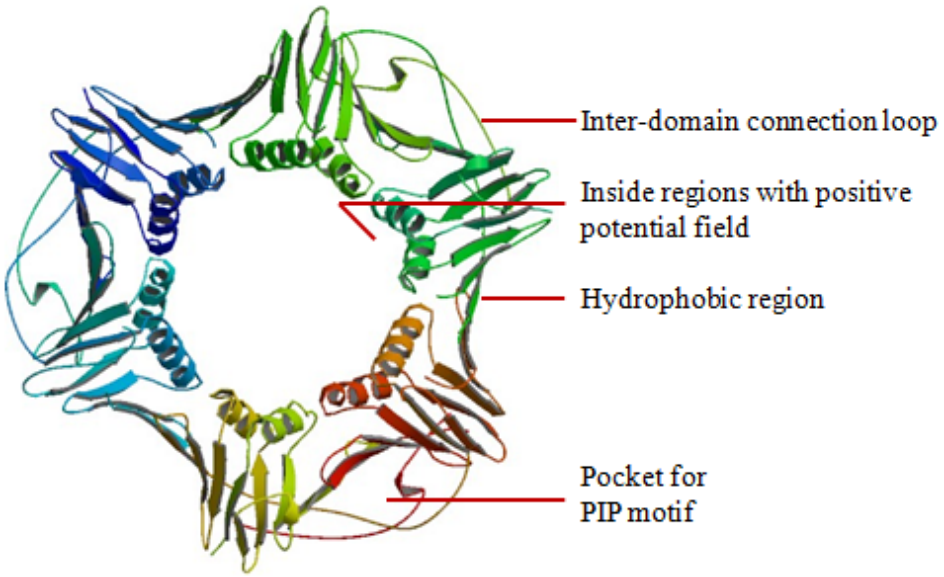


Figure 6: Front view of three-dimensional structure of human PCNA. Different subunits are coloured in red, green and blue. In trimeric form, the protein exhibits hexameric symmetry and forms basic ring structure inside enabling it to wrap around negatively charged DNA molecule. Adapted from PDB structure 1AXC (Gulbis *et al*., 1996b).

The inside and outside surfaces of the PCNA 'ring' are differentially charged. Many amino acid residues in the inside hole of the ring are basic (positively charged), that interacts with the negatively charged sugar-phosphate of DNA. On the other hand, the outside surface is the interface for protein interactions. In the trimeric form, the domains of each monomer are structurally very similar, that gives the molecule a pseudo-

hexagonal symmetry. The unique structure of PCNA allows it to coordinate and organize different partners onto DNA (Podust *et al*., 1994; Schurtenberger *et al*., 1998).

1.6.2 PCNA interacting proteins

PCNA can interact with a large number of proteins and effectively organize their functions temporally and spatially. Its proteins partners play roles not only in DNA metabolism and repair, but also in chromatin remodelling and assembly, cell cycle regulation, sister-chromatid cohesion, translation, and even cytoskeletal movement (Stoimenov and Helleday, 2009). A number of proteins that interact with PCNA are listed in Table 3.

It has been demonstrated that several proteins bind to PCNA using a conserved motif, the PCNA-interacting protein (PIP) motif, to interact with other proteins. This motif consists of QXX(M/L/I)XX(F/Y)(F/A), occasionally flanked by KAX sequence at the N terminus (Jonsson *et al*., 1998). It forms an unusual helical secondary structure that fits into the hydrophobic pocket of PCNA residing in the inter-domain connection loop (Bruning and Shamoo, 2004).

However, the PIP motif is not the only protein domain responsible for PCNA binding. The other two PCNA binding motifs are KA (Xu *et al*., 2001) motif and AlkB homologue 2 PCNA-interacting motif (APIM) (Gilljam *et al*., 2009; Stoimenov and Helleday, 2009). These two domains have been found in a small group of PCNA-interacting proteins.

Table 3: A list of proteins that interact with PCNA.

Process	Protein	PCNA binding motif	Reference
DNA replication	DNA polymerase δ p66	PIP motif	(Zhang *et al*., 1999)
	Flap endonuclease 1		(Warbrick *et al*., 1997)
	DNA ligase 1		(Levin *et al*., 2000)(Gilljam *et al*., 2009)
	DNA replication factor Cdt1		(Stoimenov and Helleday, 2009)
	DNA polymerase	KA motif	(Eissenberg *et al*., 1997)
	Replication factor C		(Fotedar *et al*., 1996)
	Topoisomerase II α	APIM motif	(Niimi *et al*., 2001)
	DNA ligase IV		(Gilljam *et al*., 2009)
	Pol δ interacting protein 2	N/A	(Moldovan *et al*., 2007)
DNA repair	Mismatch repair protein Msh3	KA	(Clark *et al*., 2000)
	Mismatch repair protein Msh6	KA+PIP	
	UNG2	PIP	
	hMYH		(Umar *et al*., 1996)
	DNA polymerase β		
	XPG		(Gary *et al*., 1997)
	PARP-1		(Umar *et al*., 1996)
	XPA	APIM	(Gilljam *et al*., 2009)
	Mlh1	N/A	(Umar *et al*., 1996)
	WRN		(Lebel *et al*., 1999)
	BLM		(Bachrati and Hickson, 2008)
	RECQ5		
DNA damage avoidance	DNA polymerase η	PIP	(Haracska *et al*.,2002)
	RAD 18	N/A	(Shiomi *et al*., 2007)
	REV1		(Stoimenov and Helleday, 2009)
	SHRPH		
	HLTF		(Unk *et al*., 2008)
	UBC9		(Hoege *et al*., 2002c)
Cell cycle control and survival	p21	PIP	(Stoimenov and Helleday, 2009; Waga *et al*., 1994)
	p15		

Process	Protein	PCNA binding motif	Reference
Cell cycle control and survival	p53	N/A	(Moldovan *et al*., 2007)
	Cyclin D1		(Xiong *et al*., 1992)
	CDK2		(Maga and Hubscher, 2003)
Chromatin assembly and maintenance	DMNT1	PIP	(Moldovan *et al*., 2007; Stoimenov and Helleday, 2009)
	HDAC1	N/A	
	p300		(Hasan *et al*., 2001)
Sister chromatid cohesion	ESCO1, ESCO2	N/A	(Stoimenov and Helleday, 2009)
Apoptic factors	Gadd45	N/A	(Vairapandi *et al*., 2000)
	MyD118		

1.6.3 Post-translational modifications of PCNA

Like many other proteins, post-translational modifications of PCNA are important for regulating its functions by affecting its binding affinity with different partners, leading to degradation, sequestration from pathway or other effects. So far, PCNA has been found to be ubiquitylated, phosphorylated, acetylated, methylated and SUMOylated (Moldovan *et al*., 2007). It has been demonstrated that ubiquitylated and SUMOylated PCNA could have important roles in post-replication DNA repair pathway (Lee and Myung, 2008). During PCNA ubiquitylation, a small protein called ubiquitin is attached to a highly conserved lysine residue at position 164 (K^{164}). Based on the number of molecules added, it can be either mono-ubiquitylation or poly-ubiquitylation (Figure 7). In yeast, mono-ubiquitylation is carried out by Rad6/rad18 dependent pathway and poly-ubiquitylation is mediated by Ubc13/Mms2 and Rad5 dependent pathway (Lee and Myung, 2008). Like ubiquitylation, SUMOylation involves attachment of a small protein called SUMO (small ubiquitin like-modifier) by Ubc9/Siz1 pathway (Hoege *et al*.,

2002a). Interestingly, the conserved K^{164} is the site of this modification as well. However, in yeast an additional SUMOylation site is present at position 127 which is not present in higher eukaryotes. Poly-SUMOylation has also been described in yeast (Windecker and Ulrich, 2008).

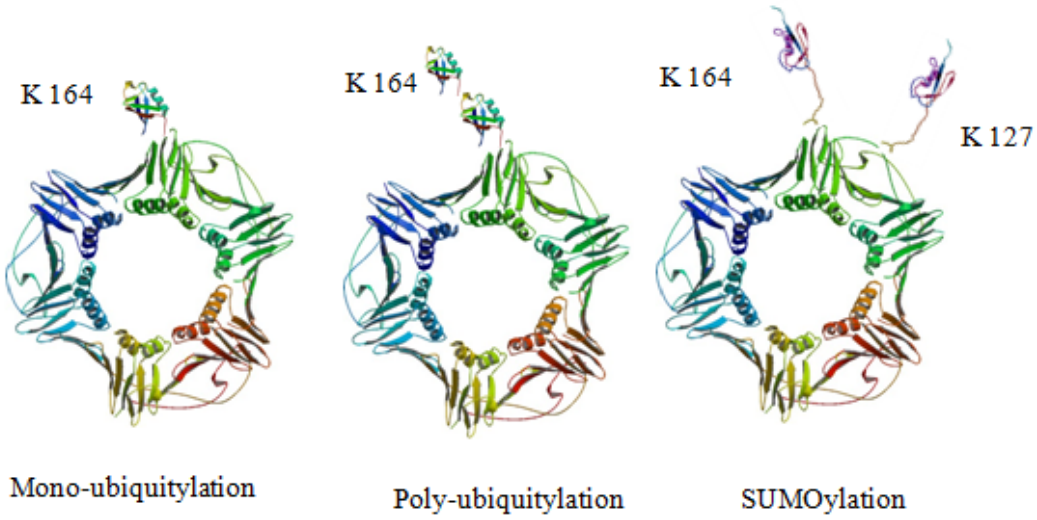


Figure 7. Post-translational modifications of PCNA. PCNA mono-ubiquitylation and poly-ubiquitylation can occur at K164 and K 63 respectively. SUMO can be attached at K 164 and K127 in yeast. The K127 site is not present in mammals. The modifications can occur in all the monomers.

These modifications have profound regulatory functions. For example, attachment of ubiquitin or a SUMO molecule results in changes in surface property of the protein and induces change in interacting partners (Suzuki *et al.*, 2009). In addition, SUMOylation has been demonstrated to inhibit the binding of Eco1 protein to PCNA, thus blocking the establishment of sister-chromatid cohesion in S-phase (Bergink and Jentsch, 2009).

1.6.4 Role of PCNA modifications in post-replication repair

DNA replication machinery cannot proceed through damages present in single-stranded templates as they cannot be recognized or correctly processed by the replicative DNA polymerases. In such circumstances, the progressing replication fork might be stalled and eventually fall apart. However, stalled replication forks can activate pathways that allow completion of replication at damaged sites facilitating the completion of cell cycle (Lehmann, 2006). There are two main mechanisms involved in post-replication repair: (1) the homologous recombination or possibly template switching ensuring error-free replication; and (2) employing the specialized, damage-tolerant DNA polymerases capable of carrying out replication across the damaged template in a process called trans-lesion synthesis (TLS) in an error-prone manner. The switch between the two pathways relies on post-translational modification status of PCNA (Suzuki *et al*., 2009) (Figure 8).

Mono-ubiquitylation of PCNA has been shown to directly enhance its affinity for trans-lesion synthesis polymerases Polη and Rev1 (Guo *et al*., 2006; Parker *et al*., 2007). At damaged DNA sites where replication is blocked, it promotes the switch between replicative and trans-lesion synthesis polymerase to facilitate error prone DNA replication. This pathway allows some level of DNA damage tolerance and this mechanism is now established for eukaryotic Y family of polymerases (Bi *et al*., 2006). On the other hand, PCNA can be poly-ubiquitylated at the same lysine residue. PCNA poly-ubiquitylation leads to the use of genetic information from newly synthesized sister chromatid at blocked replication forks either by template switching or by a mechanism involving homologous sister chromatid invasion, DNA synthesis, and the resolution of the Holliday junction (Lawrence, 1994). Another possible mechanism in this pathway

could be reversal of the replication fork. However, these pathways involve production and resolution of complex intermediates. This indicates the involvement of a complex mechanism that is as yet to be discovered (Sogo *et al*., 2002).

Unlike ubiquitylation, effects of SUMOylation are less characterized. SUMOylation of PCNA is commonly present in yeast cells, and it has been only detected in few higher eukaryotic cells, such as chicken DT40 cells (Arakawa *et al*., 2006; Frampton *et al*., 2006; Leach and Michael, 2005). In yeast, SUMOylation has been demonstrated to occur in S phase in a damage independent manner. However, effects of blocking PCNA SUMOylation have been observed under conditions of DNA damage (Hoege *et al*., 2002b). The Role of SUMOylation of PCNA became evident when it was found to prevent the resolution of damaged DNA sites by homologous recombination at stalled replication forks, favouring the trans-lesion synthesis pathway (Watts, 2006). Since SUMOylation and ubiquitylation occur at the same site, the switch can be easily controlled by PCNA modification (Figure 8). In addition, SRS2 (suppressor of RAD6 mutant 2) can be recruited via PCNA to damage sites. SRS2 is a helicase capable of dissolving or preventing the formation of recombinogenic Rad51 filaments on single-stranded (ss) DNA, acting as an anti-recombinase. Hence PCNA/SRS2 employs regulation of unwanted HR at damaged sites (Veaute *et al*., 2003). Thus this pathway is a safeguard mechanism to minimize recombination and allows error prone synthesis mediated by ubiquitylated PCNA (Papouli *et al*., 2005; Watts, 2006).

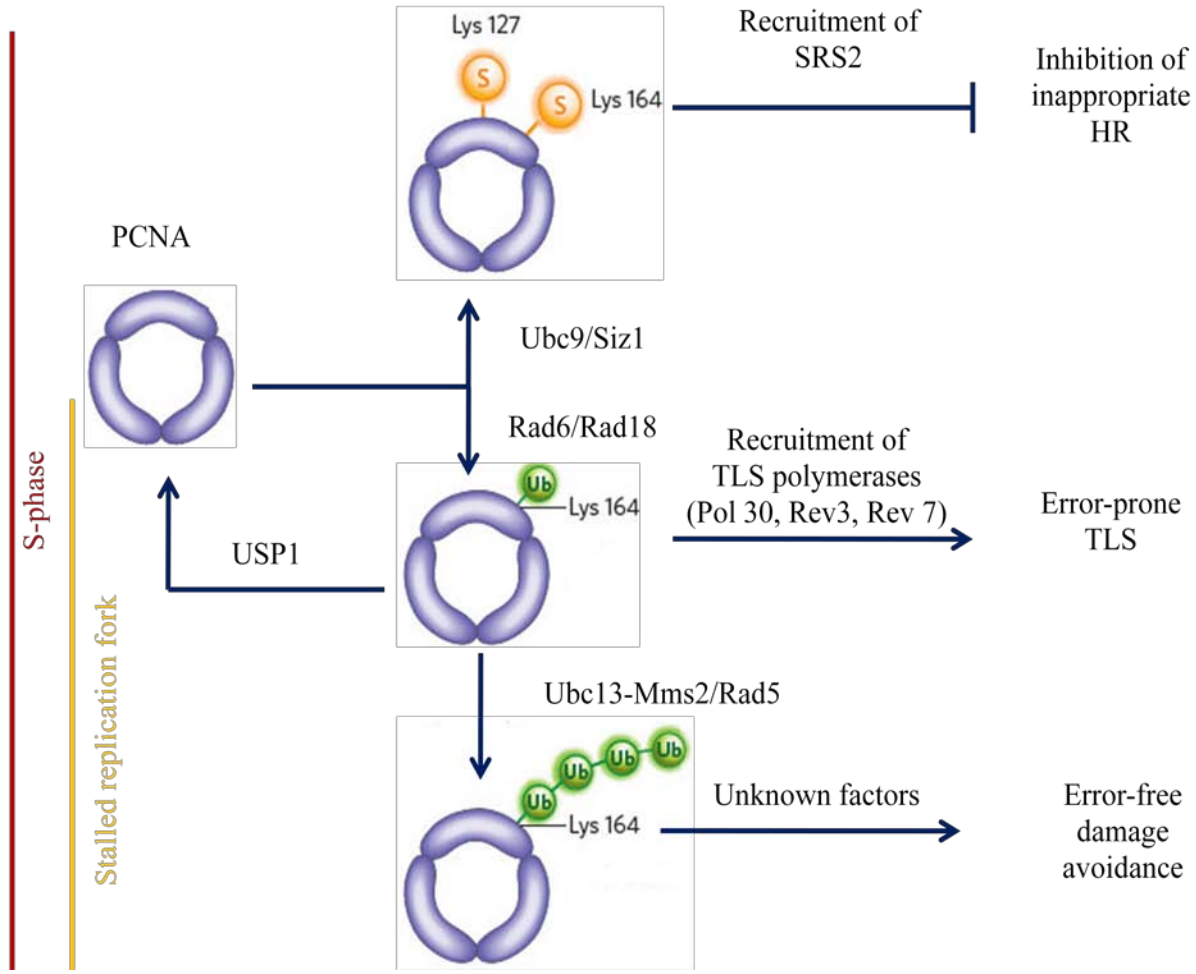


Figure 8: Regulation of post-replication repair by PCNA modifications. PCNA mono-ubiquitylation recruits trans-lesion synthesis (TLS) polymerases to facilitate error-prone DNA damage bypass. Alternatively, poly-ubiquitylation of PCNA promotes error-free damage avoidance through complex mechanisms. SUMOylated PCNA recruits SRS2 to inhibit inappropriate recombination. Error-free repair of the damage is also possible via RAD51 dependent recombination pathways, which is negatively regulated by SUMOylated PCNA. The proteins responsible for the modifications are described in figure and text. The picture is adapted from (Lee and Myung, 2008).

1.7 Human diseases associated with genomic instability

Several helicases have been found to be associated with human diseases including so called 'chromosome instability syndromes' (Table 4). The BLM gene was the first helicase demonstrated to be associated with genomic instability and cancer predisposition (Ellis *et al*., 1995). In addition to BLM, other members of the RecQ family helicases have also been found to be associated with such disorders-RecQ4 (Kitao *et al*., 1999), WRN (Ozgenc and Loeb, 2006) and BLM (Chu and Hickson, 2009) which give rise to Rothmund-Thomson syndrome, Werner syndrome and Bloom's syndrome respectively. These proteins interact with various protein partners and act as part of multi-enzyme complexes, having role in several pathways including replication and HR (Bachrati and Hickson, 2008).

Table 4: Human disorders associated with helicases.

Syndrome (gene)	Clinical Features	Cancer predisposition	Reference
Bloom's syndrome (BLM)	Dwarfism, skin lesion due to inappropriate pigmentation, infertility, diabetes type II	Most types, particularly, non-Hodgkin's lymphoma, leukemia, breast, colon	(Chu *et al*., 2009)
Werner's syndrome (WRN)	Premature ageing, growth retardation, soft tissue calcification	Mainly cancers of mesenchymal origin	(Ozgenal *et al*., 2006)
Rothmund-Thomson Syndrome (RECQ4)	Short stature, skeletal abnormalities, dystrophic nails and teeth, hypogonadism	Mainly osteosarcoma	(Kitao *et al*., 1999)
RAPADILINO syndrome (RECQ4)	Radial hypoplasia and aplasia, diarrhoea, dislocated joints, limb malformation	Lymphoma and osteosarcoma	(Siitonen *et al*., 2003)

Syndrome (gene)	Clinical Features	Cancer predisposition	Reference
Baller-Gerold syndrome (RECQ4)	Radial aplasia and hypoplasia, short stature, craniosynostosis	Not reported	(Dietschy *et al*., 2007)
Xeroderma pigmentosum (XP; XPA-XPG)	Skin abnormalities on sun-exposed areas, photosensitivity, cataract	Skin cancer, Premalignant actinic keratoses, Melanoma	(Cordonnier and Fuchs, 1999)
Trichothiodystrophy (XPB, XPD,TTD-A)	Photosensitivity, intellectual impairment, short stature	Not reported	(Cordonnier and Fuchs, 1999)
Cockayne syndrome (XPB, XPD, XPG, CSA, CSB)	Growth deficiency, neurological disorders, photosensitivity, skeletal abnormalities	Not reported	(van Brabant *et al*., 2000)
FanconiAnemia (FANCJ and other complementation group genes)	Congenital anomalies, progressive bone marrow failure	All types, specifically breast cancer	(Wu *et al*., 2009)

In vitro studies revealed that they can preferentially act on substrates that mimic replication forks, bubbles or Holliday junctions (Vindigni and Hickson, 2009). Indeed Bloom's syndrome is characterized by an almost tenfold increase in sister chromatid exchange due to its role in resolution of HR intermediates (Chaganti *et al*., 1974). The Werner's syndrome protein WRN plays essential roles in DSB repair (Chu and Hickson, 2009).

Unlike RecQ family helicases, BRIP1/BACH1 plays role in repairing intra-strand crosslink (ICL) in DNA. It is defective in Fanconi anaemia complementation group J (FANCJ) (Niedernhofer *et al*., 2005). Genomic instability in some syndromes is observed due to DNA damage response. For example, mutations in XPD and XPB are associated with human diseases related to transcription and base excision repair (BER),

such as xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy (Oksenych and Coin, 2010).

1.8 Involvement of RTEL in the development of human brain tumors

Recently, two studies have shown that mutations in human RTEL gene are associated with susceptibility to gliomas. Using a genome-wide association study (GWAS), two mutations located within intron 12 and intron 17 of RTEL on chromosome 20 were only found in human patients with gliomas (Wrensch *et al*., 2009; Shete *et al*., 2009). In addition, variation in copy number of this gene was also identified in this brain tumor (Shete *et al*., 2009). Since genomic instability plays an important role in tumorigenesis (Aaltonen *et al*., 1993) and RTEL is an important factor in the maintenance of genomic integrity (Ding *et al*., 2004), these human genetic studies highly implicate that RTEL could be a new genetic factor involved in the development of tumors. Our on-going transgenic studies on manipulating the expression level of RTEL in mice could allow us to address this hypothesis.

1.9 Project rationale and hypothesis

RTEL, a putative helicase, has been shown to be an essential protein in the maintenance of genomic stability and telomere integrity (Ding *et al*., 2004). Recently it has been demonstrated to suppress HR by acting as an anti-recombinase (Barber *et al*., 2008). Mutations in RTEL have been associated with development of human tumors (Wrensch *et al*., 2009; Shete *et al*., 2009). However, the biological pathway(s) in which this protein is involved is largely unknown. Several helicases have been found to interact with DNA replication and repair related proteins to regulate specific pathways (Lohman

and Bjornson, 1996). Finding the protein(s) that interact with RTEL and characterizing the interaction(s) will provide a greater understanding of the role of RTEL at molecular level, specifically in the maintenance of genomic stability.

Thesis hypothesis: The interaction between RTEL and PCNA is essential for the maintenance of genomic stability.

Thesis aims: The major aims of this project were to find out RTEL-interacting proteins and to find out the role of RTEL/PCNA interaction in the maintenance of genomic stability. The specific research objectives of the project are as follows:

1) Identification of RTEL-interacting proteins by yeast two-hybrid

2) Characterization of the RTEL/PCNA interaction

3) Establishment of transgenic mouse model to identify the significance of RTEL/PCNA interaction *in vivo*

Chapter 2: Materials and methods

2.1 Recombinant DNA technology

2.1.1 Polymerase chain reaction (PCR) amplification

Polymerase chain reaction (PCR) was carried out using a high fidelity Takara LA *Taq* kit (Takara Inc., Madison, USA; #TAK RR002A,M,B,C). The kit included the *Taq* polymerase along with other reagents like buffer, $Mg^{2+}$and dNTPs. Filter sterilized nano-pure water was used for PCR reactions. The starting amount of DNA used was 10 ng for purified DNA and 100 ng for genomic cDNA. For primers, 100 ng were added in all of the reactions. The total reaction volume used was usually 50 µl. The reaction cycles were optimized for different samples; however, the following program was used as a standard:

Temperature	94°C	94°C	(A*-5)°C	72°C	72°C
Time	1 min	30 sec	30 sec	30 sec/ 1 min	10 min
No. of cycle		X35	X35	X35	

*A is annealing temperature suggested by primer composition

2.1.2 Purification of PCR products

The PCR products were separated by running on 1% agarose gel at 100 V till the desired separation range was achieved. Using AlphaImager (BioRAd Inc.), the corresponding bands were visualized with aid of ethidium bromide under ultra-violet illumination. The PCR products were purified using a gel purification kit from Qiagen following manufacturer's instructions.

2.1.3 Restriction digestion and ligation

All the restriction enzymes used were from New England Biolabs. Volumes of restriction digestion reactions were set according to desired use and incubated at 37°C for at least one hour. For cloning purposes, 4 µg of DNA was digested for each product. For ligation reaction, the Takara ligation kit (Takara Inc., Madison, USA; #6021) was used. Usually the reaction was set for overnight at 16°C. The ratio of the DNA fragments for the ligation varied depending on the amount of DNA.

2.1.4 Transformation and screening for positive clones

For bacterial transformation, heat-shock competent DH5α cells (Invitrogen, Canada; #18258-017) were used. Heat shock was carried out at 37°C for 30 seconds. The cells were then incubated in LB media without selection for 1 hour and plated on LB-agar plates with selection (ampicillin) for overnight at 37°C. For isolation of plasmids from the clones, miniprep kits were used from (Sigma, #PLX15/Fermentas, #K0503). Plasmid DNA was isolated using manufacturer's instructions. The identity of the clones was revealed by restriction digestion and DNA sequencing (see section 2.2.8).

2.1.5 List of primers used in this project

Table 5: List of primers in this project

Process involved	Primer Name	Sequence
Y2H screen	RtelRTp1F	5′ gttgtgccgcaagaaggtagca 3′
	RtelRTp2R	5′ gcatccaacaggtaattgtatggca 3′
	RtelRTp3R	5′ agatcacaactgttcccttcaggt 3′
	RtelRTp4R	5′ gataatctccagtcctgaagccaca3′
	RtelRTp5R	5′ ctttgcgatgtcttccagctcca 3′
	RtelRTp6R	5′ tccagtgattccaaaatgcagcct 3′
	pAS1RtelC p1	5′ gtctagacaccgccatgactatggg 3′

Process involved	Primer name	Sequence
Y2H screen	pAS1RtelCp2	5′ cctcgagctattgcttactccgatg 3′
	pAS1 seq primer	5′ gacatcatcatcggaagagag 3′
Creating RTEL PIP mutants	RtelCtermp1	5′ ggactgctagctgccttagaataca 3′
	RtelCtermp2	5′ tcaatggtgatggtgatgatgacc 3′
	RtelQ/Ap1	5′ ccaagaaacctgagaagaccgcgagtaagatctcatcc 3′
	RtelQ/Ap2	5′ ggatgagatcttactcgcggtcttctcaggtttcttgg 3′
	RteII/Ap1	5′ ctgagaagacccagagtaaggcctcatccttcttctttagac3′
	RtelI/Ap2	5′ gtctaaagaaggatgaggccttactctgggtcttctcag 3′
	RtelF1/Ap1	5′ ccagagtaagatctcatccttatttagacagaggcc 3′
	RtelF1/Ap2	5′ ggcctctgtctaaataaggatgagatcttactctgg 3′
	RtelF2/Ap1	5′ gtaagatctcatccttcttgagacagaggccagatg 3′
	RtelF2Ap2	5′ catctggcctctgtctcaagaaggatgagatcttac 3′
	RtelFF/AAp1	5′ gtaagatctcatccttgttgagacagag 3′
	RtelFF/Aap2	5′ gatgaggccttactcgcggtcttc 3′
Creating 5’ and 3’ Probes for Southern analysis	Rtelki 5 probe p1	5′ gcaacattgctcgcgtggtgccc 3′
	Rtelki 5 probe p2	5′ ctgtggtcacacaggagatggcccc 3′
	Rtelki 3 probe p1	5′ ttgggatggatggacaccaagttctgg 3′
	Rtelki 3 probe p2	5′ cctgtatgggtctggctgcaaagcc 3′
PCNA expression vectors	mPCNA RT p1	5′ctcatctagtcgccacaactccgcca 3′
	mPCNA RT p2	5′ cctcaggacacgctggcatctcagga3′
	mPCNA flag p1	5′ ggaattccgccaccatgtttgaggca 3′
	mPCNA flag p2	5′ aggatccaaatgcttcctcatcttca 3′
	mPCNAKRmutp1	5′ gttgtgatatcctgtgcaaggaatggggtgaagttttc 3′
	mPCNAKRmutp2	5′gaaaacttcaccccattccttgcacaggatatcacaac 3′
MBD6 expression vectors	MBD6 5-RT p2	5′ ggttaaagcttccatccgagtgggaag 3′
	MBD6 x3flag p1	5′ gccaccatggatggagacaatgcgagca 3′
	MBD6 x3flag p2	5′ tctagatggggccagtttcctcctct 3′

2.2 Yeast two-hybrid screen

2.2.1 Materials

a. Plasmids

Plasmid Name	Vector size (bp)	*E. coli* selection	*S. cerevisiae*	Genbank accession no.	Source/Reference
pACT2	8.1	Amp	-Leu	U29899	Clontech (#638822)
pAS1	7.1	Amp	-Trp	-	R. D. Gietz

b. cDNA libraries

Species	Organ	Vector	Source
Mouse	Testis	pCAT2	Clontech (# 638848)

c. Yeast (*S. cerevisiae*) host strains

Yeast strain	Genotype	Source
KGY37	MATa, ade2 , gal4, gal80, his3-Δ200, trp1-Δ901, leu2 ::pUC18 Ura3::GAL1-lacZ, LYS2::UASG17 MERS(x3) GAL1-HIS3	R.D. Gietz
AH109	MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$Gal2_{TATA}$-ADE2, URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ	(James *et al*., 1996)

2.2.2 Bait vector construction

The full length RTEL bait expression construct for yeast two hybrid screens was generated by cloning RTEL cDNA into the *NheI/BamHI* sites of the pAS1 vector. The bait vector constitutively expresses the recombinant protein fused with DNA binding domain of Gal4 under yeast ADH1 promoter. In addition, the recombinant protein is fused with a HA tag to facilitate its detection and purification. The vector also contains selectable markers in yeast- tryptophan and has bacterial origin of replication and selectable marker ampicillin. Primers used for construction of different RTEL bait vectors are listed in section 3.1.5. Bait vectors used in this project are listed in Table 6.

Table 6: List of Bait vectors used in this study

Name	Portion of RTEL expressed	Product size (kDa)	Comment
pAS1-Rtel	Full length	150	Expression level very low
pAS1-Rtel-2101-3625 (RTEL C-terminal)	C terminal part	57	Good expression level
pAS1-Rtel-2500-3625	C terminal part	43	Very good expression level

2.2.3 Prey vector library amplification

In this study, a mouse testis MATCHMAKER cDNA library (Clontech CAT.# 638848) was used for Y2H screens. This library was chosen because RTEL is highly expressed in mouse testis and we expect that screening the library derived from this tissue could allow us to identify relevant RTEL-interacting partners. The source of the library was testes of normal mice, pooled from 8-12 weeks BALB/c mice. The cDNA library was constructed in the pACT2 vector with as average 2.0 kb insert size. There were estimated 3.5 million independent clones. Library amplification was performed as described in the manufacturer's user manual with the following exception: 15 cm plates were used for ease of handling. The library was amplified 10 times more than the suggested requirement, using more plates to get proper complexity. Phenol/chloroform extraction was carried out to isolate the cDNA library with final concentration of 13.92 mg/ml. For confirmation of library diversity, library preparation was run on 1% agarose gel. Since the size of pACT2 vector alone is 8.1 kb, adequate library diversity was determined by presence of smear at 8 kb region in the gel.

2.2.4 Transformation of S. *cerevisiae*

S. cerevisiae cells were transformed by using lithium acetate/single-stranded carrier DNA/PEG method. This high efficiency transformation method was adapted from a previously described protocol by R. D. Gietz (Gietz *et al*., 1995; Gietz, 2006).

Briefly, specific yeast strain was inoculated with a single colony into 5 ml broth of YPAD or appropriate synthetic complete omission medium (SC-) and incubated on a shaker (200 rpm) at 30°C. The following day, the appropriate volume of overnight culture for $2.5X10^8$ cells was sub-cultured into 50 ml of fresh YPAD or SC- media. The inoculum was grown on shaker (200 rpm) at 30°C for approximately 4 hours (two cell divisions) so that the final concentration was at least $2X10^7$cells/ml. The cells were then harvested by centrifugation at 3000 *g* for 5 min, followed by washing with 25 ml of sterile double-distilled water. The cells were re-suspended in 1.0 ml of 100 mM LiAc. Cells were again centrifuged and re-suspended in sterile water such that the final volume is 500 µl. 50 µl of the cell suspension was centrifuged and used for each reaction.

For each transformation, 240 µl of 50% (v/v) PEG, 36 µl 1M LiAc, 50 µl denatured salmon sperm DNA and 1 µg of DNA to be introduced were added to the cell pellet on ice and mixed by vigorous vortex. The transformation mix was then incubated at 40°C in a water-bath for 40 min. The cells were then centrifuged and re-suspended in 1.0 ml of sterile double-distilled water. Appropriate amount of the cells were plated SC-agar plates to allow the growth of the transformed cells under selection.

2.2.5 Library screen transformations

Large scale Yeast two hybrid (Y2H) screens were carried out in both solid and liquid media. 60X or 120X scale up of the transformation was carried out. The entire cell pellet was combined to one and diluted in sterile water for transformation.

2.2.5.1 Culture plates-based Y2H screening

Yeast cells were transiently co-transfected with a plasmid expressing the gene of interest and the library were scaled up to 60X and re-suspended into 50 ml of sterile water. 500 µl of the transformation suspension was plated onto each 25 cm plates lacking leucine (-leu), tryptophan (-trp) and histidine (-his), supplemented with 25 mM 3-Amino-1,2,4-triazole (3-AT) and incubated for up to 7 days at 30°C. The positive colonies were selected for X-gal staining, DNA digestion pattern and sequencing analysis.

2.2.5.2 Liquid culture-based Y2H screening

Yeast cells transiently co-transfected with the plasmid expressing gene of interest and the library were scaled up to 120X and re-suspended in sterile water. Finally the transformed cells are mixed with 1200 ml of media (-Leu-Trp-His) with 0.5 mM 3-Amino-1,2,4-triazole (3-AT) and 1.5 ml of the suspension was added to each well of a 96 well chamber plates. The cells were then incubated at 30°C on shaker (300 rpm) for 72 hours. The cultures were diluted twice in SC-Leu-Trp-His with 0.5 mM 3-AT media (first dilution 1/10, second dilution 1/100). Each dilution was grown for 24 hours. To determine Y2H positives, OD_{600} was measured and colonies were selected as positive based on growth.

2.2.6 β-galactosidase assay

Colonies selected were further screened fo r β-galactosidase activity. β-galactosidase assay was carried out either directly on agar-plates with X-gal or indirectly by colony lift assay. Colonies turned blue when X-gal was added to the plate along with the media. In colony lift assay the colonies were replicated on Whatman filter paper, by pressing them gently against the surface of the agar plate. Cells attached to the filter paper were then ruptured by repeated freeze (-80°C) thaw cycle, 3 times. The filter papers were then soaked with Z buffer (60 mM $Na2HPO_4$, 40 mM $NaH_2PO_4.H_2O$, 10 mMKCl, 1 mM $MgSO_4$.7H2O, 39 mM 2-mercaptoethanol) with X-gal (1mg/ml) and incubated for 2 hours at 30°C. Positive colonies appeared blue due to β-galactosidase activity.

2.2.7 Plasmid recovery from *S. cerevisiae*

Plasmids were extracted from yeast cells following a protocol of Hoffman and Winston (Hoffman and Winston, 1987). A single colony of the putative positive yeast clone was inoculated into 5 ml of SC (-Trp-Leu-His) media and incubated overnight on shaker (200 rpm) at 30°C. Cells were harvested from the culture by centrifuging at 5000 g for 5 min and re-suspended in yeast cracking buffer [2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mMNaCl, 20 mMTris, pH 8.0, 10 mM EDTA. Equal volume of glass beads (Sigma G-8772) was added to the cells and vortexed for 30 seconds. The DNA was then extracted by phenol: chloroform and re-suspended into 30 µl of sterile water.

2.2.8 Clone identification

pACT2 plasmids harbouring putative positive clones from Y2H screen were extracted from yeast cells and re-transformed into *E.coli* for subsequent screening and

sequencing of the cDNA insert. For this purpose, preparation and electroporation of competent DH5α cells were performed by following protocol from Dower *et al.* (Dower *et al.*, 1988). 50 µl of DH5α *E. coli* cells and 15 µl of the DNA solution (previous section) were used for transformation into *E. coli* by electroporation. The suspension was then placed into an ice cold 1 mm electroporation cuvette (Molecular BioProducts, #5510). For electroporation by Gene pulser (Bio-Rad), the following set up was used: 1.25 kV, 25 µF and 400Ω. Following electroporation, the cells were immediately re-suspended into 1.0 ml LB broth and incubated at 37°C on shaker for 1 hour. All the cells were pelleted and re-suspended in 100 µl LB. The suspension was plated on LB agar plates with Amp (100 µg/ml) and incubated for 16-20 hours. Colonies were picked, grown and subjected to mini-prep for preparation of plasmid DNA using mini-prep kit (Sigma, Fermentas). Restriction digestion was then carried out to identify whether the clones were same or different based on the size of the insert. Clones with different digestion pattern were then sent for sequencing using the L4 sequencing primers. DNA sequencing was performed at the TCAG sequencing facility, Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON. Identity of the clones were determined by using homology based bioinformatics tools such as BLAST.

2.2.9 Preparation of protein extracts from yeast cells

Yeast strains KGY37 or AH109 were transformed by the method mentioned in the section 2.2.4 and incubated at 30°C for 7 days on agar plates with specific nutritional selection. A single colony was inoculated into 5 ml SC- media and incubated for approximately 36-48 hours. 40 mg of cells were harvested, washed with sterile water and re-suspended in 400 µl of sterile water. 400 µl of 0.2 M NaOH was added and incubated

for 10 minutes at room temperature to lyse the cell walls. Cells were pelleted by centrifugation at 13,000 rpm for 1 minute and re-suspended in 100 µl sample loading buffer [0.06 M Tris pH 6.8, 5% (v/v) glycerol, 2% (w/v) SDS, 0.0025% (w/v) bromophenol blue, 4% (v/v) β-mercaptoethanol and boiled for 5 min in a water-bath. 45 µl of the solution was used for western blotting.

2.3 Mammalian cell culture and transfection

2.3.1 Mammalian cell culture

HEK 293 (kindly provided by Dr. E. Leygue and Dr. L. Simard, University of Manitoba), HeLa (kindly provided by Dr. B. Triggs-Raine, University of Manitoba) and mouse embryonic fibroblast (MEF) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco), supplemented with 5% FBS, 0.3% D-Glucose, 2mM L-Glutamine, 1000 units/ml penicillin and 100µg/ml streptomycin. BHK cells were maintained in Modified Eagle's Medium alpha (MEM alpha, Gibco), supplemented with 5% FBS, 0.3% D-Glucose, 2mM L-Glutamine, 1000 units/ml pencillin and 100µg/ml streptomycin. Cells were grown in a humidified atmosphere containing 5% CO_2 in a water-jacketed incubator at 37°C.

2.3.2 Mammalian expression vectors

For performing western blot, immunofluorescence (IF) and immunoprecipitation (IP) studies in mammalian cells, several constructs were generated that contained epitope tags. This was helpful in cases where there was no antibody available for the protein of interest. cDNA was prepared and when necessary the products were sub-cloned to pGEMT-easy vector (Promega). Primers used for constructing the vectors are listed in

section 2.1.5. MBD6 and PCNA were cloned into p3XFLAG-CMV-14 vector (Sigma) that adds C-terminal FLAG-tag to the recombinant protein. HA tag was added to RTEL and RTEL mutants by cloning into the pCDNA3 vectors (Invitrogen). For creating V5 tagged RTEL and RTEL mutants, the cDNA was cloned to pCAGIpuro plasmid (Ding Lab). All the plasmids were constructed by using PCR based strategy described in section 2.1. Midi preparations of the plasmids were carried out using a kit from Qiagen Inc. following manufacturer's instruction to ensure high quality of the plasmids.

2.3.3 Transfection

Transfection was carried out using DNAfectin (abm Inc. #G061) or Lipofectamine (Invitrogen, #11668-019), following manufacturer's instruction. At the time of transfection, cells were 80% to 90% confluent. The incubation time varied depending on the type of application.

2.3.4 Preparation of protein extracts from mammalian cells

Cells were scrapped off the plate after washing with 1X PBS using a scrapper. Depending on the type of application, cells were then lysed with different types of lysis buffers. After incubating with the lysis buffer for 10 minutes on ice cells were subjected to sonication using a sonic dismembranator (Fisher) with the setting: Power 1, 10 second pulse. After sonication, the preparation was incubated for 30 minutes at 4°C with shaking. Soluble proteins were collected as supernatant after centrifugation at 16,000 rpm for 15 minutes. Protein concentration of the lysate was measured using BCA Protein Assay Kit (Pierce, #23225) following manufacturers instruction. For Western blot analysis, cells were lysed in RIPA buffer (50 mMTris-HCl, 150 mMNaCl, 1 mM EDTA, 1% Triton x-100, 1% Sodium de-oxycholate, 0.1% SDS). 30 µg – 50 µg of total protein

was re-suspended in 2X Laemmli buffer (4% SDS,20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M TrisHCl pH 6.8) and boiled in boiling water-bath for 5 minutes.

2.3.5 Immunoprecipitation (IP)

Cell lysate was prepared using IP lysis buffer (50 mMTris-HCl pH 8.0, 150 mM NaCl,1% NP-40, 1mM EDTA) or HEPES IP lysis buffer (10 mM HEPES pH 8.0, 2 mM EDTA, 0.1% NP40,5 mM DTT, 1 mM PMSF), supplemented with protease inhibitor cocktail (Roche). The protein lysate (500 µg - 1 mg) was pre-cleared with 10 µl of Protein A-Sepharose beads (Sigma). Overnight incubation was carried out with 40 µl of beads and antibody. Antibodies and dilutions that were used are listed below:

Table 7: List of antibodies used in this project

Antibody	Species	Dilution used for IF	Dilution for western blot	Amount used for IP (µl)	Source
Anti-V5	Mouse	1/750	1/3000	2.5	Invitrogen
Anti-V5	Rabbit	1/750	1/1000	2.5	AbCam
Anti-FLAG	Mouse	1/750, 1/1000	1/3000	2.5	Sigma
Anti-HA	Mouse	--	1/1000	2.5	Sigma

The beads were then collected by centrifugation at 5000 rpm for 5 minutes and washed 5 times with the IP lysis buffer. Finally the beads were re-suspended in 2X Laemmli buffer and boiled followed by western blot analysis.

2.3.6 Western blot analysis

SDS-poly-acrylamide gel electrophoresis (SDS-PAGE) was applied to separate proteins based on their molecular weight under denaturing conditions, using Mini-PROTEAN system (Bio-Rad, #1653302). The gels were run at 100 V for approximately 2 hours. Using a semi-dry transfer system, the proteins were transferred to Hybond-ECL nitrocellulose membranes (GE healthcare, #RPN68D) following manufacturer's instructions. The transfer was done at 9 V (3.0 mA per cm^2) for 1 hour and 15 minutes. After transfer, the membrane was stained with Ponceau S to check the quality of the transfer. Blocking was done for 1 hour at room temperature with 5% skim milk in .005% Tween-TBS (TBST). Primary antibody dilutions were made in the block and incubated with the membrane for overnight at 4°C. Following 3 washes with TBST, secondary antibody (diluted in block) was applied. After 3 washes with TBST, membranes were developed using Amersham ECL (GE healthcare) following manufacturer's instruction.

2.3.7 Immunofluorescence (IF) studies

After transfection, approximately $1X10^4$ cells were seeded on cover slips to obtain 50% - 60% confluency. Cells were first fixed with 4% paraformaldehyde at room temperature for 15 minutes. Alternatively, they were fixed with methanol by keeping the cells at -20°C for 5 minutes. The cover slips were then washed with PBS three times and treated with 50 mM NH_4Cl for 10 minutes at room temperature. The cells were then permeabilized with 0.2% Triton X-100. Following three washes with PBS, the blocking was carried out using 3% BSA in PBS. Blocking was carried out at room temperature for 1 hour. Dilutions of the primary antibody was made in block and incubated at 37°C for 1

hour. After 3 washes with PBS, secondary antibody was applied (diluted in block). The cells were then stained with 0.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) for 1 min. The cover slips were then washed with PBS and mounted with ProLong Gold Antifade reagent (Invitrogen). Imaging was carried out using Axio Imager (Zeiss) or Axioplan 2 Imaging (Zeiss) microscope and analysed by Axiovision software (Zeiss).

2.4 Generation of *RtelI1169A* (PIP mutant) knockin mice

2.4.1 General Principle

To specifically introduce RTEL-PIP domain mutation (*RtelI1169A*) into the mouse genome, a ES cell mediated transgenic approach was carried out. In our approach, we applied a specific gene-targeting vector to introduce *RtelI1169A* point mutant into ES cell genome by homologous recombination. The gene targeting strategy is described in Figure 9.

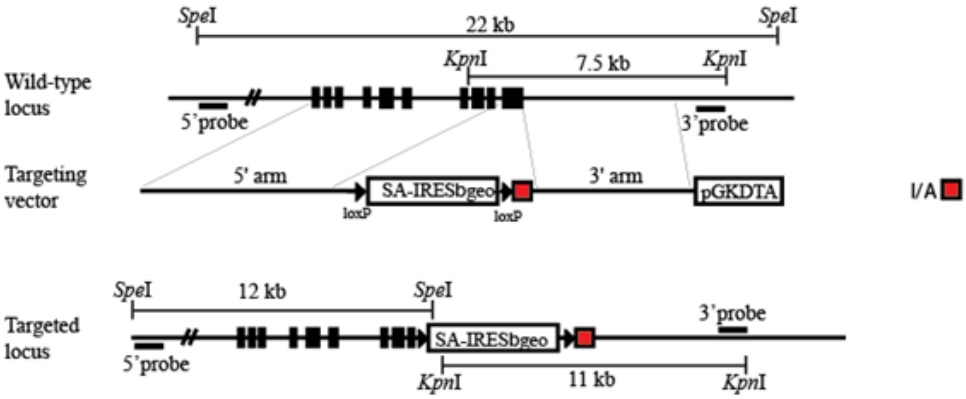


Figure 9: Gene targeting strategy. Schematic representation of the partial Rtel genomic locus, the gene-targeting vector and the mutated locus with exon 34 for the PIP domain mutant I1169A. The *loxP* flanked SA-IRESβgeo cassette can be deleted by *Cre* recombinase excision, forming the RtelI1169A knockin allele.

A plasmid based target vector was created and inserted into the R1 *Rtel*$^{-/-}$ ES cell genome by electroporation. Cells containing vector integration was selected by G418 and analyzed by southern hybridization. The positive ES cells were used for ES cell-diploid aggregation to generate chimeric blastocysts. The blastocysts were surgically implanted into the uterus of pseudo-pregnant foster mother. The chimeric mice were bred with ICR/CD1 mice for testing germline transmission which were then further bred to homozygotes for characterization and phenotyping. This procedure is summarized in figure 10. To delete the loxP flanked SA-IRESbgeo selection cassette, these mice were further breed with pCXDdx-cre mice that ubiquitously express Cre. This approach allowed us to establish a mouse allele with RtelI1169A (PIP mutant) knockin.

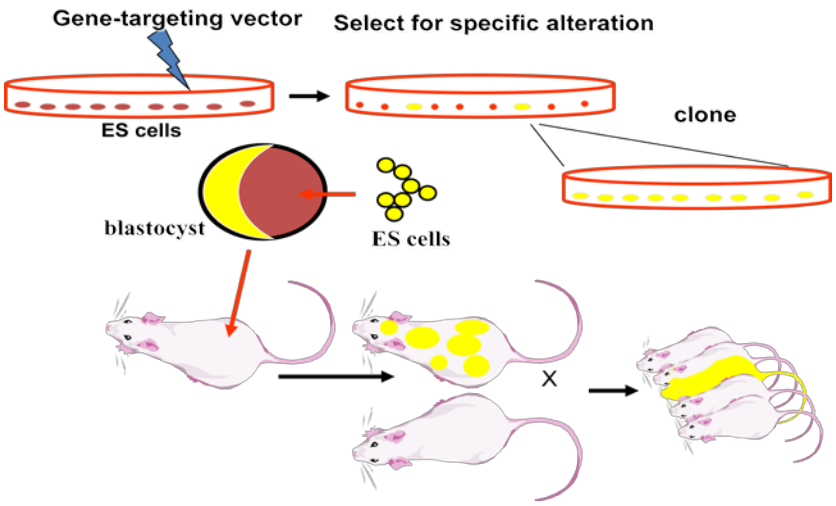


Figure 10: A gene-targeting approach for establishing *RtelI1169A* allele in mouse genome. Linearized gene targeting vectors were electroporated into ES cells. The ES cells were selected for the correct integration of the vector and allowed to form chimeric blastocysts. The blastocysts were then implanted in a pseudo pregnant mother. The chimeric mice were further bred to homozygotes.

2.4.2 Generation of gene targeting vector

The *RtelI1169A* knock-in vector was made based on a PCR-based cloning strategy as described previously (Wu and Ding, 2007). Briefly, the mouse *Rtel* genomic fragments required for 5' and 3' arms of homology as well as exon 36 (the last coding exon of *Rtel* which contains PIP motif) were PCR-amplified from the genomic DNA of R1 ES cells with a high fidelity polymerase (Clontech, #639241). After validation by DNA sequencing, a PCR based mutagenesis approach was used to change nucleotides atc to gcc. A V5 tag was also inserted into exon 36 in which the coding sequence of V5 was in frame fused with the last codon of *Rtel*. This modified exon 36 was then ligated with 3' arm followed by a *pGK-DTA* negative selection cassette. 5' arm of homology was inserted into a cloning vector that contains a *lox*P flanked *SA-IRESβgeo* cassette. Using the restriction enzymes and the cloning strategy as described in Figure 11, the DNA fragments were isolated, and assembled together to generate the *RtelI1169A* knock-in vector.

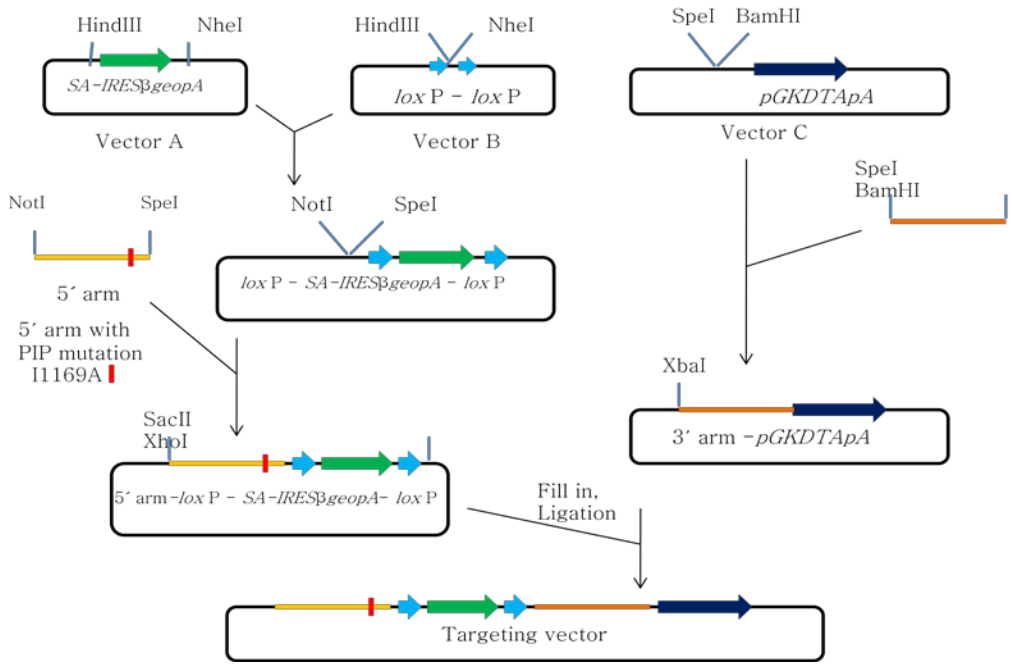


Figure 11: Overall strategy to clone the gene targeting vector for *RtelI1169A* knock-in

The primers used for generating the vector are listed below:

Primer name	Sequence
RTELV5KiP1	5′ tcaaagcctcagttcctacagac 3′
Rtelki 3 arm p1	5′ gaatgttggttatggtagggcactgaacagtgtg 3′
Rtelki 3 arm p2	5′ gacatgacctgtcctccactcatttgcgtgag 3′
Rtelki 5 arm p1	5′ gaggacttgaactgacagccactcattcac 3′
Rtelki 5 arm p2	5′ cccaggatccttgcctgtatccctgaatagag 3′
Rtelki 5 arm p3	5′ ggcaaggatcctgggaacccaaagtatatc 3′
Rtelki 5 arm p4	5′ gcatgaattccctaggggaggtctcttgg 3′
Rtelki 5 arm p5	5′ agggaattcatgctagaggcttggcac 3′
Rtelki 5 arm p6	5′ gcaagggtaggagtcttccagtgggc 3′
Rtel Ex 32 p1	5′ tctcgcactgttgctatctttagggccctcgatgtccaaga 3′
Rtel Ex 32 p1a	5′ gctaacaggaagcccataagtttctcgcactcgttgctatc 3′
Rtel Ex32p2	5′ ccctattgcttactccgatgaggcttcataagctc 3′

2.4.3 Electroporation of embryonic stem (ES) cells

Electroporation of ES cells was carried out using a GenePulser machine (BioRad, #1652108). Briefly, ES cells were grown on gelatin coated plates and a total of 1X 10^7 cells were collected. These cells were mixed with 20 µg of linearized targeting vector and the electroporation was performed based on the instruction of manufacturer. The transfected ES cells were plated on 10 cm gelatine coated plates with knockout media (Invitrogen, 10829018), supplemented with LIF (leukemia inhibitory factor). After 48 hours, 250 ug/ml of G418 were added and selected for 10-14 days. The G418-resistant colonies were picked and cultured in 96 well-plates.

2.4.4 Southern blot analysis to identify the correct targeted ES clones

To identify the correct targeted ES clones, Southern blot hybridization was performed. For isolation of genomic DNA, embryonic stem (ES) cells were lysed in lysis

buffer (1M Tris-HCl, pH 8.5, 0.5 M EDTA, 20% SDS, 5M NaCl) with Proteinase K, at 55°C for overnight. Phenol -chloroform extraction was performed and the DNA was dissolved in water. Overnight digestion was carried out with restriction enzyme. DNA extractions in 96-well plate were digested with SpeI for the 5' probe, and KpnI for the 3' probe. The whole procedure of Southern hybridization is described in section 3.4.6.

2.4.5 ES-diploid aggregation for establishing *RtelI1169A* knockin allele

E2.5 (8 cell stage) embryos were collected from ICR/CD1 mice. The fallopian tube was cut out and embryos were flushed out in M2 media. The embryos with good morphology were selected under microscope by mouth pipette and gathered in a 6 cm plate on drops of M2 media. After several wash with the media, the zona of the embryos were removed by incubation in Acid Tyrode's.

The zona-removed embryos were aggregated with the gene-targeted ES cells. Each embryo was aggregated with a clump of ES cells (containing 8-10 cells) in a well which was made by a sterilized needle on M2 media drops covered with mineral oil. The ES-diploid aggregates were cultured at 37° C till blastocyst stage (about 24 hours). The blastocyst stage embryos were collected by pipette filled with M2 media and surgically implanted to pseudo-pregnant mice.

2.4.6 Molecular Analysis

2.4.6.1 Southern hybridization

Southern hybridization was carried out using a modified version of Bart Herman's protocol (Carmeliet *et al*., 1999). Digested genomic DNA was then run on 0.8% agarose

gel at 100V. The gel was then treated with denaturing solution (1M NaCl, 0.5M NaOH) for 20 mins followed by 20 mins in neutralizing solution (3M NaCl, 0.5M Tris-HCl pH7.4). Afterwards it was washed with 10XSSC for 20 mins. The separated DNA was then transferred to Hybond-N, Nylon membranes (Amersham) overnight by capillary transfer using 10X SSC (3M NaCl, 0.3M sodium citrate pH7.0). The membrane was then exposed to UV light for 15 minutes to cross-link DNA to membrane.

The DNA probes were PCR-amplified using mouse genomic DNA. PCR products were then subcloned into pGEM-Teasy vector. The probes were isolated and labelled using random priming method following manufacturer's instructions.

Pre-hybridization was carried out for 4 hours at 65°C with 2mM denatured salmon sperm DNA in 5X SSC (1.5M NaCl, 75mM sodium citrate), 10X Denhart's solution (2% ficoll, 2% polyvinylpyrrolidone, 2% BSA) and 0.1% SDS. Hybridization with the radioactive probe was then carried out overnight at 65°C with $5x10^6$ cpm/ml probes. To wash out non-specific hybridized probes, membranes were washed under increasingly stringent conditions [SSC 1X (0.3M NaCl, 15mM sodium citrate) SDS 0.1%; then SSC 0.1X (0.03M NaCl, 1.5mM sodium citrate) SDS 0.1%] at 65°C. The membranes were then exposed to the phosphor-imaging screens, and images were taken using a Molecular Imager FX (Bio-Rad).

2.4.6.2 RT-PCR

RNeasy plus kit (Qiagen) was used to isolate RNA from derived *RtelI*^{1169}*A* knockin ES cells and MEF cells following manufacturer's instruction. As the controls, wt ES or MEF cells were applied. To obtain cDNA, reverse transcription reactions were performed on DNAse treated total RNA. Total RNA were incubated for 30 minutes at

37°C with 0.5U of DNAse (Sigma). After inactivation of the enzyme by 1.5 mM EDTA, the RNA samples were then transcribed using SuperScript III reverse transcriptase (Invitrogen), incubating for 50 minutes at 42°C with RT buffer mix (0.5mM dNTPs, 10mM DTT, 50mM Tris-HCl pH8.3, 75mM KCl, 3mM $MgCl_2$). Again the enzyme was denatured by incubation for 15 minutes at 70°C. PCR reaction was then carried out using primers listed in section 3.4.2. PCR conditions of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds in buffer supplied by the manufacturer were used to generate amplicons.

2.4.6.3 Western blot

For determining the expression of RTEL-V5 and RTELI1169A-V5 protein, western blot was done on ES cells following a protocol described in section 3.3.5.

2.5 Cytogenetic analysis

2.5.1 Preparation of metaphase spreads

Metaphase spreads were prepared from ES or MEF cells. In both cell lines, the cells were first treated with 10 nM nocodazole for 24 hours, then trypsinised and harvested in PBS. The suspension was centrifuged for 25 min at 5,000 rpm. Cell pellets were suspended in hypotonic solution (0.075 M KCl) and incubated for 30 min at room temperature. Cells were then centrifuged and cell pellets were suspended in the fixative (Methyl alcohol/glacial acetic acid, 3:1, v/v) drop by drop gradually increasing the amount with 5 min interval and incubated at room temperature for 20 minutes. Drops of the suspension were allowed to spread on freshly prepared slides by quickly treating the

slides with high temperature (55°C) and acetone treatment. The slides were then incubated with dye and washed with PBS followed by air drying.

2.5.2 Quantitative FISH (Q-FISH) analysis

Slides with metaphase spreads were incubated with PBS for 5 min and prefixed with 3.7% formaldehyde in PBS. To remove any proteins binding with the chromosomes, pepsin treatment was carried out followed by 3 washes with PBS. For pepsin treatment, slides were incubated with 0.01 M HCl with 1.0 g/ml pepsin for 10 minutes. The slides were then washed and fixed with 3.7% formaldehyde in PBS, followed by 3 washes with PBS at room temperature. For dehydrating, the slides were kept in 70%, 90%, 100% ethanol for 2 minutes at room temperature. After air drying, 8.0 µl of Cy3-$(CCCTAA)_3$ PNA-probe (DAKO, #K5325) was applied to the selected area on slides and covered with 22 mm X 22 mm cover-slips. All steps were done afterwards avoiding direct exposure to light. To avoid evaporation, the cover slip was sealed with rubber cement. Using a HYBrite thermo-cycler, the slides were denatured at 80°C for 3 minutes and hybridized for 2 hours at 30°C. The slides were then washed with 70% formamide in 10 mM Tris-Cl, pH 7.4 at room temperature for 15 minutes each. The slides were then washed with PBS and SSC (0.3M NaCl, 15 mM sodium citrate)and stained with DAPI. Slides were kept in 70%, 90%, 100% ethanol for 2 minutes at room temperature for dehydration and air dried. Finally, the cells were mounted with Vectashield (VectorLabs, #H-1000).

Images were acquired using a microscope equipped with epifluorescent optics specific for each fluorochrome. Well spread, nicely stained metaphases were imaged, 20 metaphases per slide. Images were captured by ZeissAxioplan 2 Imaging microscope

using Spectra Cube (ASI) with a 63X 1.4 oil objective. To perform PC analysis, the Case Data Manager 4.0 software (ASI) was used.

2.6 Bioinformatics analysis

For bioinformatics analysis various tools available from National Commission for Biotechnology Information (NCBI) and European Molecular Biology Laboratory (EMBL) were used.

For determining identity of the Y2H positives, basic local alignment tool (BLAST) was used from NCBI, with different options and parameters specific to the query sequence. (Altschul *et al*., 1990). CLUSTALW2 software was used from EMBL to align different proteins for finding conserved domains or homology (Larkin *et al*., 2007).

Chapter 3: Results

3.1 Identification of RTEL-interacting proteins by a yeast two-hybrid (Y2H) screen

To identify novel interacting partners for RTEL *in vivo*, yeast two-hybrid (Y2H) screen was employed. Two bait constructs were used for the screens, one for the full length RTEL protein and the other for C-terminal portion of RTEL (amino acids 2101-3733). Both solid and liquid screening methods were used to screen a cDNA testis library with approximately 3.5 X 10^6 clones. In addition, two different host strains were used- KGY37 and AH109 (Gietz lab). Table 8 summarizes the results of the screens.

pACT-2 plasmids harbouring the cDNA fragments from positive clones were isolated and used to confirm the interaction with full length RTEL using β-gal staining method. Putative positives were sequenced and their identity was revealed by using Basic Local Alignment Tool (BLAST) from NCBI. The top candidate proteins are listed in Table 9.

Table 8: Summary of yeast two-hybrid screens

Bait	Y2H screen media	Y2H positives	Reconfirmed Y2H positives with full length RTEL	No. of transformants screened (approximately)	No. of clones in library (approximately)
Rtel full length	solid	23	19	$4.8X10^8$	$3.5X10^6$
	liquid	6	2	$3.36X10^9$	
RtelC-terminal	solid	7	3	$1.62X10^9$	
	liquid	29	22	$1.39X10^{10}$	

Table 9: Top Y2H positives for RTEL in yeast two-hybrid screens

Y2H positive	No. of times isolated	Sub-cellular location	GenBank Accession number	Comments
RAN binding protein 9 (RANBP9)	19	Nuclear/ cytoplasmic	NM_019930.2	GTP binding protein; associated with nuclear transport
Cathepsin L preproprotein	2	Lysosome	NM_009984.3	Involved in antigen presentation and processing
Testis specific serine kinase 2	2	Cytoplasm	NM_009436.2	Serine/Threonine protein kinase with unknown fuction
MBD6	3	Nuclear	NM_033072.2	Consists of a conserved methyl-CpG-binding (MBD) domain. Functions are not characterized
Casein kinase II beta subunit	3	Golgi apparatus, endoplasmic-reticulum	NM_009975.2	Functional protein consists of 3 subunits and regulates metabolic pathways, signal transduction, transcription, translation, and replication. The beta subunit is involved in the regulation of the protein.

3.2 Characterization of RTEL/MBD6 interaction

3.2.1 Cellular localization of MBD6

From the Y2H positive candidates, only Methyl-CpG-binding Protein 6 (MBD6) was found to be localized in nucleus, making it a strong candidate. Therefore, MBD6 was selected for further characterization to determine whether it can indeed interact with RTEL.

As a first approach, we have applied immunofluorescence assay to demonstrate that MBD6 is localized in nucleus (Figure 12).

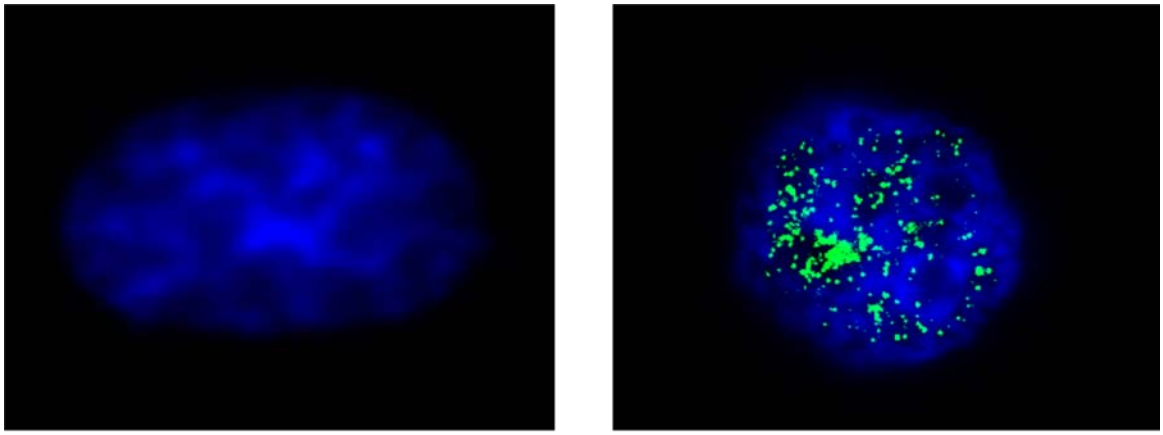


Figure 12. Nuclear localization of MBD6. FLAG-tagged MBD 6 was transfected into HeLa cells. 24 hours after transfection, cells were fixed and the cellular localization of the protein was revealed by indirect immunofluorescence. Green: anti-FLAG antibody; Blue: DAPI.

3.2.2 To verify the interaction between RTEL and MBD6

To verify if MBD6 interacts with C-terminal region of RTEL, co-immunoprecipitation (Co-IP) studies were done. HEK293 cells were transiently transfected with FLAG/myc tagged MBD6 and HA-tagged RTEL C-terminal. As shown in Figure 13, immunoprecipitation of RTEL C-terminal or MBD6 could pull down MBD6 or RTEL-C-terminal, indicating these two proteins could interact with each other.

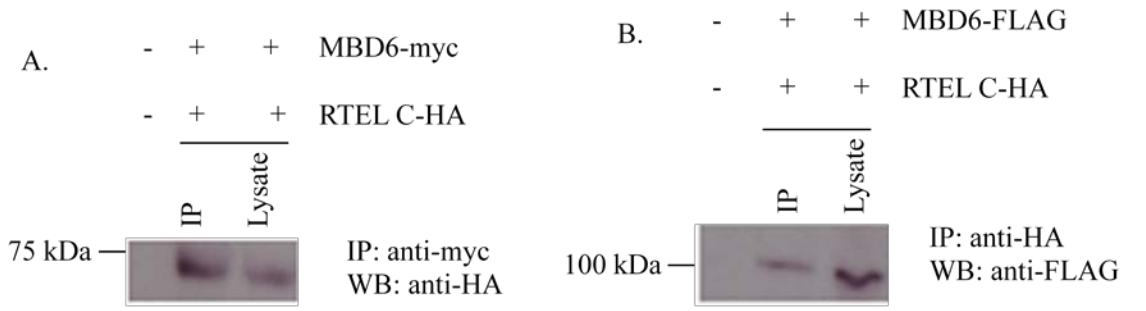


Figure 13. Co-IP to demonstrate the interaction between the RTELC-terminal and MBD6. HA tagged RTELC-terminal construct and FLAG/myc tagged MBD6 were transfected in HEK 293 cells. IP was carried out by both anti-myc **(Fig. A)** and anti-HA **(Fig. B)** antibodies followed by western blot with anti-HA and anti-FLAG antibody respectively.

However, when we carried out IP with full length RTEL-V5, no specific bands corresponding to MBD6-FLAG was detected. When IP was carried out MBD6-FLAG, the antibody pulled down RTEL non-specifically. A representative IP result is described in Figure 14. Based on these findings, we concluded that MBD6 is not a true RTEL-interacting protein.

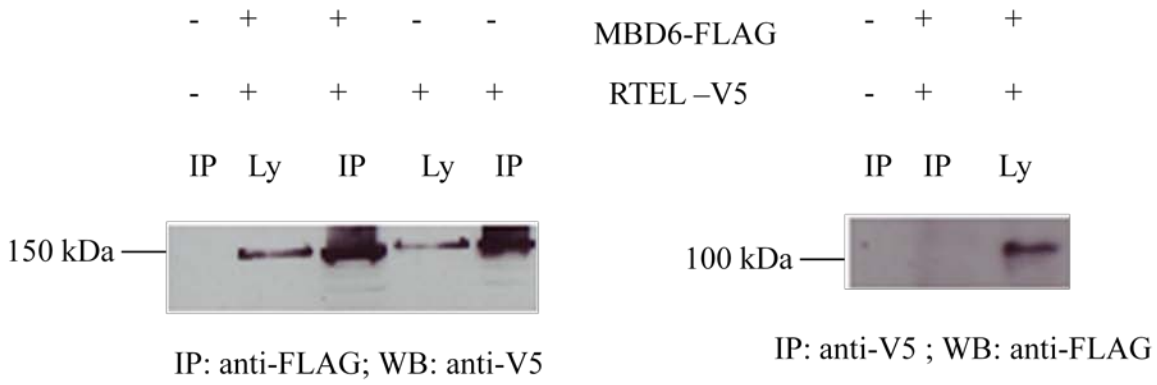


Figure 14. Co-IP studies could not demonstrate the interaction between full length RTEL and MBD6. V5- tagged RTEL and FLAG-tagged MBD6 were transfected in HEK 293 cells. IP was carried out by both anti-FLAG and anti-V5 antibodies followed

by western blot with anti-FLAG and anti-V5 antibody respectively. IP: immunoprecipitation; Ly: cell lysate.

3.3 Identification of RTEL-interacting proteins by bioinformatics

3.3.1 Identification of PCNA-interacting protein (PIP) motif in RTEL

To further search for RTEL-interacting proteins, a bioinformatics approach was applied to identify the protein motifs/domains in RTEL protein that are potentially responsible for protein interaction. From CLUSTAL W from EMBL (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Larkin *et al*., 2007), a well-conserved PCNA-Interacting Protein (PIP) motif was identified, with the consensus sequence Q-X-X-I-X-X-F-F in both mouse and human RTEL proteins (figure 14).



Figure 15. Identification of a conserved PIP motif in RTEL. CLUSTALW alignment of RTEL proteins from mouse, human and bovine origin demonstrates the conserved PIP motif. Of the 8 amino acids, 7 are identical in all three species and the last phenylalanine (F) is conservatively substituted by leucine (L) in human and bovine."*" indicates identical, ":" indicates that conserved substitutions, "." shows that semi-conserved substitutions are observed. The conservation is denoted by colors- Red: small (Small + hydrophobic including aromatic Y), Blue: Acidic, Magenta: Basic, Green: hydroxyl+amine+basic-Q, Gray: others

3.4 Characterization of RTEL/PCNA interaction

3.4.1 Co-IP to demonstrate that RTEL interacts with PCNA via PIP motif

PCNA was envisioned as a possible interacting protein for RTEL because of the presence of the conserved PCNA binding motif, PIP box (encoded by exon 34 at C terminal) in RTEL. Co-IP was employed to demonstrate that RTEL interacts with PCNA (Figure 16 B). To further study if this interaction is mediated via PIP motif, a PCR-based mutagenesis method was used to introduce a series of point mutants in the RTEL PIP box (Figure 16 A). As demonstrated by co-immunoprecipitation studies, some of mutations, such as *RtelQ1166A* and *RtelII1169A*, can completely abolish the interaction between RTEL with PCNA. A representative IP data is presented in Figure 16. Immunoprecipitation was carried out using anti-V5 antibody, followed by western blot with an anti-PCNA antibody.

3.4.2 Immunofluorescence (IF) studies

To further confirm the interaction between RTEL and PCNA, indirect immunofluorescence studies were carried out. FLAG-tagged PCNA and V5-tagged RTEL were found to be co-localized in HeLa cells (Figure 17 A). Both RTEL and PCNA form punctate staining patterns, however, the patterns are different for RTEL and PCNA. While PCNA forms punctate pattern only in S-phase,(Schermelleh *et al*., 2007), such patterns were observed in case of RTEL throughout the cell cycle. When V5-taggedRTEL-PIP mutants were co-expressed with FLAG-tagged PCNA, no co-localization was observed (Figure 17 B).

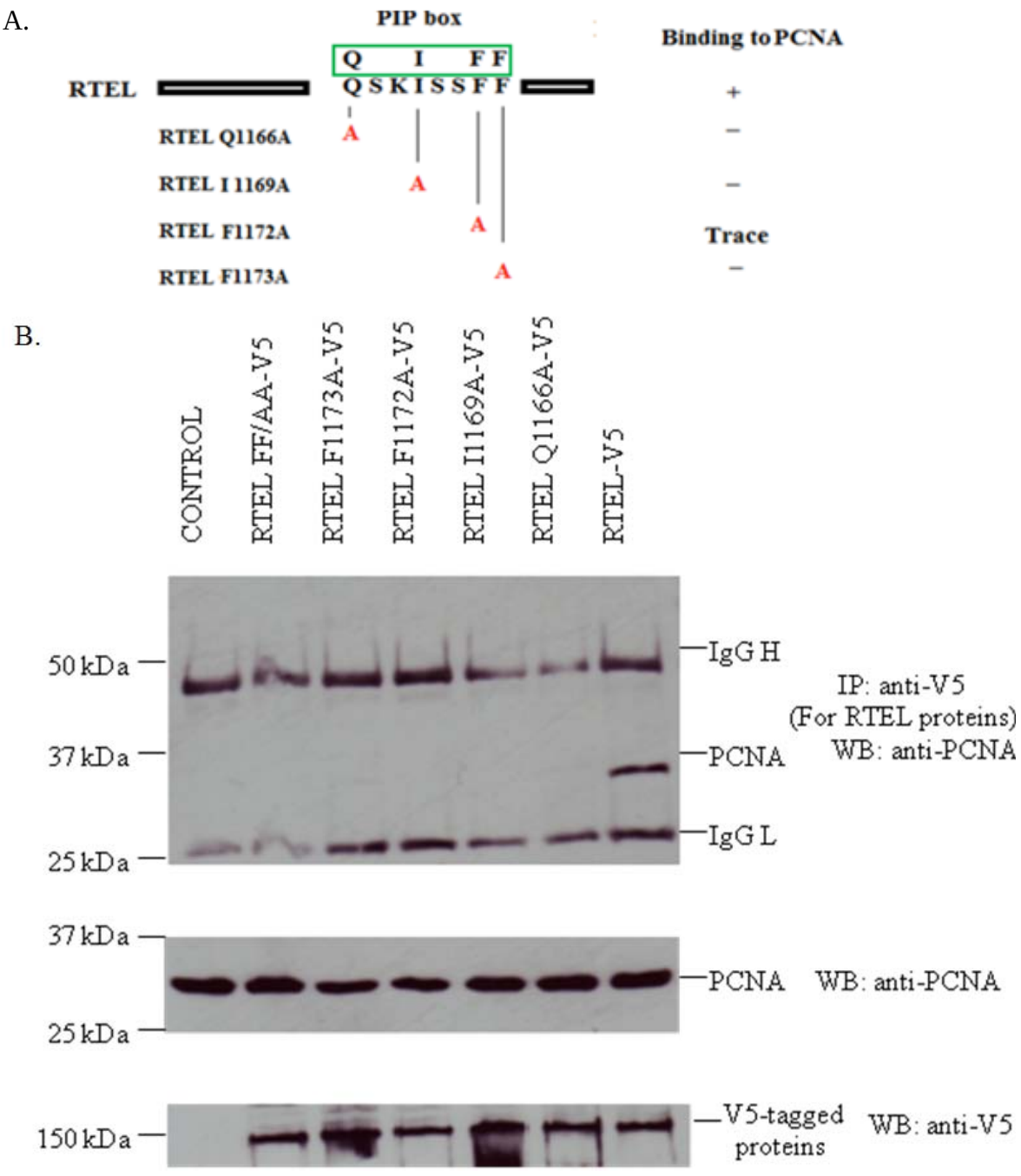


Figure 16: RTEL interacts with PCNA via PIP motif. A. A diagram shows the conserved PIP box and several amino acids in this motif replaced by the indicated amino acids using PCR based mutagenesis. **B.** Western blots demonstrating that several specific mutations in the PIP box of RTEL, such as I1169A, can completely abolish the interaction of RTEL with PCNA.

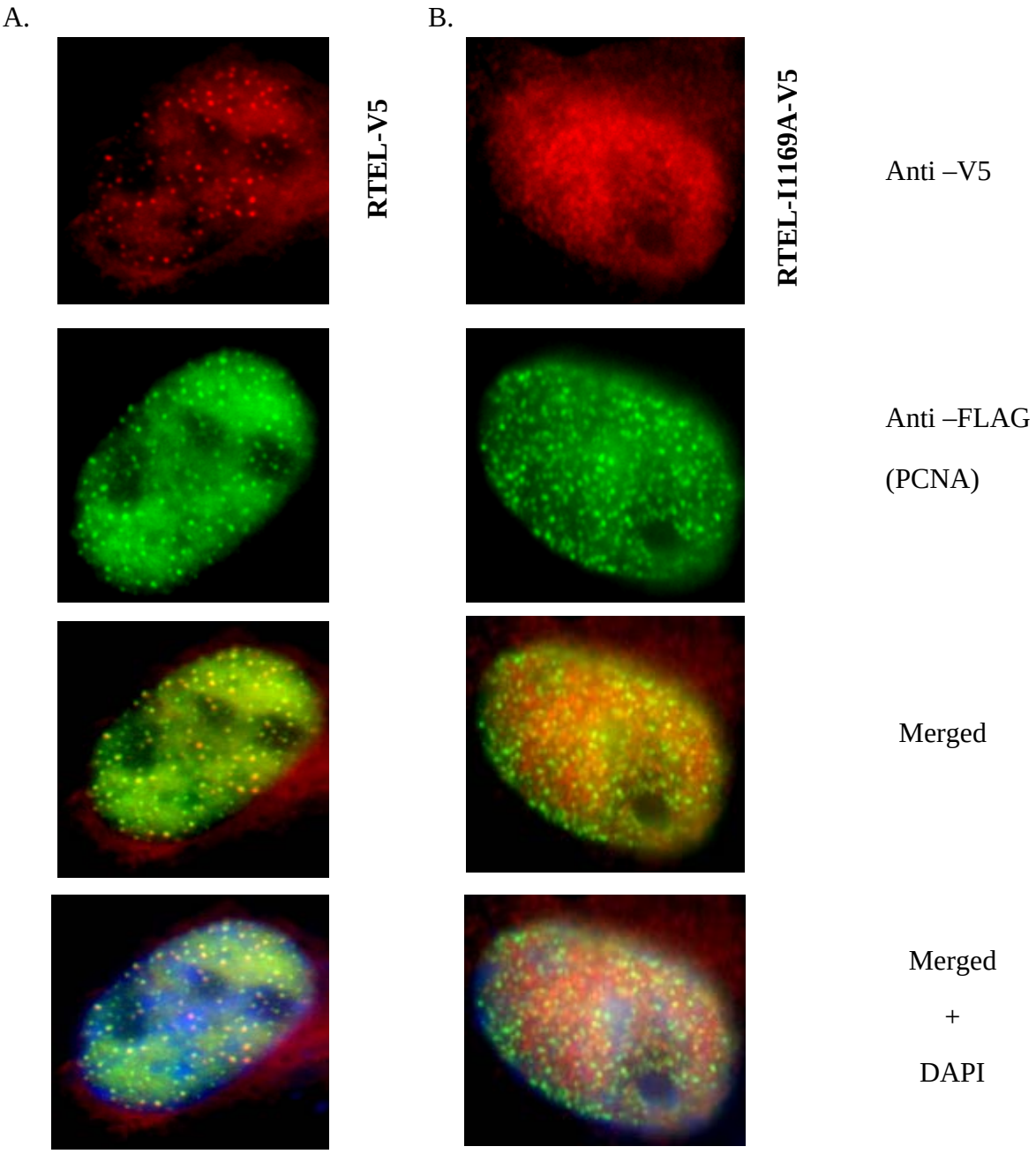


Figure 17. Co-IF analysis of RTEL/PCNA interaction. (A) V5 tagged RTEL (red) co-localizes with FLAG-tagged PCNA (green) (B) V5-tagged RTEL PIP mutant - RtelI1169A (red) does not co-localize with FLAG-tagged PCNA (green).

3.5 Establishment of genetic tools to characterize RTEL/PCNA interaction

To determine the physiological relevance of RTEL/PCNA *in vivo*, a mouse embryonic stem cell (ES)-mediated transgenic approach was applied to specifically introduce a RTEL PIP mutation (RTELQ1166A) into *Rtel* locus in mice. This approach allowed us to establish a mouse mutant that lacks the interaction between RTEL and PCNA. Further phenotypical characterization of this mutant mouse will help us to understand whether this interaction is essential for the maintenance of genomic stability and its involvement in mouse development.

3.5.1 Generation of the gene-targeting vector for knocking in *RtelI1169A* mutation in mouse genome

The targeting vector was generated based on our published cloning method (Wu and Ding, 2007). The 5′ arm and 3′ arms in this vector aided the homologous recombination to specifically replace exon 34 with a modified exon 34 with a specific mutation to generate RTEL protein with Q1166A mutation. As demonstrated in Figure 16, we have showed that RTELQ1166A completely abolish RTEL/PCNA interaction. Therefore, from this gene-targeting strategy, we expected to generate a mouse mutant that can completely abolish RTEL/PCNA interaction *in vivo*. To facilitate detecting RTELQ1166A protein in mice, we have inserted a V5 tag on the C-terminus. To account for the effect of V5 tag on our phenotyping analysis, we have also created a mouse allele in which only V5 tag was inserted on the C-terminus of RTEL. Using the restriction digestion pattern, we confirmed that these gene-targeting vectors are correct (Figure 18). We have also verified these vectors by DNA sequencing.

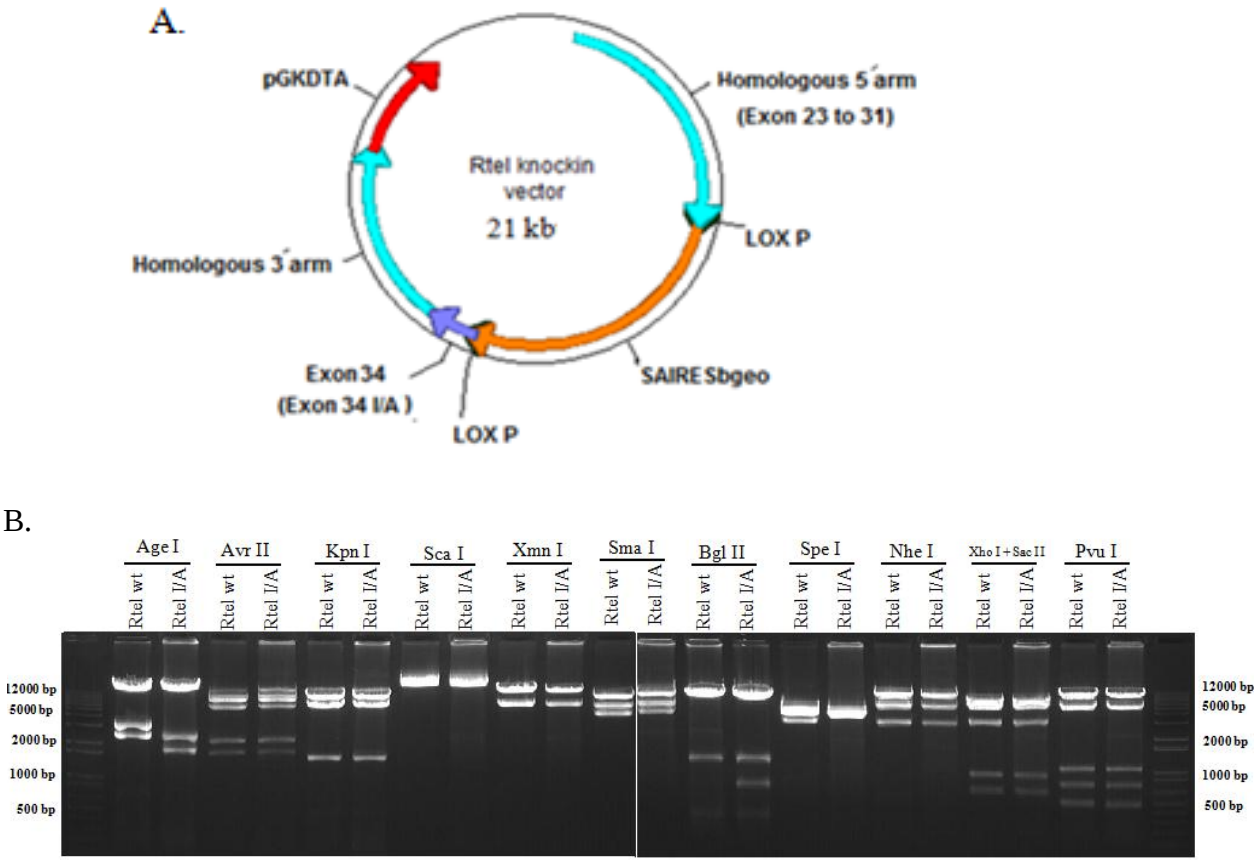
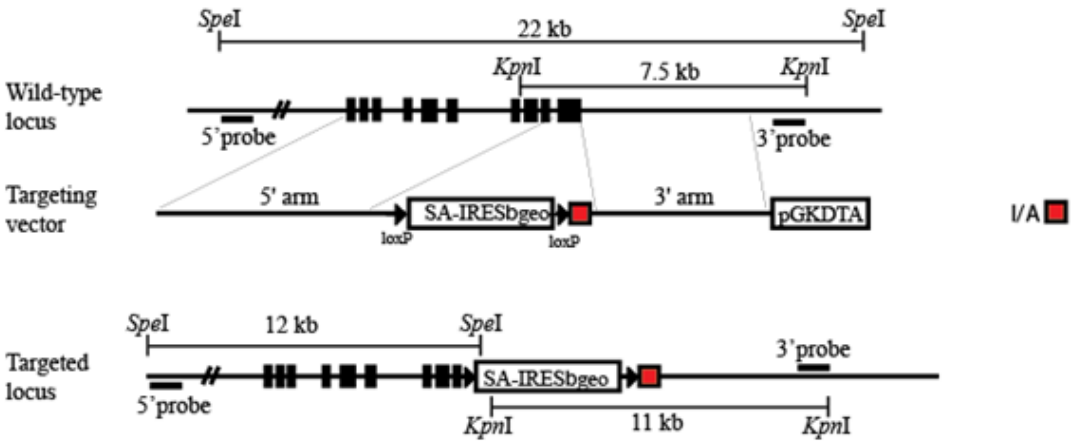


Figure 18. Generation of the gene targeting vector to knockin a PIP mutation in mouse genome. (A) Schematic diagram of the gene targeting vector, showing the PIP mutation on exon 34 of RTEL, selection cassettes and the homologous arms. (B) Restriction digestions of knock-in vectors for *RtelV5* allele and *RtelI1169AV5* allele, showing the predicted pattern based on DNA sequence of vectors. .

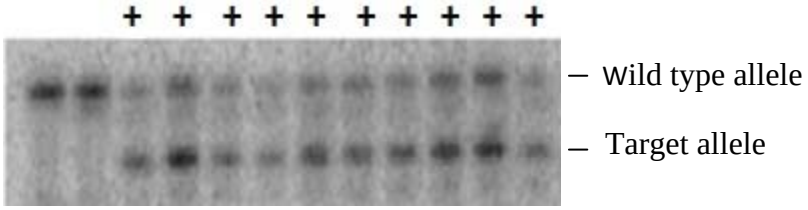
3.5.2 Knocking-in *RtelI1169A-V5* allele into mouse genome

To specifically knockin the PIP mutation, *RtelI1169A*, into the mouse genome, the gene-targeting vectors were linearized and electroporated into ES cells. Correct homologous recombination in ES cells was confirmed by Southern blot analysis (Figure 19). From this study, we have obtained multiple ES clones for producing transgenic knock-in mice.

A. gene-targeting strategy



B. Southern blot with 5´ probe



C. Southern blot with 3´ probe

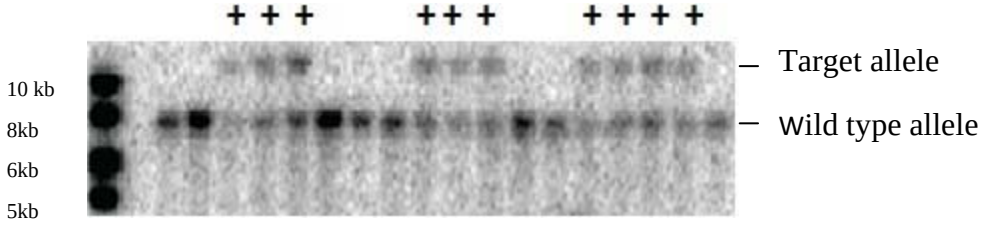


Figure 19. Knock-in RtelI1169AV5 allele into mouse *Rtel* locus. A. Schematic representation of the partial Rtel genomic locus, the gene-targeting vector and the mutated locus with exon 34 for the PIP domain mutant I1169A. **B,C.** Southern blot analysis of ES clones with insertion of targeting vector. The genomic DNA was digested with *SpeI* and *KpnI* and probed with 3´ and 5´ probes, respectively. '+' denotes positive integration of the vector. Note the high gene-targeting frequency (>60%) in this experiment.

3.5.3 ES cell diploid aggregation

To generate the RtelI1169-V5 and RtelV5 mouse alleles, an ES cell-diploid aggregation was applied. 8-10ES cells was aggregated with E2.5embryos as illustrated in figure 24. After 24 incubation at 37 °C with 5% CO_2, the aggregates developed into blastocytes (Figure 24C), which were then transferred into pseudo-pregnant ICR/CD1 females for developing into off springs, i.e. chimeras.

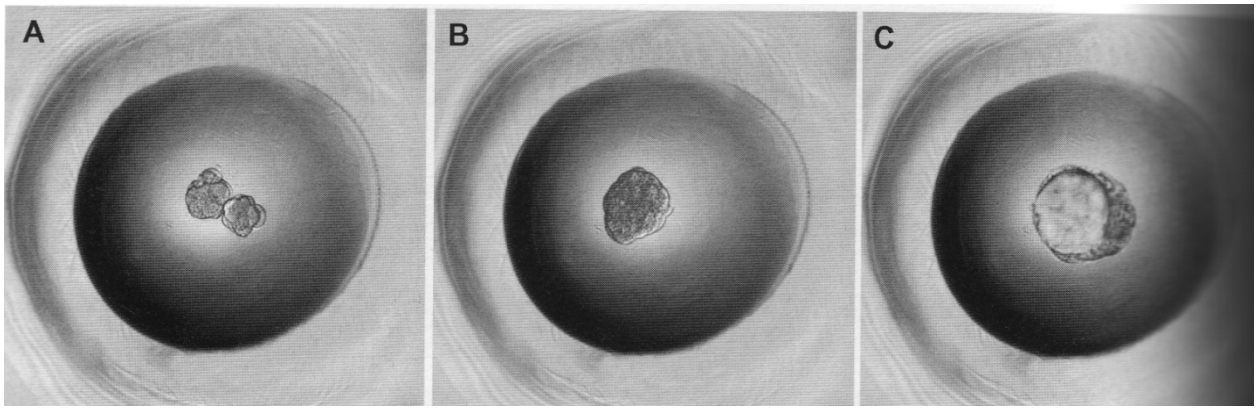


Figure 20. ES-diploid aggregation assay to generate Rtel1169V5 and Rtel V5 chimeras. A. aggregates of zona-removed E2.5 mouse embryos and targeted ES cells. B. Fusion of ES cells with embryos C. Formation of blastocyst after incubation for 24h. The bastocytes were transferred into pseudo-pregnant mice for further development.

3.5.4 Characterization of *RtelI1169AV5* and *RtelV5* alleles in established transgenic mice

Using the above approaches, we have generated two transgenic mice lines with two different alleles in the *Rtel* locus of the genome- Rtel I1169AV5 and RtelV5. We have first confirmed the presence of the RTELI1169A-V5 mutation by sequencing of genomic DNA (Figure 21 A).

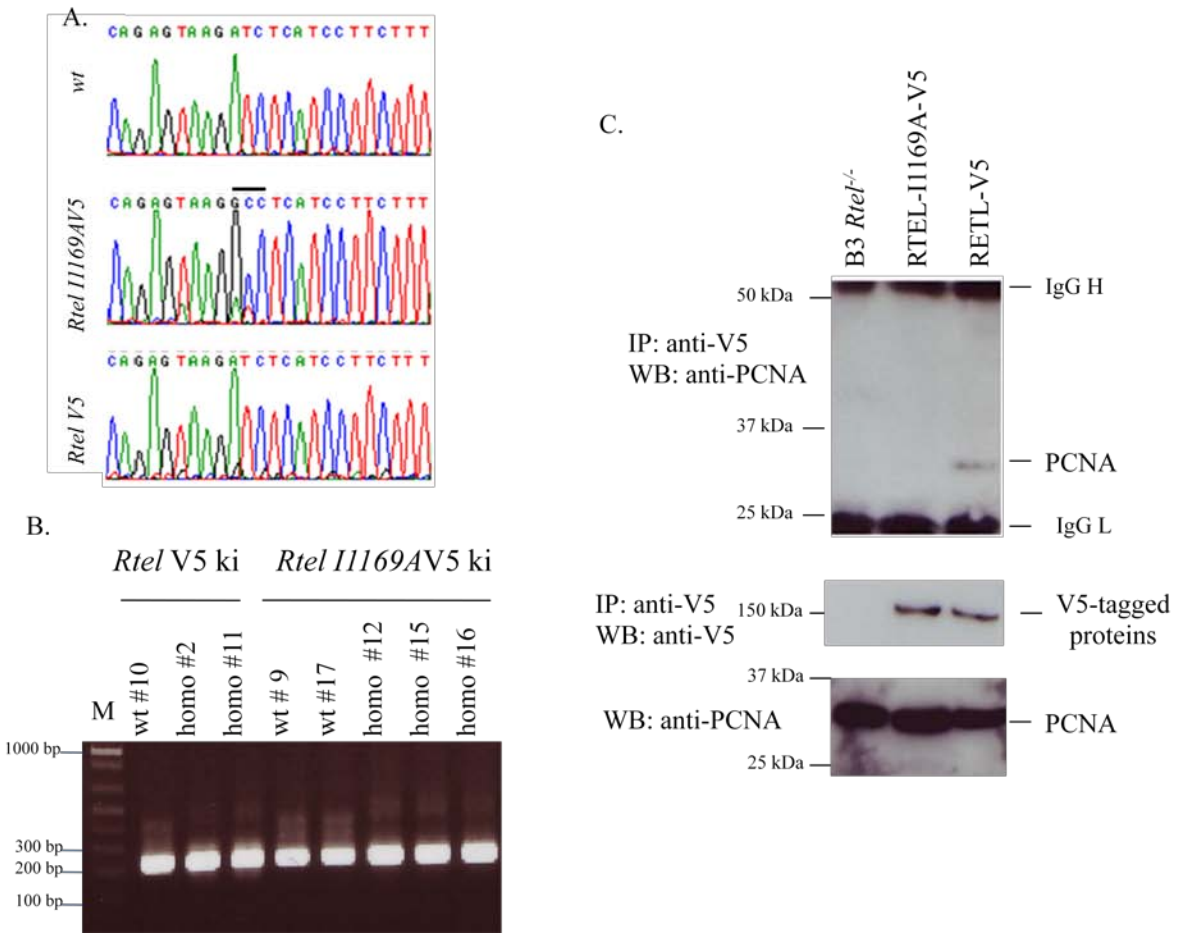


Figure 21: Characterization of *Rtel V5* and *Rtel I1169V5* alleles in established transgenic mice. **A.** Sequence confirmation of mice homozygous for *RtelI1169A* allele. The DNA sequence encoding the 1169 amino acid residue of RTEL in different alleles was confirmed by direct DNA sequencing using primer RtelV5ki p1 and RtelV5ki 4a (Table1). The Figure shows a chromatogram of DNA sequence for coding the PIP motif from different alleles. The I1169A mutation is highlighted by bar**. B.** RT-PCR showing expression of wt, *Rtel V5 ki* and *Rtel1169A V5 ki* alleles. **C.** Consistent with previous co-immunoprecipitation experiment results, western blot demonstrating that knocking-in RtelI1169A-V5 mutation can abolish the RTEL/PCNA interaction in mouse ES cells derived from $Rtel^{1169A/I1169A}$ mice.

To demonstrate that these mouse alleles indeed can express the V5 tagged *Rtel* or V5 tagged *RtelI1169A* mutants, we have applied RT-PCR to determine expression of the alleles at RNA level (Figure 21B). The RT-PCR results demonstrate that under endogenous conditions the alleles are expressed. Moreover, the expression level of *RtelV5* and *RtelI1169AV5* alleles were similar. To determine the expression level of the alleles at protein level, western blot was carried out. In contrast to RNA level, the protein level was very low in ES and MEF cells (data not shown). This might indicate a high turnover rate of the proteins. However, immunoprecipitation experiments results demonstrated the expression of the alleles (Figure 20 C). Co-immunoprecipitation experiments were carried out where RTELV5 and RTElI1169AV5 proteins were immunoprecipitated with an anti-V5 antibody, followed by western blot with an anti-PCNA antibody. The results suggested that the insertion of I1169A mutation can abolish the interaction between RTEL and PCNA *in vivo* (Figure 20 C). Taken together, these results demonstrate the *Rtel-V5* and *RtelI1169A-V5* alleles have been established in the mouse genome.

3.6 Chromosomal phenotypes in *Rtel*$^{I1169A/I1169A}$ mutant mice

To determine the chromosomal features of the MEF cells derived from *Rtel*$^{+/+}$ and *Rtel*$^{I1169A/I1169A}$ background mice, metaphase spreads of the chromosomes were prepared and stained with DAPI. Table 10 lists the chromosomal phenotypes.

Table 10: Summary of cytogenetic observations in *Rtel*$^{I1169A/I1169A}$ cells.

	Metaphases Analyzed	Aneuploidy (% Cells)[a]	Fragile Telomere (Frequency per Metaphase)[b]	Chromosomal Breaks/Fragments (Frequency per Metaphase)	End-to-End Associations/Fusions (Frequency per Metaphase)[c]
Wild type	44	29%	4 (0.09)	2 (0.04)	0
Rtel$^{I1169A/I1169A}$	62	56%	26 (0.41)	11 (0.17)	5 (0.08)

[a] Expressed as percentage of metaphase with other than 40 chromosomes (each Robertsonian fusions counted as two chromosomes).

[b] Chromatid ends with doublet signal was scored as a fragile telomere

[c] Expressed as total number of end-to-end associated chromosomes per metaphase, including dicentric chromosomes, telomere associations and Robertsonian fusions.

For exploring the telomeric phenotypes, quantitative-FISH (Q-FISH) analysis were done on cells using a Cy3-$(CCCTAA)_3$ PNA-probe for telomeres. Figure 22 describes representative images of wild type and PIP mutant cells. The results indicate a loss of telomeric signals in mutant cells. In wild type cells, only a few cells had low telomere signals, whereas in mutant cells the frequency of such cells was much higher. In addition, several chromosomal abnormalities were observed in the cells, such as end-to-end fusion, fragmentation and fragile telomere phenotype. Fusions occur due to

telomeric dysfunction. Fragile telomeres can result from replication problem in telomeres.

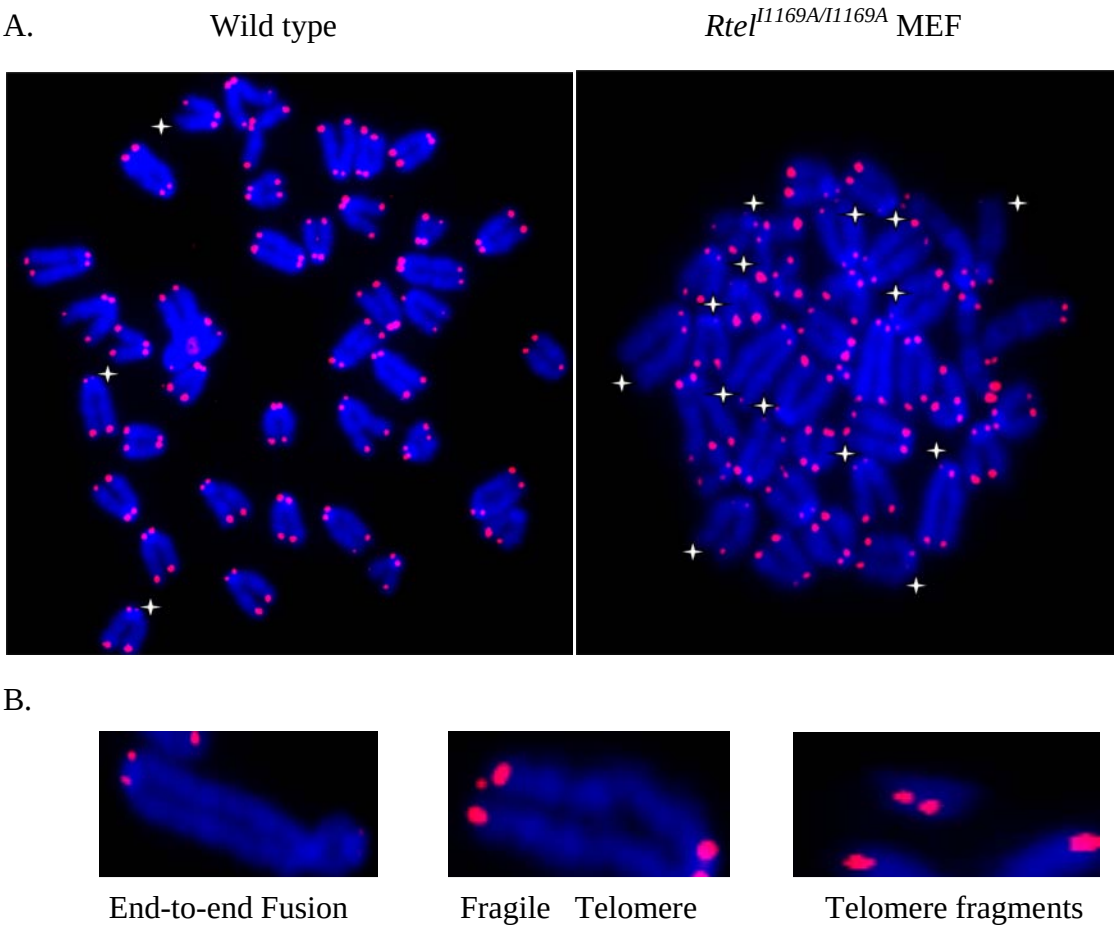


Figure 22: Chromosomal and telomeric abnormalities in *Rtel*$^{I1169A/I1169A}$ mouse. MEF cells from wild type and *Rtel*$^{I1169A/I1169A}$ mice were used to prepare metaphase spreads. Telomeric PNA probes are stained in red and chromosomes in blue for DAPI. **A.** Wild type cells have reduced telomeric signal loss, compared to *Rtel*$^{I1169A/I1169A}$ cells. The mutants have a heterogeneous population of telomeres. Asterisks denote lack of telomere signals. **B.** Several chromosomal abnormalities were observed in *Rtel*$^{I1169A/I1169A}$ cells.

Chapter 4: Discussion

4.1 Identification of RTEL-interacting proteins

4.1.1 Y2H screen failed to establish a functionally important partner for RTEL

Y2H is a widely used technique for determining protein-protein interaction (Gietz, 2006). This assay exploits the modular nature of eukaryotic transcription factors (e.g. GAL4) which consist of two separable domains, a sequence-specific DNA-binding domain (BD) and a transcription activation domain (AD). The protein of interest, for example RTEL in this study, can be fused to BD domain to create a "bait" protein, whereas the interacting proteins are expressed as fusions to the AD, creating "prey" proteins. If a prey protein interacts with the bait protein in the same cell, the BD and AD are reunited, and able to activate transcription from a GAL4-responsive promoter which is linked to a LacZ reporter. In order to improve the accuracy of this screening assay, a genetically engineered strain of yeast is often applied. In these yeast cells, the biosynthesis of certain nutrients (usually amino acids or nucleic acids) is lacking. Therefore, the yeast cells will only survive on media that lacks these nutrients once they re-synthesize these nutrients from both plasmids that express bait and prey proteins. However, even with these elegant designs, this screening system often gives false positives. The exact rate of false positive is not known, but estimates are as high as 50% (Deane *et al*., 2002). The reason for this high false rate lies in the principle of the screen: the assay investigates the interaction between over expressed fusion proteins in the yeast nucleus. Over-expression of the proteins might create a favourable condition for the proteins to interact that does not occur physiologically (Gietz, 2006; Uetz and Hughes,

2000). Moreover, a mammalian protein is sometimes not properly modified in yeast (e.g. missing post-translational modifications such as phosphorylation), which can also lead to false results. Finally, some proteins might specifically interact when they are co-expressed in the yeast, although in reality they are never present in the same cell or cell component at the same time. Due to the combined effects of all error sources the overall confidence of the yeast two-hybrid assay is rather low. Even though, Y2H data has been shown to be similar quality to data generated by the alternative approach of co-affinity purification followed by mass spectrometry (AP/MS) (Yu *et al*., 2008). Due to the simplicity of Y2H screening, Y2H is largely considered as the first choice for identifying protein-protein interactions.

In this study, we have applied the Y2H approach to search for RTEL-interacting proteins. Because of high the expression of RTEL in testis, we have specifically chosen mouse testis library (provided by CLONTECH) for screening. Initially, a full length RTEL was used as a bait. However, Y2H positives out of this screening were functionally irrelevant. Sequence analysis revealed that most of them were not in correct reading frame with Gal4 AD domain. None of other positives showed functional relevance, suggesting that they are most unlikely to be RTEL-interacting proteins. The failure of this experiment could be due to very low expression of RTEL bait protein in yeast cells. To circumvent this problem, we have further used the RTEL C-terminal fragment as a bait to increase the expression of bait protein in yeast cells. Indeed, RTEL C fragment showed a high level of expression in these cells and several candidates were identified from this screening.

Among these candidates, RAN binding protein 9 (RANBP9) was isolated frequently (19 times). RNABP9 plays a role in translocation of RNA and proteins through nuclear pore complex involved in cellular trafficking and immune response (Bai *et al*., 2003; Murrin and Talbot, 2007). No evidence is available to demonstrate that this protein to be involved in the maintenance of genomic stability. Furthermore, RNABP9 was also found to be one of the common false positives in Y2H screens (unpublished data). Therefore, we did not perform further experiments to verify its interaction with RTEL.

From this screening, only Methyl-CpG-binding 6 (MBD6) was found to be a potential candidate. Methyl-CpG-binding (MBD) proteins form a distinct class of proteins with a conserved MBD motif that can bind to methylated DNA sites (Klose and Bird, 2006). MBD proteins can act in gene expression by recruiting histone deacetylases (HDACs) and other chromatin remodelling factors to methylated CpG sites (Ballestar and Wolffe, 2001). Recently, MBD proteins have also been shown to be involved in the maintenance of genomic stability (Klose and Bird, 2006). Therefore, MBD6 may interact with RTEL *in vivo*. However, using co-IP experiments, we failed to show that RTEL can interact with MBD6 (Figure 14), which ruled out the possibility of MBD6 being a binding partner for RTEL.

In summary, the Y2H screening was unsuccessful and failed to identify RTEL-interacting proteins. In this study, we have used two different screening methods- solid screening and liquid screening. Liquid screen provides better experimental conditions since it minimizes the growth time and facilitates better diffusion of nutrients and selection agents such as 3AT. Despite all of our troubleshooting and efforts, the Y2H

screening failed probably due to its inherent limitations. As discussed above, there are several drawbacks for using yeast in protein-protein interaction studies. Many post-translational modifications do not occur properly in yeast. Moreover, some proteins do not interact in the environment of yeast nucleus (Koegl and Uetz, 2007). The low expression of RTEL in yeast can also be a reason. In many cases an interaction could not be found by Y2H when the expression of bait is low. It is worth to consider that Y2H was not able to find a suitable interacting protein for RTEL's yeast homolog, SRS2 (Krejci *et al*., 2003a). Its interaction with RAD51 was shown by *in vitro* binding assays, not by Y2H experiments (Krejci *et al*., 2003b).

4.1.2 Identification of RTEL interacting proteins using bioinformatics tools

To identify RTEL interacting proteins, we have also applied *in silico* approach, including homology based search for conserved protein-interacting motifs. A conserved motif was identified in RTEL called PIP motif. Since this motif aids in the interaction with PCNA in many proteins, PCNA was considered as a potential interacting partner for RTEL.

4.1.3 RTEL/PCNA interaction is mediated by PIP motif

To verify the interaction of RTEL and PCNA via the PIP motif, we have specifically introduced several mutations into RTEL PIP motif. Using both IP and IF assays, we clearly showed that wild type RTEL can immunoprecipitate PCNA or form foci with PCNA, but not with RTEL containing PIP mutations (Figures 16 and 17). These data strongly indicate that RTEL can form a protein complex with PCNA *in vivo*.

4.2 Establishment of transgenic mouse model for characterizing RTEL/PCNA interaction *in vivo*

To investigate the functional significance of RTEL-PCNA interaction, we have applied a mouse transgenic approach to specifically knock-in a RTEL PIP mutation, *RtelI1169A* to abolish RTEL/PCNA interaction in mice. The proper integration of the mutant allele was verified by southern blot and PCR analysis. DNA sequencing was employed to confirm the integration of correct mutation in the *Rtel* locus. In addition, RT-PCR studies demonstrated that there is no significant difference at the expression level for the mutant allele compared to the wild type allele (Figure 20).

The absence of embryonic lethality in the $Rtel^{I1169A/I1169A}$ mouse is not surprising; rather it might be an indication that RTEL plays multiple functions *in vivo*. It is important to note that telomere dysfunction may not be exhibited in early generations. For example, the $mTerc^{-/-}$ mouse, lacking expression of the enzyme 'telomerase' did not exhibit telomeric dysfunction phenotypes immediately. This was thought to be due to abundance of longer telomeres in mice. However, with successive crossing, the telomeres were found to face gradually shorten. At generation 4, cytogenetic abnormalities were found, such as end-to-end fusion and loss of telomeric repeats (Blasco *et al*., 1997). In addition, marked decrease in the number of hematopoietic precursor cells, reduced mitogen induced proliferation of B and T lymphocytes and splenic atrophy were observed (Lee *et al*., 1998). If RTEL/PCNA interaction is involved in telomere replication or maintenance, such phenotypes may be observed in later generations. It will be interesting to see what phenotype it develops in the tissues with high expression of RTEL, such as testis. The significance of the interaction in cell proliferation and maintaining genomic stability can

be further validated if any chromosomal instability disorder phenotypes are observed. The *Rtel*$^{I1169A/I1169A}$ mouse might develop gradual weight loss and other phenotypes, which will indicate defects in DNA replication.

4.3 Cellular aspects of RTEL/PCNA interaction

4.3.1 RTEL/PCNA interaction in Replication

PCNA is called the maestro of replication fork due to its key role in controlling DNA metabolism. In addition to its role as a processivity factor or clump loader, it has a regulatory function during replication. Recently, PCNA SUMOylation has been shown to suppress the recombination mediated pathways, such as RAD52 pathway in yeast (Pfander *et al*., 2005). Though it is not well characterized in mammalian cells, SUMOylation of PCNA has been reported in chicken cells and thought to occur in higher eukaryotes (Arakawa *et al*., 2006). Interestingly, RTEL has been demonstrated to have an anti-recombinogenic activity, like its yeast homolog SRS2 (Barber *et al*., 2008). PCNA might interact with RTEL and suppress HR events to ensure faithful DNA replication.

In this study, the RTEL/PCNA interaction has been demonstrated by using co-IP and immunofluorescence (IF) studies. In contrast to the RTELI1169A mutant, RTEL formed specific foci in the nucleus, in almost all the cells observed. PCNA, on the other hand is known to form specific foci during S-phase of cell cycle, assumed to be replication sites. The co-localization of RTEL/PCNA foci indicates possible role of this interaction in replication. Further studies are required to validate if the interaction is S-phase specific. Cells could be stained with bromo-deoxyuridine (BrdU) to verify the cellular stage. Also, co-IP studies with synchronized cells specifically at S-phase or

blocking replication with agents like aphidicolin can verify if this interaction is specific for or affected during replication. S-phase specific interaction of RTEL and PCNA would denote yet another regulatory role of PCNA during replication by suppressing HR in higher eukaryotes. Even more interesting would be to investigate if RTEL binds to SUMOylated PCNA.

SUMOylation of proteins, including PCNA has been shown to influence non-covalent interaction with other proteins (Kerscher, 2007). Several conserved motifs has been described that mediate the interaction with SUMO or SUMOylated proteins. Initially, a conserved motif with consensus sequence S-X-S was considered responsible for binding to SUMO (Minty *et al*., 2000). However, subsequent studies established other consensus sequences for the binding, such as V/I-X-V/I-V/I (Song *et al*., 2004). Though the S-X-S motif is now believed not to be necessary for direct binding to SUMO, it has been proposed that this motif might be necessary for stabilizing the interaction with SUMO-modified proteins (Hannich *et al*., 2005; Hecker *et al*., 2006). Interestingly, there is an S-M-S motif six amino acid upstream of the PIP motif in RTEL that is conserved in both mouse and human. Given RTEL's homology with yeast SRS2 and presence of such motif, it can be speculated that RTEL might interact with SUMOylated PCNA. However, in this study, no higher molecular weight bands corresponding to SUMOylation have been observed for PCNA. This might be due to the fact that the level of SUMOylated PCNA was undetectible owing its low abundance and/or activity of de-SUMOylating enzymes in cell extracts. It will be interesting to see if mutations in the conserved serine residues can affect the interaction in direct binding experiments, such as GST pull down or co-IP. Also the SUMOylation site in PCNA (K^{164}) can be mutated to find its affects on

the interaction by co-IP or IF studies. Along with these mutational studies, cells could be transfected with tagged-SUMO substrate to detect the modified PCNA. Direct binding studies can be employed to see if SUMO can compete with PCNA for binding to RTEL to find out if RTEL interacts with PCNA-SUMO.

Moreover, the transgenic model for abolishing the interaction between RTEL/PCNA provides tools to investigate the role of this interaction during replication. The MEF cells with *Rtel*$^{I1169A/I1169A}$ background can be used for checking cellular growth or proliferative changes at specific stage of cell cycle using colony forming assay or flow cytometry. Given the possible role of RTEL/PCNA interaction in replication, the cells could have reduced growth rate, cell cycle arrest, and/or hypersensitivity to DNA damage agents like methyl methane sulphonate (MMS) or radiation.

PCNA is SUMOylated even in absence of damage. Since SUMOylation and ubiquitylation of PCNA occur at the same site (K164), it is postulated that during replication SUMOylated PCNA drives replication and can prevent the recruitment of TLS polymerases during replication leading to erroneous replication or mutations. Moreover, it can recruit regulatory helicases like RTEL to prevent unwanted HR repair during replication. In yeast, the blocked of RAD52 mediated sister chromatid exchange by SUMOylated PCNA during replication serves two purposes: it blocks unwanted recombination events during replication and it suppresses HR mediated repair (Pfander *et al*., 2005). Possible role(s) of RTEL/PCNA interaction during replication are summarized in Figure 22. Thus, RTEL/PCNA interaction can have important role(s) in DNA replication.

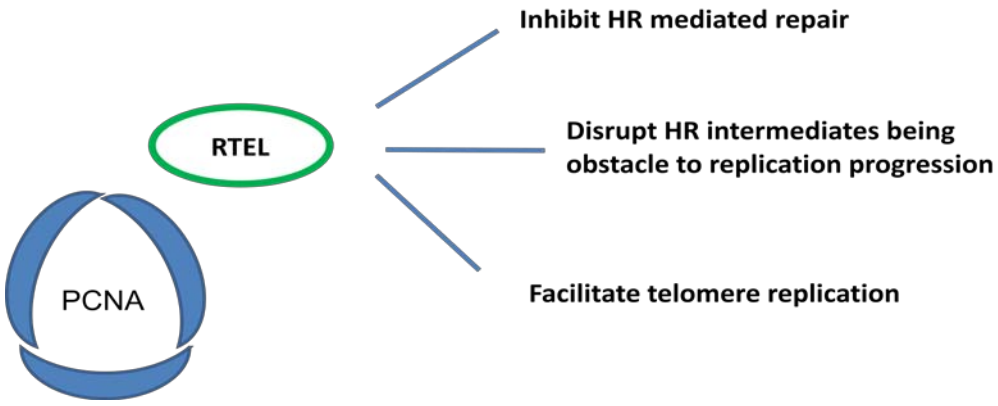


Figure 23: Possible role of RTEL/PCNA interaction in replication. With the capacity to disrupt recombination D-loops and act as an anti-recombinase, RETL can be recruited by PCNA at various stages of replication to ensure faithful replication of DNA.

4.3.2 Role of RTEL/PCNA interaction in recombination repair

HR is a central process in DNA damage response. In absence of regulatory helicases, the number of HR events increases at such damaged sites (Sung and Klein, 2006). This could lead to several events including mutations or aberrant chromosome structures. Moreover, large number of recombination intermediates can be formed exceeding the capacity of the cellular machinery to resolve them. Gross rearrangements due to HR and accumulation of toxic intermediates in cells can lead to genomic instability (Aguilera and Gomez-Gonzalez, 2008). Because of RTEL's ability to disassemble the invading 3´strand, it has been proposed to act as an anti-recombinase to control inappropriate HR events (Barber *et al*., 2008). Unlike other regulator helicases, such as BLM, RTEL cannot dissociate RAD51 from the ssDNA invasion of overhang strand during HR. Rather it can disrupt the invading 3´ overhang from the D-loops. In

addition, it can dissolve the D-loop guiding the recombination intermediate towards non-crossover products (Figure 23).

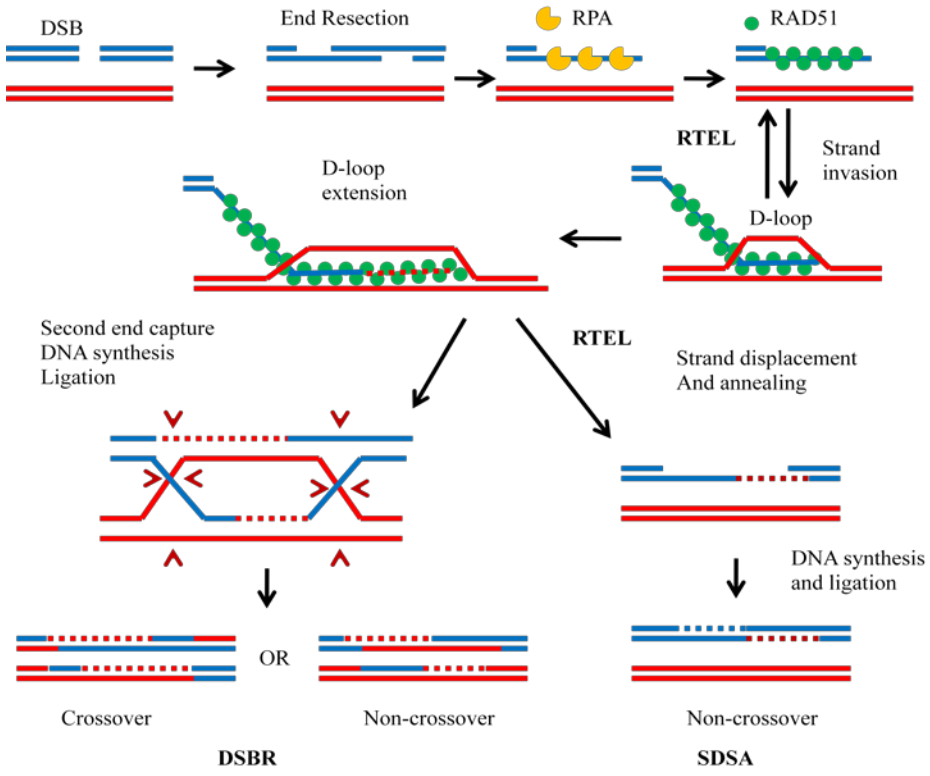


Figure 24: Role of RTEL in the HR pathway. It has been proposed that Rtel can regulate HR in at least two ways-regulations the invasion of overhang strand that act as a catalyst for HR initiation and driving SDSA that results in non-crossover products.

However, how RTEL is recruited to double strand breaks or coupled to replication and/or repair machinery is unknown. Whether RTEL is recruited to the sites of DSB, can be revealed by IF studies. It will be interesting to see if RTEL/PCNA co-localizes with γ-H2AX. The phosphorylated form of the H2AX histone which is specifically detected in response to the DNA DSBs. In addition, increase or decrease in HR events can be assessed by IF studies with RAD51 on MEF cells with *Rtel*$^{+/+}$ and *Rtel*$^{I1169A/I1169A}$

background. The PIP mutant cells could show the formation of increased RAD51 foci indicating a possible role in regulating HR. Cytogenetic studies can reveal whether gross chromosomal abnormalities are induced by disruption of the RTEL/PCNA interaction. If RTEL mediates its function in HR via PCNA, the *Rtel*$^{I1160A/I1169A}$ MEF cells could exhibit increased sister chromatid exchange. They could show hyper sensitivity to inter-crosslink agents, like mitomycin C, and *cis*-platinum (II) diammine dichloride. To investigate the proposed function of RTEL/PCNA interaction in HR, it will also be interesting to see if RAD51 knock down by siRNA can rescue the wild type phenotypes observed in the PIP mutant cells. Though related to chromosomal instability, accumulation of HR intermediates might not be associated with RTEL/PCNA interaction. In yeast, it has been demonstrated that SRS2 can resolve toxic HR intermediates independent of PCNA (Le Breton *et al*., 2008). To find out the level of recombination in *Rtel*$^{+/+}$ and *Rtel*$^{I1169A/I1169A}$ cells, recombination vectors like pNeoA and pLrec can be used. Such vectors are designed in a way that the selection marker is expressed only when HR occurs (Meyn, 1993). If RTEL controls HR, the cells with PIP mutation could exhibit increased recombination.

4.3.3 RTEL/PCNA interaction in post-replication repair

The replication of DNA is committed to completion once started. If replication is stopped due to damaged replication fork or presence of lesions for prolonged time, cells might undergo apoptosis or cell cycle arrest. Cells can employ DNA damage tolerant polymerases recruited by PCNA at damaged sites. Alternatively, the obstacle can be overcome HR mediated pathway (Lee and Myung, 2008).

Mono-ubiquitylation of PCNA recruits TLS polymerases while poly-ubiquitylation leads to HR mediated error free pathway. Though the PCNA switch is well described in yeast, little is known about its regulation in higher eukaryotes. In this study, several DNA damaging agents were used to induce DNA damage mediated ubiquitylation of PCNA, such as Methyl Methane Sulfonate (MMS), UV and hydroxyl urea (HU). Western blot and co-IP studies did not demonstrate that RTEL interact with ubiquitylated PCNA (data not shown). SUMOylation of PCNA can mediate an addition to the PCNA switch. Functionally, the SUMOylation of PCNA can be considered as PCNA mono ubiquitylation, leading to recruitment of translesion synthesis polymerases (Lee and Myung, 2008). However, SUMOylated PCNA also suppresses the RAD52 mediated pathway for HR which might function as a salvage pathway in stalled replication forks when other HR pathways are impaired (Pfander *et al*., 2005). In *C. elegans*, the abolishment of Rtel lead to increased sensitivity to several DNA damage agents, such as topo-isomerase I inhibitor camptothesin, indicating its role in stalled replication forks (Barber *et al*., 2008). The MEF cells with PIP mutants ($Rtel^{I1169A/I1169A}$) can be challenged with various agents to find out the significance of RTEL/PCNA interaction during the stalled replication forks.

4.3.4 Possible role of RTEL/PCNA interaction in telomere maintenance

4.3.4.1 Replication of telomeres

Telomeres assume specific structure to protect the chromosomal ends. It has been proposed that telomeres can be in two inter-changeable states. When cells are not replicating, the telomeres are in a closed state, maintaining the t-loop. In this state,

several proteins of the t-loop, such as TIN2, TPP, RAP1 inhibit NHEJ and HR mediated repair of telomeric sequences (Sarthy *et al*., 2009). On the other hand, the replication machinery needs to access the telomere during S phase (Figure 28). To facilitate this, the t-loop structure is disrupted and the telomere transiently acts as a double strand break. However, unlike DSBs that are repaired by HR mediated pathways, telomeres are protected from being recombined. In such an 'open state' telomerase can access the terminal end by replacing POT1. In addition, other proteins or protein complexes like MRE11 can gain access to ensure proper telomere length and formation of the protection cap (Linger and Price, 2009).

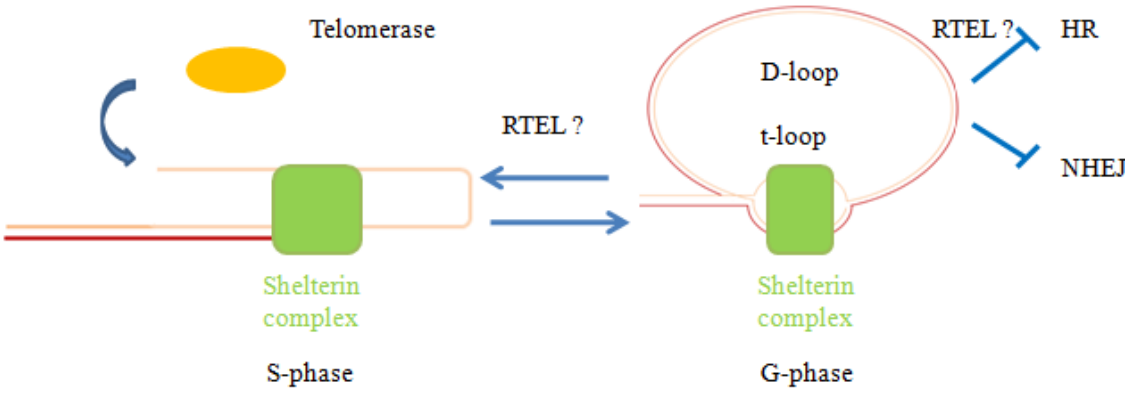


Figure 25: Inter-switchable states of telomeres and possible role of RTEL. In its closed state, the shelterin complex stabilizes the protective cap during G2 phase, protecting it from HR or NHEJ mediated repair. However, during S phase, the structure is disassembled to be 'open state' such that telomerase can access the terminal replacing POT1 in yeast.

However, which protein(s) are associated with disassembling the t-loop of telomeres, specifically during replication is not well defined. Given the absolute necessity of RTEL in telomere maintenance and its capability to disrupt d-loop structures, it is

possible that PCNA utilizes its capacity to recruit RTEL at the telomeres during replication, to control HR mediated pathways and/or to disassemble the t-loop for replication. This possibility can be explored by chromatin immunoprecipitation (ChIP) and IF couple with FISH experiments.

In addition to the replication machinery, proteins associated with recombination and repair has been shown to be associated with telomere maintenance. Interestingly, MRX (Mre11-Rad50-Xrs2), a protein complex involved in DNA double-strand break (DSB) repair, is recruited to the telomeres in late S phase. The authors have utilized their expertise in ChIP to demonstrate the binding of the complex to telomeres during S phase, when replication of telomeres occur (Takata *et al.*, 2005). While similar telomeric proteins like TIN2, Rap1 inhibits recombination and NHEJ, presence of such DSB repair machinery indicates existence of regulatory mechanisms to HR during replication of telomeres. ChIP experiments with telomeric probes can reveal if there is a difference of telomere binding between $Rtel^{+/+}$ and $Rtel^{I1169A/I1169A}$. Since there is no antibodies available for endogenous RTEL, the RTE-V5 and RTEL-I1169A-V5 alleles established in mouse genome facilitates the use of anti-V5 antibody in this purpose. IP can be done with anti V5 antibody and association with telomeres can be assessed by specific telomeric primers or probes. With specific role of RTEL/PCNA in telomeric region, there should be differential binding at telomeric region between RTEL and RTEL PIP mutant cells. In addition telomere binding can be assessed in S-phases and/or different treatments that induce DNA damage mediated by HR such as MMS, HU. In this project, Q-FISH done on MEF cells with $Rtel^{I1169A/I1169A}$ mutation showed differential telomere length compared to wild type cells. While the wild type cells have normal distribution of

telomeric signal, the cells with PIP mutation exhibit heterogeneous population of telomeres. This might be the result of disruption of telomere elongation due to faulty replication or inappropriate recombination. In the absence of a regulatory anti-recombinase, like RTEL, telomeric repeat sequences can undergo HR mediated elongation or shortening. Additional information in this regard can be obtained by immunofluorescence studies coupled with FISH. It will be interesting to see if RTEL and/or PCNA co-localizes with telomeres during replication or in response to DNA damage agents.

4.3.4.2 HR mediated repair at telomeres

In addition to DNA damage, the specialized structure of the telomeres can be a challenge for efficient replication. For example, due to repetitive GC sequences, telomeres can assume secondary structures such as G-quadruplex, which impedes the progression of replication forks. Since replication in higher eukaryotes occur in a unidirectional manner, replication must be continued at such damage sites or stalled replication forks to avoid loss of telomeric sequences (Ueno, 2010).

Indeed, telomeres can act as ‘fragile sites’ that pose a threat to the replication machinery, often associated with stalled replication forks or collapsed replication forks. Sfeir *et al.* demonstrated that at telomeric regions, there are replication dependent defects resembling defects at fragile sites (Sfeir *et al*., 2009). Characteristic chromosomal abnormality phenotypes associated with telomeric fragile sites have been observed partially in Rtel deficient ES cells (Ding *et al*., 2004). In addition, Sfeir *et al.* also demonstrated that si-RNA mediated knockdown of Rtel induces the fragile site

phenotypes associated with telomeres. Though RTEL was initially thought to be a homolog of Dog1 and have a role in resolving G-quadruplex structures, there is no experimental evidence to support this. Barber *et al.* have demonstrated in *C. eleaens* that rtel-1 mutants do not exhibit instability at G-rich sequences like dog-1 mutants (Barber *et al*., 2008). However, how RTEL is recruited to such damaged sites or if its action is co-ordinated with DNA replication and repair machinery is unknown. It is possible that PCNA recruits RTEL at the telomeric sites to repress HR and facilitate proper duplication of the telomeres. Q-FISH done on MEF cells with the $Rtel^{I1169A/I1169A}$ background reveals that many telomeres have multiple signals at the chromosomal ends. Whether this is specific fragile site related or not, can be verified by further experiments. Replication in cells can be inhibited by aphidicolin. If such signals are associated with replication related fragile sites, the treatment would increase the phenotype. Fragile telomeres often have multiple telomeric signals at metaphase spreads that are associated with telomeres that were not properly replicated resulting in failure of condensation of telomeres or broken telomeres (Sfeir *et al*., 2009). Given PCNA's role in stalled replication forks, it is worth to explore if such events occur at telomeres as well. It will be interesting to see if $Rtel^{I1169A/I1169A}$ cells have increased incidence of stalled replication forks at telomeres. This can be assessed by SMARD (single-molecule analysis of replicated DNA (Norio and Schildkraut, 2001) coupled with telomeric probes. Functional telomeres require interaction with not only proteins of shelterin complex, but also DNA-damage repair proteins, suggesting that the DNA-damage repair machinery is involved in telomere maintenance. The implication of RTEL/PCNA interaction in controlling such pathways attracts immense interest.

Chapter 5: Conclusions

The hypothesis of this project is that RTEL/PCNA interaction is essential for the maintenance of genomic stability. Previous works have shown that RTEL is an essential gene for maintaining telomere integrity and genomic stability. Therefore, in attempt to find out the molecular mechanisms mediating RTEL's function, we have used Y2H to identify RTEL-interacting proteins. Our Y2H screen results were unable to find out any functionally important protein partner for RTEL. This could be due to the inherent limitations associated with Y2H. However, we found out a RTEL-interacting protein called PCNA using bioinformatics and biochemical approaches. The experimental results presented in this thesis demonstrated that RTEL interacts with PCNA. Further, we have shown that RTEL/PCNA interaction is disrupted by introduction of several point mutations in the conserved PIP motif in RTEL. To characterize the significance of RTEL/PCNA interaction *in vivo* we have successfully introduced one of the point mutations (I1169A) in the PIP motif of RTEL into mouse genome. Our data demonstrated that the RTEL/PCNA interaction is disrupted in $Rtel^{I1169A/I1169A}$ mouse. We have observed that the MEF cells derived from $Rtel^{I1169A/I1169A}$ exhibit abnormal phenotypes which are related to genomic instability. Particularly, the telomeres were shortened in $Rtel^{I1169A/I1169A}$ cells and there were increased events of telomere doublets, end-to-end fusion and chromosomal breakage in these cells compared to wild type cells. Taken together, we conclude that the interaction between RTEL and PCNA plays important role to maintain genomic stability.

Chapter 6: Future directions

Given the critically important role of RTEL in the maintenance of genomic stability, further characterization of RTEL/PCNA interaction will provide some new insights into telomere maintenance and tumorigenesis.

Our strategy to find out functional role of RTEL in the maintenance of genomic stability was to characterize RTEL/PCNA interaction and establish a transgenic mouse model to elucidate the significance of this interaction. We have demonstrated that RTEL and PCNA do interact via PIP motif and this interaction is disrupted in the *Rtel*$^{I1169A/I1169A}$ mouse that carries a point mutation in the PIP motif of RTEL. To further study the role of RTEL/PCNA interaction in the maintenance of genomic stability, we are planning to use several approahes. Using cell survival assays, like colony formation assay, we want to investigate if there are any growth or proliferation defects in the MEF cells with PIP mutation. In addition, we are planning to use several DNA damage agents, such as MMS, UV, aphidicolin, *cis*-platinum (II) diammine dichloride and mitomycin C to induce DNA damage in the MEF cells. Survival difference between the wild type and mutated MEF cells under such conditions would direct us to specific pathways or events in which the interaction plays important role. For example, if the mutated MEF cells show higher sensitivity to aphidicolin, it would indicate that RTEL and PCNA interaction is important for replication of fragile sites in chromosomes or telomeres. In addition, we want to investigate if RTEL/PCNA foci co-localize with RAD51 which is important for HR mediated events. If PCNA recruits RTEL in HR mediated events, the foci might co-localize with RAD51. To further characterize the chromosomal phenotypes of

Rtel$^{I1169A/I1169A}$ MEF and ES cells, we are planning to use Q-FISH experiments. This will reveal if there is any difference in telomere maintenance in the mutant cells compared to wild type cells. In addition, the *Rtel*$^{I1169A/I1169A}$ mouse can be crossed with *p53*$^{-/-}$ mouse to further study the role of RTEL/PCNA interaction in tumorigenesis.

In the long term, these studies will lead to a better understanding of molecular mechanisms that regulate DNA replication and repair, telomere maintenance and genomic stability. All these processes are important for proper development and prevention of cancer. Several helicases are now targets for selective inhibition DNA repair pathways to inhibit proliferation of cancer cells. Future characterization of RTEL/PCNA interaction will ultimately lead to better treatment of cancer.

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