To characterise the role of RTEL1 DNA helicase in the maintenance of intestinal stem/progenitor cells

By

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Abstract

RTEL1 (Regulator of telomere length1) DNA helicase has been demonstrated to be vital for the maintenance of telomere length and genomic stability. However, its biological role during development is unknown. Our recent finding that RTEL1 is selectively expressed in several types of adult stem cells, suggests that RTEL1 could play an essential role in the maintenance of these cells. Depending on the function of RTEL1 in the maintenance of genomic stability, we hypothesize that RTEL1 could be required for protecting adult stem cells from genomic instability, whose dysfunction may not only impair tissue homeostasis/regeneration, but also could transform these cells to form tumors.

In this study, we have used mouse intestinal stem/progenitor cells model to address this hypothesis. With a transgenic lineage tracing assay, we demonstrated that RTEL1-expressing cells in intestinal crypts can self renew and differentiate to the progeny cells required for intestinal homeostasis. Using a conditional knockout approach, we also showed that loss of RTEL1 function could induce genomic instability in intestinal stem/progenitor cells, which significantly affected the survival of intestinal stem cells and intestinal regeneration. Finally, in this study, we also observed intestinal hyperplasia in our RTEL1 conditional knockout mice, indicating that loss of RTEL1 function may initiate intestinal tumorigenesis. All of these findings strongly support that RTEL1 could be one the key molecules necessary for the maintenance of intestinal stem/progenitor cells and this function could be important for preventing intestinal tumorigenesis.

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“Behind every act are the people who contribute immensely towards its completion and go unnoticed most of the time”. Before I list all my helping angels I would like to first thank god for giving me an experience that has completely changed me from within.

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APC	Adenomatous polyposis coli
ATP	Adenosine Triphoshpate
BLM	Bloom Syndrome Gene
CBC	Columnar base cells
CKI	casein kinase
DNA	Deoxyribonucleic acid
DSB	Double stranded breaks
DSBR	Double stranded breaks repair
ES	Embryonic stem cells
FANCJ	Fanconi anemia complementation group J
GSK3	glycogen synthase kinase 3
Hes	Hairy/Enhancer of Split
HR	Homologous recombination
ISC	Intestinal stem cells
Lgr5	Leucine-Rich Repeat Containing G Protein-Coupled Receptor 5

MEF	mouse embryonic fibroblast
NHEJ	Non- homologous end joining
NICD	Notch intracellular domain
Olfm4	Olfactomedin 4
PCNA	Proliferating cell nuclear antigen
PIP	PCNA interacting protein
POT1	protection of telomeres 1
RAP1	Repressor and activator protein 1
RNA	Ribonucleic acid
SA-IRES	Splice acceptor-internal ribosomal entry site
RTEL1	Regulator of Telomere length 1
SDSA	Synthesis dependant strand annealing
STORM	Stochastic optical reconstruction *microscopy*
TA	transit amplifying cells
TCF4	Trancription factor 4
TERT	telomerase reverse transcriptase
TIN2	TRF1-interacting nuclear protein 2
TNR	Trinucleotide repeats
TPP1	Tripeptidyl peptidase 1
TRF1	telomeric repeat-binding factor 1
TRF2	telomeric repeat-binding factor 2
UV	Ultravoilet radiation
WRN	Werner's Syndrome Gen

Chapter 1: Introduction

1.1 Overview

The regulator of telomere length 1 (RTEL1) is a DNA helicase that has been demonstrated to be essential for the maintenance of telomere length[1,2]. It has also been shown to play important roles in DNA homologous recombination, DNA replication and trinucleotide repeats expansion[3-5]. All these indicate that RTEL1 could be a key molecule involved in the maintenance of genomic integrity in the cells. However, how this function of RTEL1 involves in development is largely unknown. Recently, we found that RTEL1 is selectively expressed in several adult stem cells, including intestinal stem/progenitor cells, implicating that RTEL1 could play some important roles in the maintenance of these stem cells during development. As a part of this characterization, my research project will focus on determining the role of RTEL1 in intestinal stem/progenitor cells and whether this DNA helicase is required for intestinal homeostasis/regeneration.

To understand my research project better, in the first part of my introduction, I will discuss the current knowledge about the protein structure of RTEL1 and the roles of RTEL1 in the maintenance of genomic stability by regulating telomere length, homologous recombination and DNA replication. In the second part of my introduction, I will briefly summarize our recent findings that RTEL1 is selectively expressed in adult tissues, specifically in stem cell-zones, including intestinal crypts where the intestinal stem/progenitor cells are located. This has led us to hypothesize that RTEL1 could play some important roles in the maintenance of several adult stem cells during development. Since my project will focus on addressing the role of RTEL1 in intestinal stem/progenitor cells, therefore, in the last part of my introduction, I will discuss the recent advances on the role of intestinal stem/progenitor cells in intestinal homeostasis/regeneration. I will also discuss the role of intestinal stem cells in the formation of

intestinal tumors. This information will not only support the aims of my research, but also will help to understand the relevance of my research findings.

1.2 Protein structure of RTEL1 DNA helicase

RTEL is a DNA helicase protein[1]. DNA helicases are a large group of enzymes that unwind the double helix into a single stranded structure in an ATP dependent manner. The opening of the double helix is critical for DNA replication, DNA damage repair and transcription[6]. Besides these functions, the DNA helicase are also required for resolving abnormal DNA secondary structures that could arise due to highly repetitive sequence in the genome[7].

All the helicase proteins have conserved helicase domains which are required for performing helicase function[8]. Based on the characteristics of the helicase domains, RTEL1 is classified as the member of Superfamily 2 (SF2). Members of this family are characterized by the distinct helicase core domains, namely I (Walker A), Ia, II (Walker B), III, IV, V and VI)[9]. RTEL1 is also categorized in the DEAH-family of helicase based on unwinding polarity, nucleic acid and nucleotide preference. Furthermore, an iron-sulfur (Fe-S) motif situated between helicase motif I and II has grouped this protein in a separate subfamily along with several bacterial (DING), yeast (CHL1-orthologue to DDX11 and RAD3 - orthologous to XPD) and human proteins (DDX11/ChlR1, FancJ and ERCC2/XPD). The helicase domain and the Fe-S motif could be required for the DNA binding and helicase activity of RTEL1[10].

Apart from its helicase domains, bioinformatics analysis of RTEL1 has also suggested the presence of a Harmonin-N like domain and C4C4 RING-finger domain at the C terminal[11]. The harmonin N Like domain could be used for protein-protein interaction, whereas the RING finger domain may enable RTEL1 to display an E3-ubiquitin ligase activity. However, functional

validation of these domains has not yet been conducted. In addition, RTEL1 also contain a proliferating cell nuclear antigen (PCNA) interacting protein (PIP) motif, which has recently been demonstrated to mediate the function of RTEL1 in DNA replication[4]. Figure 1.1 summarizes all the known protein domains of RTEL1. The presence of multiple functional domains associated with helicase domain in RTEL1 protein suggests this DNA helicase protein could have diverse biological functions.

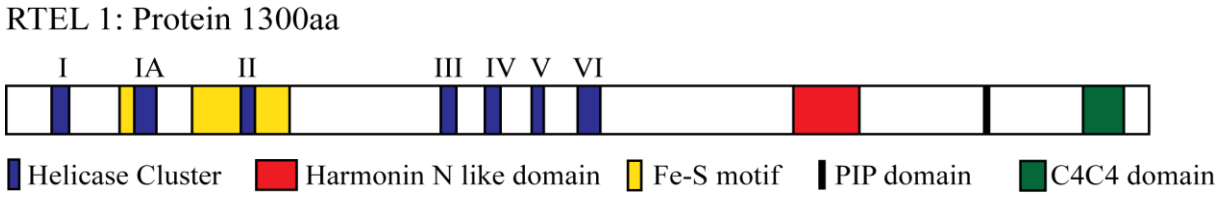

Figure 1.1 Schematic representation of the protein domains of RTEL1. Both human and mouse RTEL1 proteins are highly homologous, containing seven conserved helicase domains (I, Ia, II, III, IV, V, VI), the Fe-S motif, the Harmonin N-like domain, the C4C4 RING-finger domain and the PIP domain.

1.3 The role of RTEL1 in the maintenance of genomic stability

The RTEL1 gene was first identified by genetic linkage mapping as a candidate gene in a 5cM distal chromosome 2 regions that is responsible for the strain specific difference in telomere length between M *musculus* and *M spretus*[12]. Subsequently, the role of RTEL1 in the maintenance of telomere was revealed by loss or shortening of telomeres in the embryonic stem cells derived from constitutive RTEL1 knockout mouse[1]. Research over a period of 10 years has revealed that its biological function is mediated mainly by the ability of RTEL1 to act as a helicase in resolving different DNA secondary structures, namely the D-Loop, G quadruplex (G4) DNA and trinucleotide hairpin (TNR) structures[13]. The resolution of D-Loop helps RTEL1 in suppressing DNA homologous recombination as well as telomere replication. Resolution of G4 DNA helps RTEL1 to prevent fragility of telomere and the resolution of TNR hairpin helps

to prevent TNR expansion and overall chromosome fragility[5]. In the following sections, I will discuss these functions of RTEL1 individually.

1.3.1 Function of RTEL1 in the maintenance of telomeres

1.3.1.1 The structure of telomeres

A telomere is the physical end of chromosome which is characterised by the presence of specific repeat sequences, for example, the TTAGGG repeats in mouse and human cells[14]. The nature and the length of telomeric repeats may vary among different species[15]. The telomere is also characterized by a 3' G rich overhang, which can invade the double stranded region of the telomeric DNA to form a lariat structure, known as the T-loop (Figure 1.2A). Electron microscopy and STORM (Stochastic Optical Reconstruction Microscopy) have revealed the existence of such lariat structure at the telomere[16,17]. The formation of T-loop protects the chromosomes end from being recognized as DNA double strand breaks (DSBs) and prevent them from subjecting to the DNA repair machinery. Treatment of the telomeric ends as DSBs is detrimental to the cell because that will impair the integrity of the chromosome. In addition, the 3' telomeric overhang can also behave as a "sticky-end" to induce chromosomal fusions which can also lead to chromosomal instability (see discussion below)[18]. Therefore, the T-loop structure in the telomere is essential for the maintenance of genomic integrity in eukaryotic cells.

Telomeres are also characterized by the high G-C contents which can form a G quadruplex (G4) structure[2]. This G4 structure is formed by intermolecular Hoogsteen base-pairing between an array of four Gs that are hydrogen bonded (Figure 1.2B). The G4 structures need to be resolved during telomeric DNA replication. If it is unresolved, it will block the replication fork progression. Several DNA helicases, such as RTEL1 and BLM, have been demonstrated to be required for resolving G4-DNA structure during telomere replication[13].

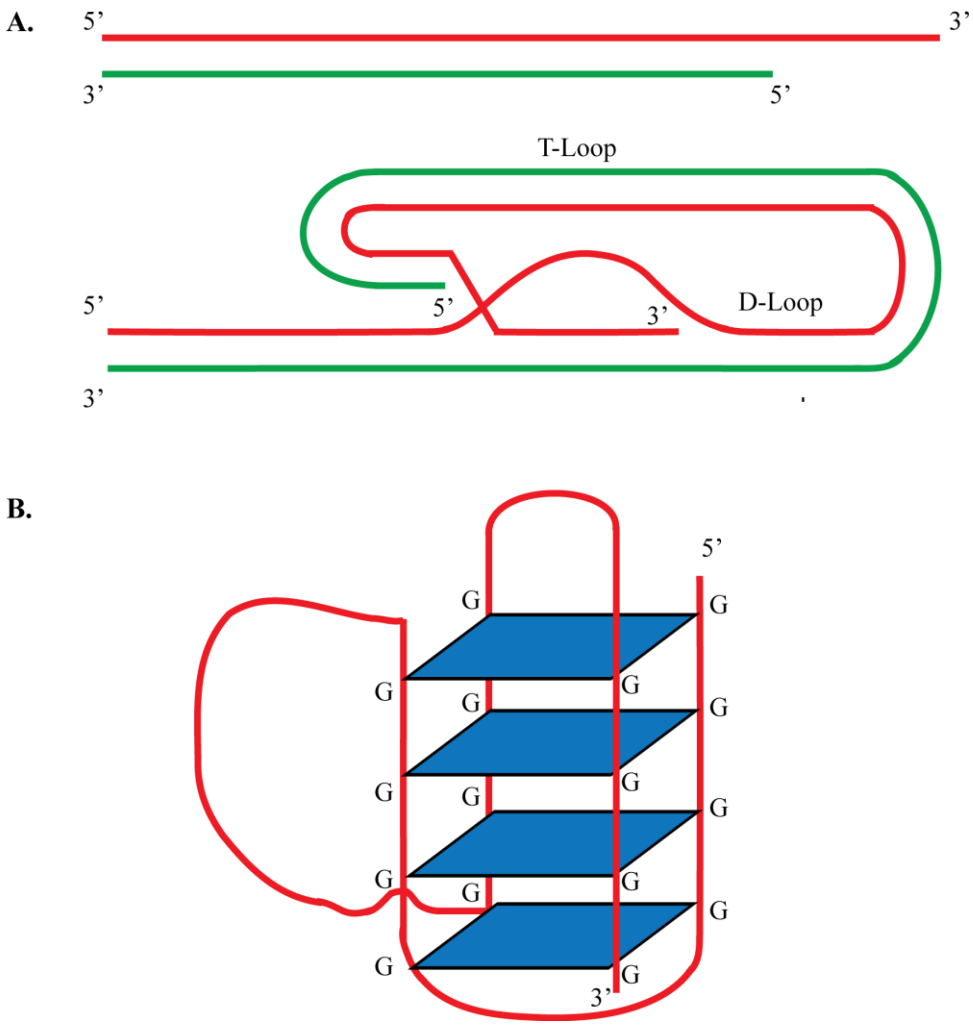

Figure 1.2 Schematic representation of the DNA structures formed in telomeres. (A) The 3' G rich overhang invades into the DNA duplex to form a DNA/protein complex structure called the T-Loop, which is essential for protecting the ends of the chromosome from being detected as DSBs. **(B)** The formation of G4 DNA structure by intermolecular Hoogsteen base-pairing between an array of four Gs in telomeres.

1.3.1.2 Telomere shortening: End replication problem

In most human or mouse cells which lack telomerase activity, telomere shortens at each cycle of DNA replication due to an inherent problem of DNA replication. DNA replication is mediated by DNA polymerase enzymes which synthesize new DNA in the 5'-3' direction. DNA replication also requires a template strand and a free 3' OH group on the complementary strand.

This free 3’ OH group on the complementary strand is provided by another group of enzymes called RNA polymerase which can synthesize short stretches of complementary RNA fragment on the template strand. When a double helix opens up for DNA replication, the 3’-5’ template strand, known as leading strand, is synthesized continuously by the 5’-3’ polymerization activity of the DNA polymerase. However, the 5’-3’ strand, known as lagging strand, is synthesized discontinuously by DNA polymerase because it requires a short RNA primer to be synthesized on the freshly uncoiled lagging strand. The cycle of RNA primer synthesis and subsequent DNA polymerization continues on the lagging strand as the replication progresses. Thus DNA synthesis on the lagging strand is discontinuous and these discontinuous fragments are called Okazaki fragments[19]. Later on the RNA primers are removed and the Okazaki fragments are joined by DNA ligase to create a continuous complementary strand. However, the newly synthesized DNA strand gets shortened after a cycle of replication because of the removal of RNA primer at the 5’ end which will not be able to be filled in by the DNA polymerase (Figure 1.3). When the cells undergo multiple divisions (> 50 divisions)[14], this end replication problem will result in critically short telomeres.

1.3.1.3 Telomere maintenance: de novo synthesis of telomeres by telomerase

Most of the eukaryotes overcome the telomere shortening due to the end-replication problem by *de novo* addition of telomeric sequences at the 3’ end of the chromosomes[20]. This is regulated by a unique ribo-nucleoprotein complex, known as telomerase. Telomerase consists of telomerase reverse transcriptase (TERT) enzyme and a RNA component which provides a template for the addition of repeat sequences at the 3’ end of the chromosomes[21] (Figure 1.4). It has been demonstrated that unicellular organisms have an unlimited amount of telomerase activity, whereas the multicellular organisms, such as human and mouse, have a differential

requirement for telomerase activity. For example, in mammals, high expression of telomerase was found in the germ cells, stem cells, and during early embryogenesis[22] when cell proliferation is an important pre-requisite for growth and development of the organism. In contrast, lack of telomerase expression was found in most of the differentiated somatic cells. The lack of telomerase in the somatic cells leads to the erosion of telomere after cycles of DNA replication and eventually the telomere reach a critical length which causes cells to stop dividing (replicative senescence). However, if somatic cells are transformed, the expression of telomerase will be induced to enable long telomeres and an indefinite life span[23].

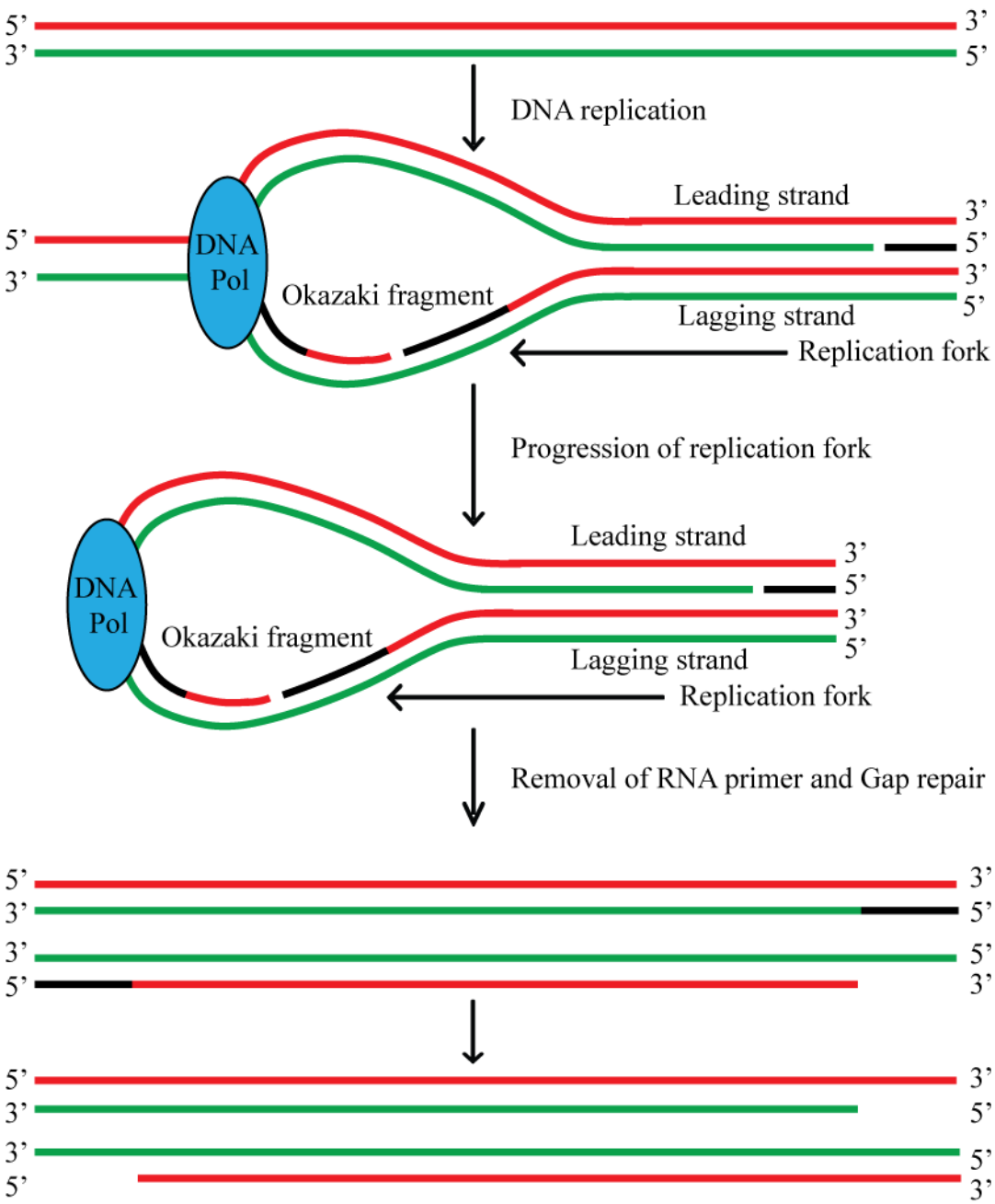

Figure 1.3 Flowchart illustration of telomere shortening caused by DNA replication. During DNA replication, the parental DNA is replicated which is initiated by the RNA primers (labelled as black). After replication, the RNA primers will be removed and the left DNA gaps will be filled in by the DNA polymerase. However, since DNA polymerase always extends DNA in the direction of 5’ to 3’, the removal of RNA primer at the chromosomal ends will not be able to be repaired by the DNA polymerase, resulting in 3’ overhang. This will lead to telomere shortening.

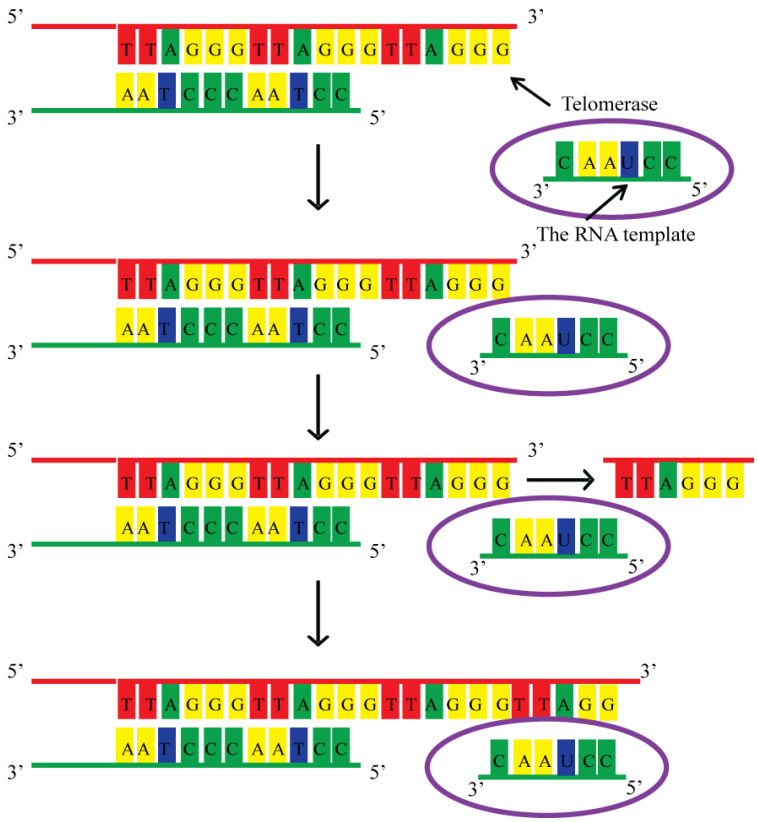

Figure 1.4 De Novo telomere synthesis by telomerase. Telomerase is recruited at the 3' end of the telomere and the RNA component of the telomerase serves as a template for adding new repeat sequences.

1.3.1.4 Maintenance of telomeres by the shelterin complex

Besides telomerase, several other proteins have also been shown to be essential for the maintenance of telomeres. These proteins form a multiprotein complex, known as the shelterin or telosome complex[24]. The protein components of the shelterin complex have evolved considerably and show variation among different species. However, considerable homology exists between members of different species and each of them performs specific function in the maintenance of telomeres. The shelterin complex has been demonstrated to have two main functions in the maintenance of telomeres: (1) it interacts with telomere DNA to form a T-

loop/protein complex which is essential for protecting telomere ends from being recognised DSBs; and (2) it regulates the activity of telomerase and recruits other factors to facilitate telomere extension[25].

In mammals, the shelterin complex consists of telomeric repeat-binding factor 1 (TRF1; also known as TERF1), TRF2 (also known as TERF2), repressor and activator protein 1 (RAP1; also known as TERF2IP), TRF1-interacting nuclear protein 2 (TIN2; also known as TINF2), protection of telomeres 1 (POT1) and TPP1 (also known as ACD) (Figure 1.5). Members of the shelterin complex bind the double stranded and single stranded part of the telomere to facilitate T-Loop formation and stabilize the telomere structure. TRF1 and TRF2 homo-dimerize and bind to the telomeric dsDNA through their DNA-binding Myb domain[26]. The yeast Rap1 also binds telomeric dsDNA but in mammals it does not bind telomeric DNA directly and retain in the telomere via its interaction with TRF2[27]. Mammalian POT1 and TPP1 bind specifically to the single stranded telomeric G rich 3' tail[28]. Mammalian TIN2 is the crucial bridging component that links between the ds-DNA binding TRF1 and TRF2, as well as connect them to the ss-DNA binding POT1 and TPP1[29]. The exact sequence of events that leads to the formation of shelterin complex and the T-loop structure in the telomere is yet unknown, however, it has been shown that knockdown of individual members of the shelterin complex lead to the de-protection of chromosomal end and initiate DNA damage response pathway[30].

It has been demonstrated that TRF1 and TRF2 are required for the formation and the maintenance of the T-loop structure at the 3' end and prevents end to end joining[31]. When TRF2 was inactivated, there was a high incidence of chromosomal abnormalities in terms of fusions. The other components of shelterin complex have also been found to prevent inappropriate homologous recombination and Non-homologous end joining at telomere ends[32]. In addition to

stabilising telomeric structure, the shelterin complex also plays an essential role in recruiting other factors, such as Mre11, BLM and WRN. These factors play an essential role in telomere maintenance and replication[33].

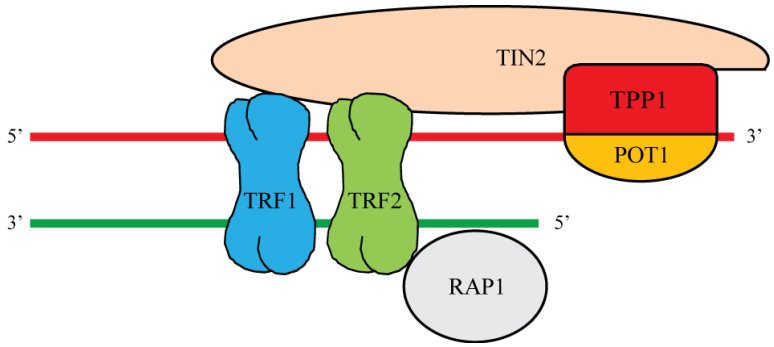

Figure 1.5 Diagrammatic representation of the shelterin complex at the telomere. TRF1 and 2 bind to double stranded DNA, whereas TPP1 and POT1 bind to single stranded DNA regions of the telomere. TIN2 helps form a link between TRF1 and 2 and also between TPP1 and TRF complex. RAP1 binds to TRF2 subunit of the shelterin complex.

1.3.1.5 Maintenance of telomeres by the DNA helicase proteins

As the telomere contains several DNA structures that require unwinding for replication, it is expected that DNA helicases could also be required for the maintenance of telomeres. In yeast cells, at least 4 DNA helicases, Sgs1, Dna2, Rrm3 and Pif1, have been found to be required for the regulation of telomere length[34]. In human cells, the WRN, BLM (homologous to yeast Sgs1) and FANCJ helicases have also been known to be involved in the regulation of telomere length by resolving the G4 structure of telomeres[35]. In addition, BLM and FANCJ could also interact with members of the shelterin complex to facilitate telomere replication[34,36]. As discussed in the following sections, we and others have provided strong evidences to indicate that RTEL1 is an important DNA helicase involved in the maintenance of telomeres.

1.3.1.6 Telomere dysfunction in RTEL1 knockout cells

In order to determine the *in vivo* role of RTEL1, we have applied a mouse knockout approach to mutate RTEL1 in mice. RTEL1$^{-/-}$ mice were embryonic lethal and died at embryonic

stage day10.5 (E10.5) with multiple defects in vascular system, neural tube and allantois[1]. Further analysis of RTEL1$^{-/-}$ ES cells revealed an increased frequency of loss of telomeres on the chromosomal ends. This telomere dysfunction was also found to lead to other chromosomal defects, such as chromosomal fusions, breaks and translocation in RTEL$^{-/-}$ cells[1]. All these indicate RTEL1 is essential for the maintenance of telomere length.

1.3.1.7 Requirement of RTEL1 in the resolution of T-loop of telomeres

To further characterize the role of RTEL1 in the maintenance of telomere length, our lab has also generated a conditional knockout of RTEL1 mouse model which allows us to knock out RTEL1 function in the cell- or tissue-specific manner[37]. Similar to RTEL1 deficient ES cells, conditional knockout of RTEL1 in mouse embryonic fibroblast (MEF) cells displayed a severe telomere dysfunction phenotype[2]. This defect was further found to be caused by the incapacity of RTEL1 in resolving T-loop structures of telomeres[2]. In the absence of RTEL1, unresolved T-loop will stimulate SLX4 nuclease activity, resulting in the nucleolytic cleavage of T-loop DNA in the form of a circle known as T circle. This leads to loss of telomeres as observed in RTEL1 deficient mouse embryonic fibroblast (MEF) cells or ES cells[2].

1.3.1.8 Requirement of RTEL1 in the resolution of G4 structures during telomere replication.

Both RTEL1 deficient ES and MEF cells also displayed a telomere fragility phenotype characterized by the extended telomere or duplicated telomere signals on a single chromatid[2]. The increased fragile telomeres observed in RTEL1 deficient cells could be explained by the fact that G4 DNA can be formed by single stranded telomeric repeats (TTAGGG). During replication, the unresolved G4 DNA structure will impede the replication fork and induce fragility[2]. In consistent with this, treatment of RTEL1 deficient cell with a G4 DNA stabilizing

drug TMPyP4 was found to dramatically increase the fragile telomere phenotype, demonstrating an important role of RTEL1 in resolving G4 structure during telomere replication.

1.3.1.9 The current mechanism of RTEL1 in the maintenance of telomere length

Taken together, the current studies indicate that RTEL1 could play two distinct functions to maintain telomere length. The first function is to dissemble T-loop structure of telomere which is important for the extension of telomeres by telomerase. During telomere replication, if RTEL1 is not present, the unresolved T-loop structure will stimulate SLX4 nuclease activity, leading to the cleavage of T-loops and telomere erosion (Figure 1.6). The second function is to resolve G4 structure during telomere replication. It is most likely both RTEL1 and BLM are involved in this process, which facilitate telomere replication (Figure 1.7).

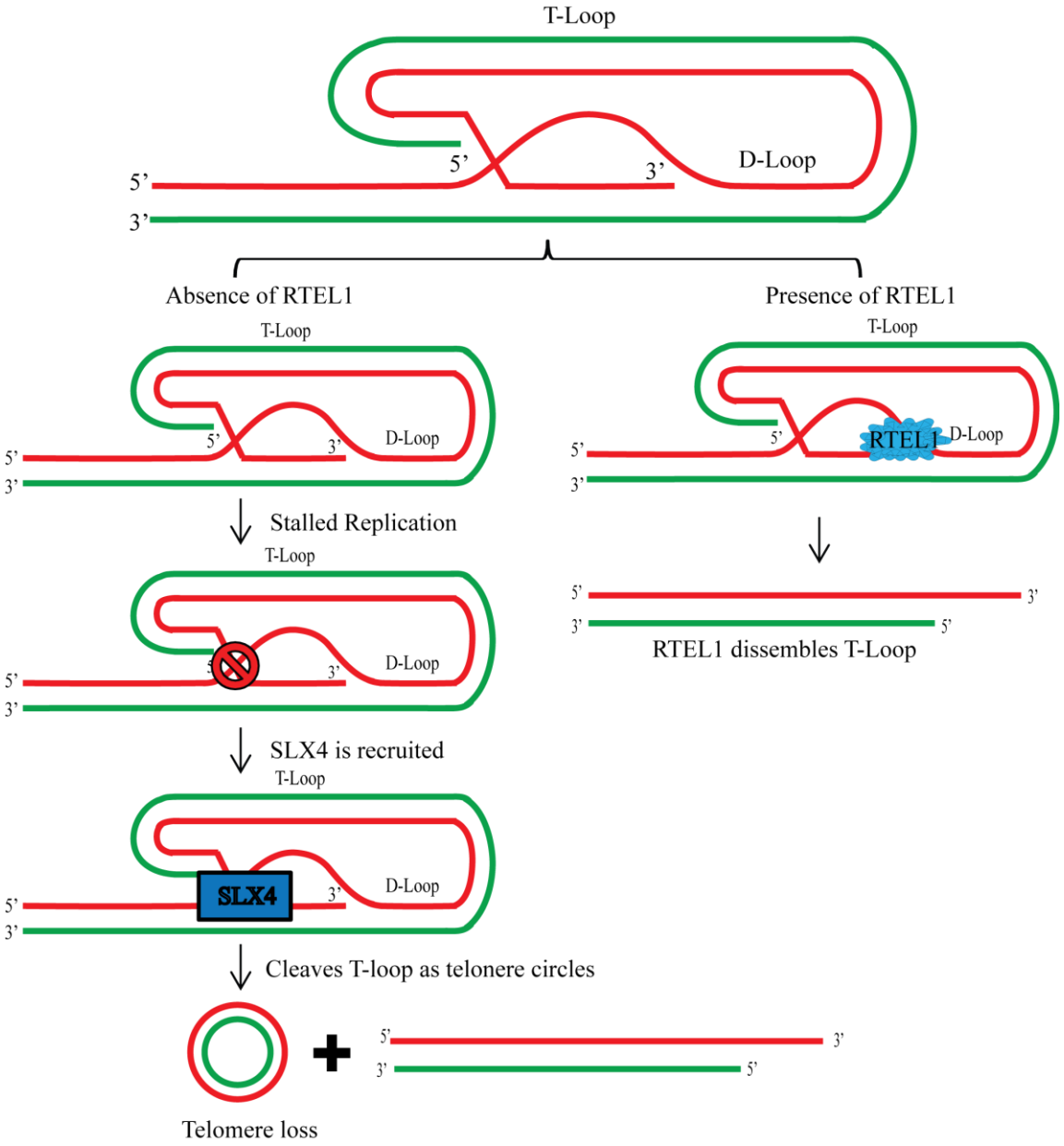

Figure 1.6 A flowchart summarizing the role of RTEL1 in the resolution of T-Loop structure. RTEL 1 (as shown by blue circle) efficiently resolves T-loop structure for efficient replication at telomeres. However, in the absence of RTEL1, the unresolved T-loop will recruit SLX4 nuclease which cleaves T-loop as telomere circles, leading to telomere shortening.

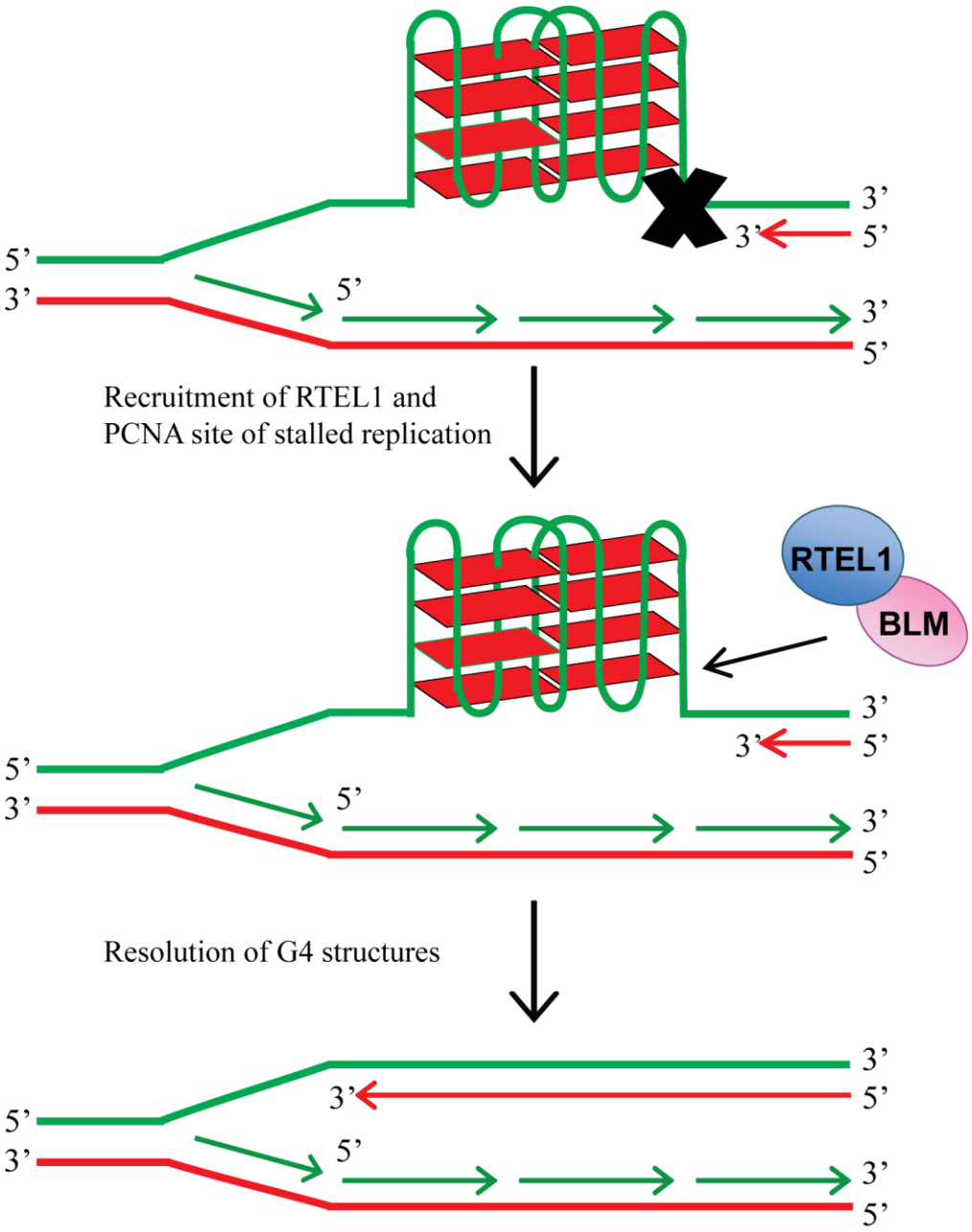

Figure 1.7 The mechanism of RTEL1 in the resolution of G4 DNA structures. The high GC contents of telomeres will lead to the formatiom of G4 quardruplex. This unique structure will block the replication of telomeres. Both RTEL1 (marked as blue circles) and BLM (red circles) helicases have been found to be able to resolve G4 structure, facilitating telomere replication.

1.3.2 Function of RTEL1 in DNA homologous recombination

1.3.2.1 Overview of DNA damages and repair pathways

Because of the reactive oxygen species produced by normal cellular metabolism, a cell could face approximately 30,000 DNA lesions per day[38]. In addition, many environmental factors, such as ultraviolet light (UV), ionizing radiations and various genotoxic compounds, can also induce DNA damage in the cells[39].This DNA damage could be the result of single DNA-strand breaks, insertions, deletions, dimers and DSBs, which can be repaired by different repairing machineries depending on the nature of damages (Figure 1.8).

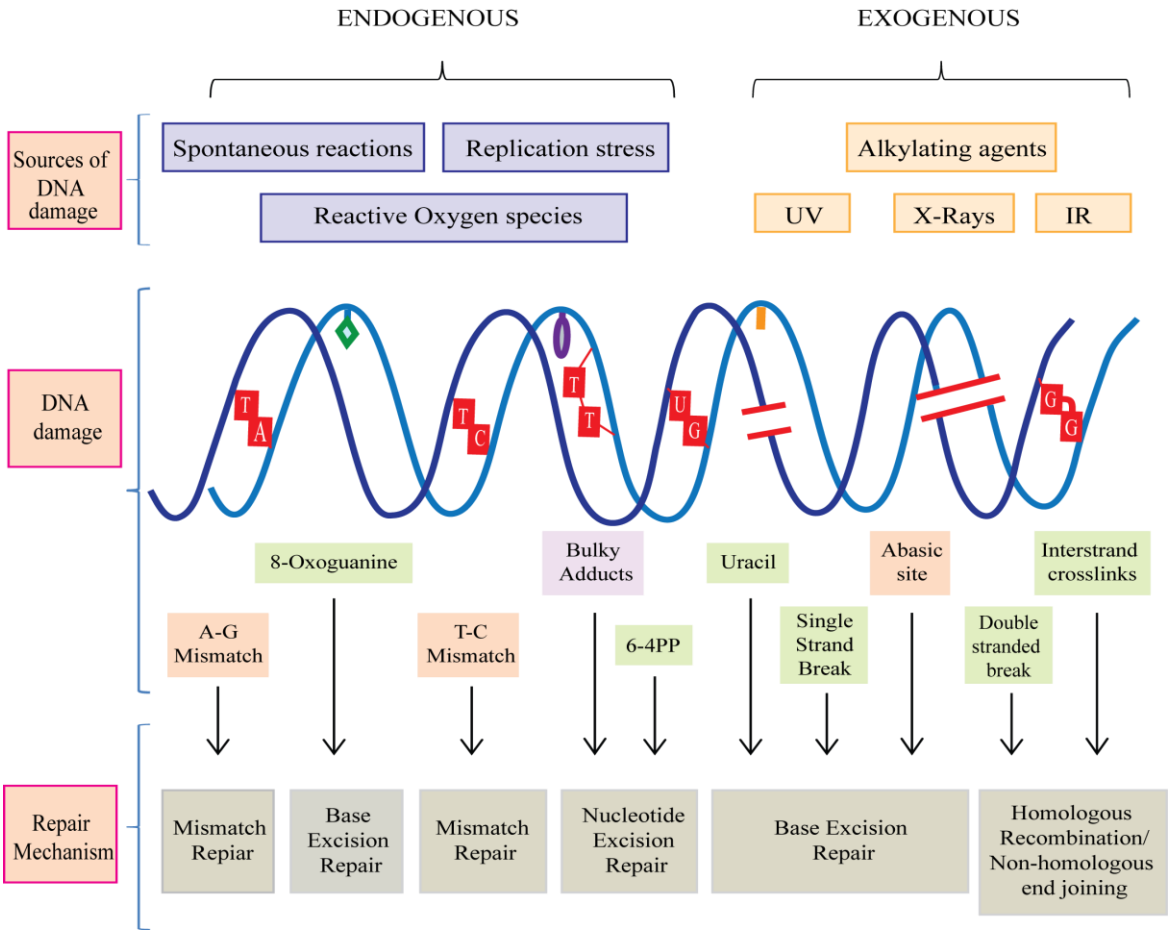

Figure 1.8 Flowchart depicting the various sources, outcome and repair of DNA damages. The top panel outlines the major sources for inducing DNA damages in cells. The middle panel summarizes the types of DNA lesions that a cell may face, and the bottom panel represents the pathways to repair DNA damages.

DSBs are the most common form of DNA damage present in the cells. They can be induced by irradiation or other chemical agents, or UV, but often arise as a result of normal cellular metabolism. DSBs are deleterious to cells, which could cause cell death if not repaired. In addition, if DSBs are not repaired correctly, they will induce DNA deletions, translocations and fusions. One consequence of this genomic instability is cellular transformation[40].

DSBs can be repaired by two major pathways. The non-homologous end joining (NHEJ) repair pathway will utilize several proteins (such as Ku70, Ku80 and DNA-PKcs) for end-processing to remove damaged or mismatched nucleotides, and re-ligate them via DNA ligase IV[41]. Since the end-processing step in NHEJ frequently removes additional nucleotides from broken ends, this repair pathway is generally considered as error-prone[42]. In contrast, the second major DNA repair pathway, homologous recombination (HR), which relies on the exchange of nucleotide sequences between two similar or identical molecules of DNA, is able to accurately repair DSBs. It has been shown that stem cells have more proficient HR activity than other cells in order to maintain a stable genomic integrity [43].

1.3.2.2 The process of homologous recombination in DNA repairing

Although HR varies among different organisms and cell types, most HR pathways involve the same basic steps which include: (1) Recognition of DSBs by the MRN complex; (2) 5' to 3' resection of DSB ends to generate 3′ overhangs; (3) Binding of Replication protein A to the 3' overhang to recruit Rad51, forming the nucleoprotein filament; (4) Invasion of the nucleoprotein filament into the complementary strand in search for homology, forming a displacement loop (D-Loop) structure. (5) Extension of invaded 3' overhang strand by synthesizing new DNA, which changes the D-loop to a cross-shaped structure known as a Holliday junction (Figure 1. 9).

Following these initial steps, there are two downstream pathways (Figure 1.10). In the DSBR (Double Strand Breaks Repair) pathway, the second 3' overhang (which does not involve in strand invasion) also forms a Holliday junction with the homologous DNA strand. These double Holliday junctions are then cut by nicking endonucleases to release the recombination products. The DSBR pathway most often results in DNA crossover which is important for meiosis[44]. But this crossover could induce aberrant recombination during the repair of DNA. Therefore, it is not commonly used in the cells for repairing DNA damages.

In the second pathway, namely the synthesis dependent strand annealing (SDSA) pathway, the extended 3' overhang is released as a Holliday junction slides in a process called branch migration. The released 3' overhang with a newly synthesized DNA sequence will then be able to anneal to another end of DSBs (Figure 1.11). This SDSA (synthesis-dependent strand annealing) repair pathway leads to yield a non-crossover repair product that can avoid crossover and thus the possibility of deleterious genome rearrangement[45]. Thus SDSA is considered as the predominant HR-based repairing pathway in somatic cells[46].

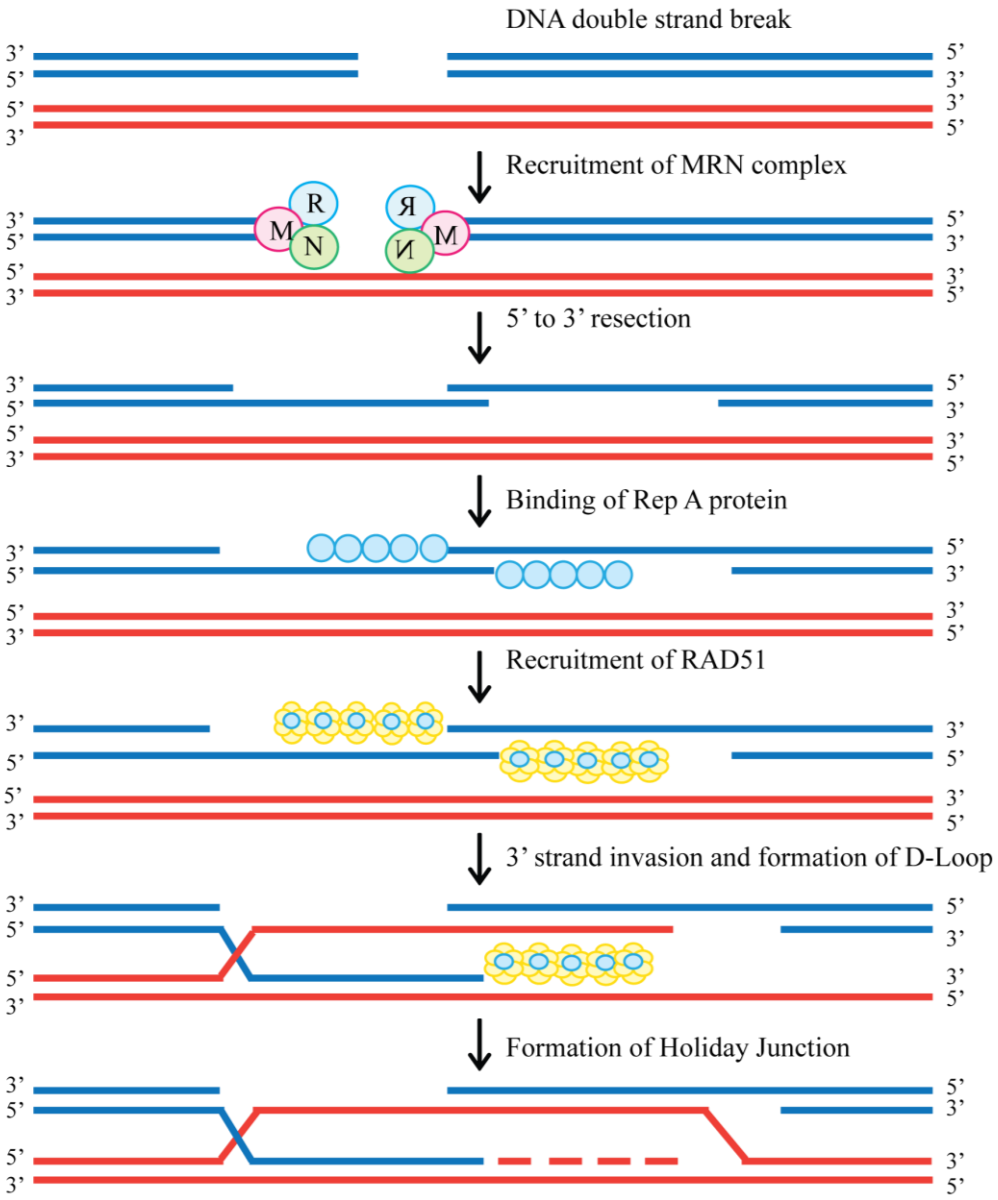

Figure 1.9 A diagrammatic representation of double stranded break repair by homologous recombination. The MRN complex is recruited at the site double strand break and 3' resection is produced. Replcation A protein binds at the site of 3' resection and recruits Rad51. Strand invasion occurs and leads to the simultaneous formation of D-loop and this is soon replaced by the formation of Holliday junction.

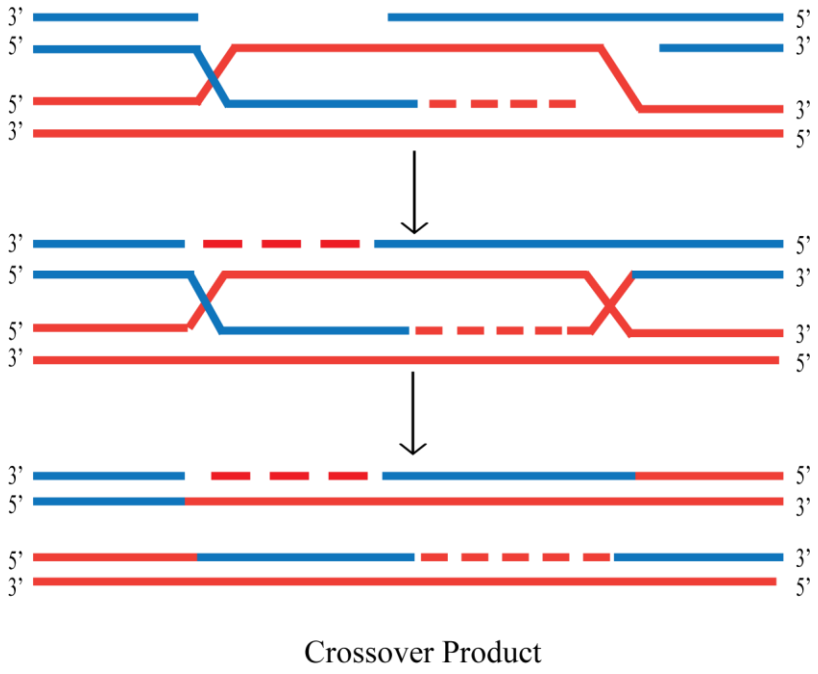

Figure 1.10 Double Strand Break Repair (DSBR) Pathway. In this pathway, nicks created by endonucleases at Holliday Junction results in DNA crossover.

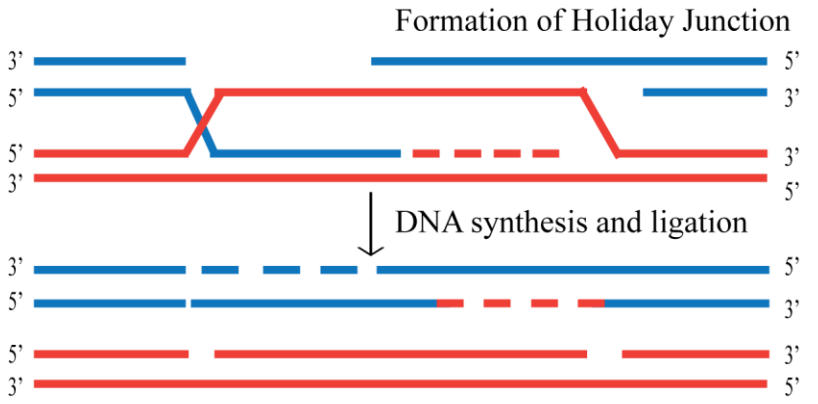

Figure 1.11 Synthesis dependant strand annealing (SDSA) Pathway. The 3' end that undergoes branch invasion undergoes DNA synthesis which on branch migration is ligation to the other end of the double strand break.

1.3.2.3 The role of RTEL1 in HR

Purified RTEL1 protein has been found to be able to dismantle pre-formed D-Loop structure *in vitro*[3]. In addition, RTEL1 was further found to preferentially disrupt 3' invasion of D-loop in presence of replication protein A[47]. The dismantling of D-Loop by RTEL1 should promote SDSA mediated DNA repair. Indeed, a significantly increased crossover was found in RTEL1 deficient germ cells. Similarly, a high recombination activity as reflected by the increased sister chromatid exchanges has also been identified in RTEL1 deficient ES cells[48]. All these indicate that RTEL1 could be an important factor to suppress HR.

1.3.3 Function of RTEL1 in DNA replication

1.3.3.1 Interaction of RTEL1 with proliferating cell nuclear antigen (PCNA)

The evidence for the involvement of RTEL1 in DNA replication emerged from a mass spectrometry analysis which revealed RTEL1 can form a protein complex with PCNA[4]. This evidence was further strengthened by identifying a PCNA-interacting (PIP) motif in RTEL1 protein, and by the immunofluorescence analysis which revealed co-localization of RTEL1 and PCNA as discrete replication foci in the S phase cells[4]. Through a mutagenesis approach, several key amino acids in the PIP motif have also been identified to be essential for RTEL1/PCNA interaction[4]. All these strongly indicate that RTEL1 can interact with PCNA, a critical factor for DNA replication, in the cells.

1.3.3.2 The role of RTEL1/PCNA in DNA replication

PCNA has been demonstrated to be a DNA clamp that binds to DNA polymerase and prevents this enzyme from dissociating from the template DNA strand[49]. PCNA has also been shown to load other replication fork proteins to facilitate the synthesis of the nascent DNA on the template strand (leading and lagging). Therefore, PCNA has been considered as a master protein

in DNA replication. The interaction of RTEL1 with PCNA suggests that RTEL1 may also play an important role in DNA replication**.**

To determine the role of RTEL1 in DNA replication, our lab has applied a mouse transgenic approach to generate a knock-in mouse allele with a single amino acid mutation to abolish RTEL1-PCNA interaction *in vivo*[4]. This mutant mouse allele showed reduced replication fork extension rate, increased origin usage due to fork stalling and overall replication fork[4]. This mutant mouse allele also showed defects in the replication of telomeres which leads to the formation telomere fragility[4]. Taken together, our work indicates that RTEL1 is also a DNA helicase associated with DNA replicasome required for DNA replication.

The mechanism of RTEL1-PCNA interaction at the DNA replication fork still remains undefined. However, based on the capacity of RTEL1 on resolving certain DNA structures, such as G4 and loop structures, it is expected that RTEL1 could be required to remove DNA secondary structures that may form at the replication fork during replication of the repetitive sequence. In absence of RTEL1, cells may not be able to resolve these structures, eventually leading to fragile chromosome and genomic instability.

1.3.4 Function of RTEL1 in suppressing the expansion of Tri-Nucleotide Repeats (TNR)

TNR repeats are generally found interspersed as repetitive sequences in the genome of higher organism promoting chromosomal rearrangements involving repetitive DNA[50]. These repetitive sequences are known to pose a threat to the genome stability during DNA replication and repair. This is because the single stranded DNA generated during replication or repair may form hairpin structures by intra strand base pairing between TNR repeats for example $(CAG)^{n}$. Such hairpin structures are known to be repaired by the DNA repair machinery, for example DNA mismatch repair system (MMR). However, failure to repair such structures may lead to the

expansion or occlusion of the repetitive sequence which may alter the local genomic organisation and may affect gene function[5].

Knockdown of RTEL1 has recently been shown to cause 5-7 fold increase in the TNR expansion in human SV40 transformed astroglial cell line (SVG-A)[5]. RTEL1 protein has also been found to interact with TNR hairpin and unwind it[4]. This effect could facilitate the passage of replication forks during DNA replication. Thus, the role of RTEL1 in unwinding TNR hairpin structure could also contribute to the maintenance of genomic stability.

1.3.5 Possible mechanisms for genomic instability induced by RTEL1 dysfunction

As discussed above, RTEL1 has been found to be required for the maintenance of telomere length, the suppression of HR, DNA replication, and the suppression of TNR expansion. Defects in any of these pathways could lead to a global genomic instability in the cells. For example, loss of telomeres as caused by RTEL1 dysfunction has been shown to induce chromosomal fusion to form a dicentric chromosome[1]. Such dicentric chromosomes could be subjected to chromosomal breakage during anaphase segregation and lead to chromosomal instability. The second mechanism for RTEL1 dysfunction-induced genomic instability could be high HR activity as observed in RTEL deficient cells. This high HR activity has been found to increase DNA crossover, thus increasing the possibility of deleterious genome rearrangements[3]. Finally, the ability of RTEL1 to remove TNR hairpins in the highly repetitive sequences in the genome could also be important for RTEL1 to protect genomic stability. In absence of RTEL1, considerable expansion of TNR repeats could lead to chromosomal fragility and subsequent genomic instability[13].

In summary, the current studies strongly indicate that RTEL1 is an essential factor for the maintenance of genomic stability. However, how this function of RTEL1 is involved in

development is still unknown. As discussed in the following section, our recent finding that RTEL1 is selectively expressed in adult stem/progenitor cells, suggests that this DNA helicase could be important for the maintenance of adult stem/progenitor cells.

1.4 Expression of RTEL1 during development

As a first approach to understand the role of RTEL1 in development, our lab has applied several approaches to analyze the expression of RTEL1 during mouse embryogenesis and post-natal development. With an in-situ hybridization approach, we found that RTEL1 is highly and ubiquitously expressed in the mouse embryos at early developmental stages (E8.5 to E10.5)[1]. Subsequently, RTEL1 expression was found to be associated with proliferating cells during mouse embryonic organogenesis (E12.5-17.5)[1]. During postnatal development, most adult tissues showed undetectable RTEL1 expression by Northern hybridization assay. Only testis, ovary and other tissues with rapid cell division, such as spleen and thymus, showed RTEL1 expression[1]. These results indicate that RTEL1 is selectively expressed during mouse development.

To further characterize the expression of RTEL1 during postnatal development, we used RTEL1-LacZ reporter mice and the RNA in-situ hybridization assay to analyze RTEL1 in adult mouse tissues. As shown in Figure 1.12, RTEL1 expression was found to be mainly located in several regions that are proposed stem cell zones. In the brain, RTEL1 was detected in the sub-ventricular zone (Figure 1.12A). In the testis, RTEL1 mRNA was mainly found in the cells located along the base membrane (Figure 1.12B). In the intestine, RTEL1 expression was specifically located in the crypts (Figure 1.12C). All these regions have been shown to contain stem/progenitor cells which are essential for tissue homeostasis.

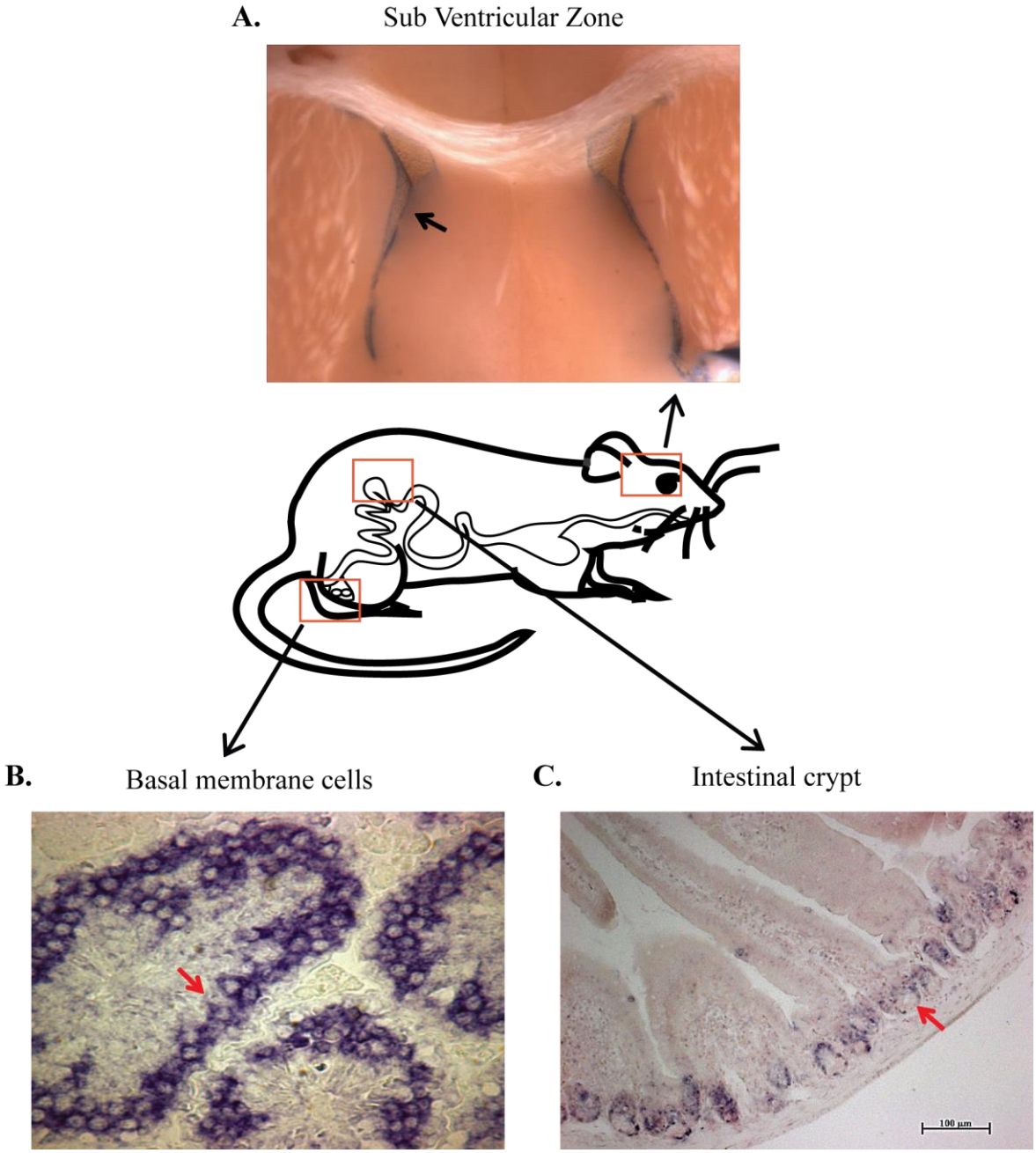

Figure 1.12 Specific expression of RTEL in the stem cell-zones of adult mouse tissues. (**A**) Expression of RTEL1 was found in the sub-ventricular zone of the brain (arrow indicates). (**B**) Expression of RTEL1 was detected in the sperm cells located along the basement membrane of the testis (arrow indicates). (**C**) RTEL1 mRNA was located in intestinal crypts where intestinal stem/progenitor cells are resided (arrow indicates).

The expression of RTEL1 in several adult stem cell-zones indicates that this DNA helicase could be required for the maintenance of stem/progenitor cells. Given the role of RTEL1 in the maintenance of genomic stability, RTEL1 may have a specific role in protecting the genomic integrity in adult stem/progenitor cells. As a part of this characterization, my research project will focus on determining whether RTEL1 is required for the maintenance of intestinal stem/progenitor cells. In the following section, I will summarize the current findings on intestinal stem/progenitor cells and the role of these cells in intestinal homeostasis/regeneration and in the formation intestinal tumors.

1.5 Function of intestinal stem/progenitor cells

1.5.1 The structure and composition of intestine

The intestine is a highly evolved organ that performs the basic function of food uptake, digestion, absorption and excretion. It is divided into two large regions, small intestine and large intestine. The small intestine constitutes of three distinct segments, namely duodenum, jejunum and ileum, and the large intestine can be divided as cecum and colon (Figure 1.13). The major function of the small intestine is digestion and absorption, whereas the function of the large intestine is limited to absorption and excretion only.

Histologically, the intestine contains three distinct cell layers, namely the outer smooth muscle layer, stromal layer and epithelial cells. The smooth muscle layer is richly supplied by nerves and performs the main function of peristalsis. Stromal layer is richly supplied with blood vessels and performs the function of nutrient absorption. The innermost intestinal epithelia cells provide the surface for absorption. In order to maximize the efficiency of the absorptive function, the intestinal epithelial layer is organized into finger like projections called the villi (Figure 1.13). Each villus is composed of three major types of epithelial cells, namely the enterocytes,

entero- endocrine cells and goblet cells, each of which has specific function. The absorptive cells or enterocytes are tall columnar cells that secrete hydrolases and help absorption. Goblet cells are the most abundant cell types which produce glycoprotein that lubricates and protects the intestinal lining from being digested by its own enzymes and acids. The less abundant entero-endocrine cells can secrete hormones, such as serotonin, substance P and secretin. In addition to these cell types, the intestine also contains the Paneth cells and the intestinal stem cells that reside at the crypts in the small intestine (Figure 1.14). The Paneth cells secrete antimicrobial peptides which form a part of the host innate immune response. These cells also secrete enzymes such as cryptidins, defensins, and lysozyme[51]. The intestinal stem cells supply a pool of cells which eventually differentiate to all the intestinal cell types required for intestinal homeostasis and regeneration.

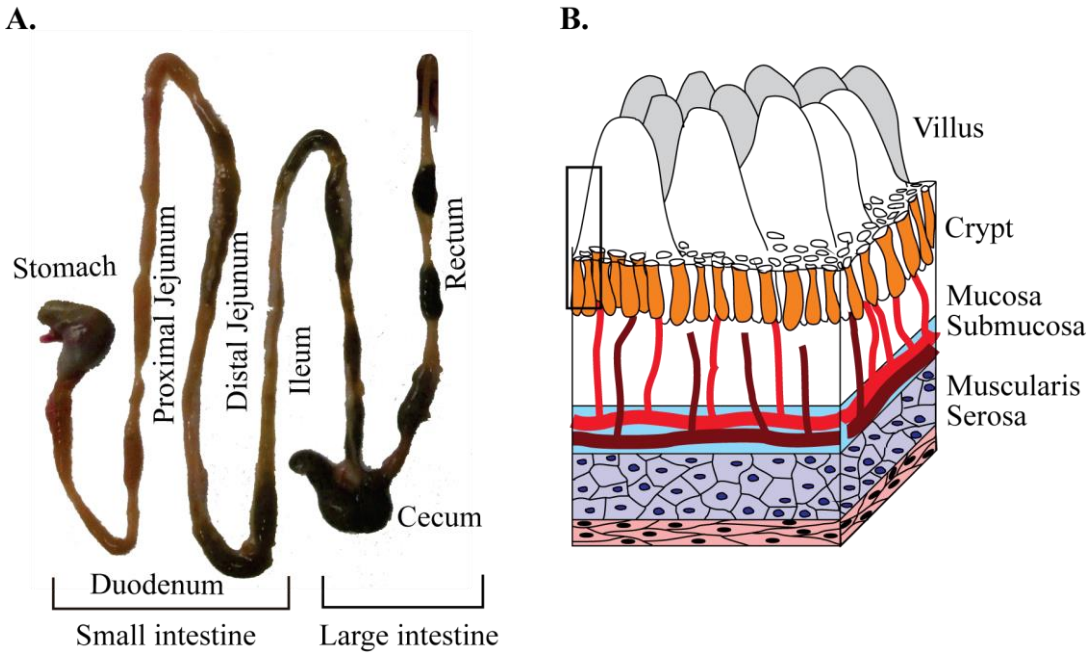

Figure 1.13 Structure and organization of mouse intestine. (**A**) Mouse intestine is largely similar to human intestine, which consists of small intestine and large intestine. Small intestine can be further divided as three segments, namely Duodenum, Jejunum and Ileum. (**B**) The vertical section of Jejunum displays the major layers of the intestine.

1.5.2 The role of intestinal stem/progenitor cells in intestinal homeostasis/regeneration

The intestinal homeostasis is achieved by matching the rate of intestinal stem cell division with the rate of cell differentiation and cell death. Due to the harsh acidic environment within the lumen, the intestinal epithelium is constantly shed off. The cells that exude from the tip of the villi have to be replaced by new epithelial cells. This replacement usually takes place every 2-5 days, and is supplied by the differentiation of intestinal stem cells that are located at the base of crypts. Intestinal stem cells can self-renew, proliferate and give rise to transit amplifying cells, which migrate along the crypt/villus axis to differentiate into different epithelial cell types of the intestine (Figure 1.14). There are two proposed locations of intestinal stem cells in the crypt: (1) +4 position (the 4th cell counting from the bottom of crypt); and (2) Columnar basal cells (CBC) resided between Paneth cells at the base of the[52] (Figure 1.14). Each crypt is estimated to have 4-6 functional stem cells[53]. Since each villus contains ~5 crypts, therefore, it could have ~20 intestinal stem cells to support a single villus. The fixed number of stem cells in the crypt is maintained by the balance between self-renewal and cell deaths/differentiation at the tip of the villi. However, the molecular mechanisms that determine whether a stem cell undergoes self- renewal or differentiation still remains unanswered.

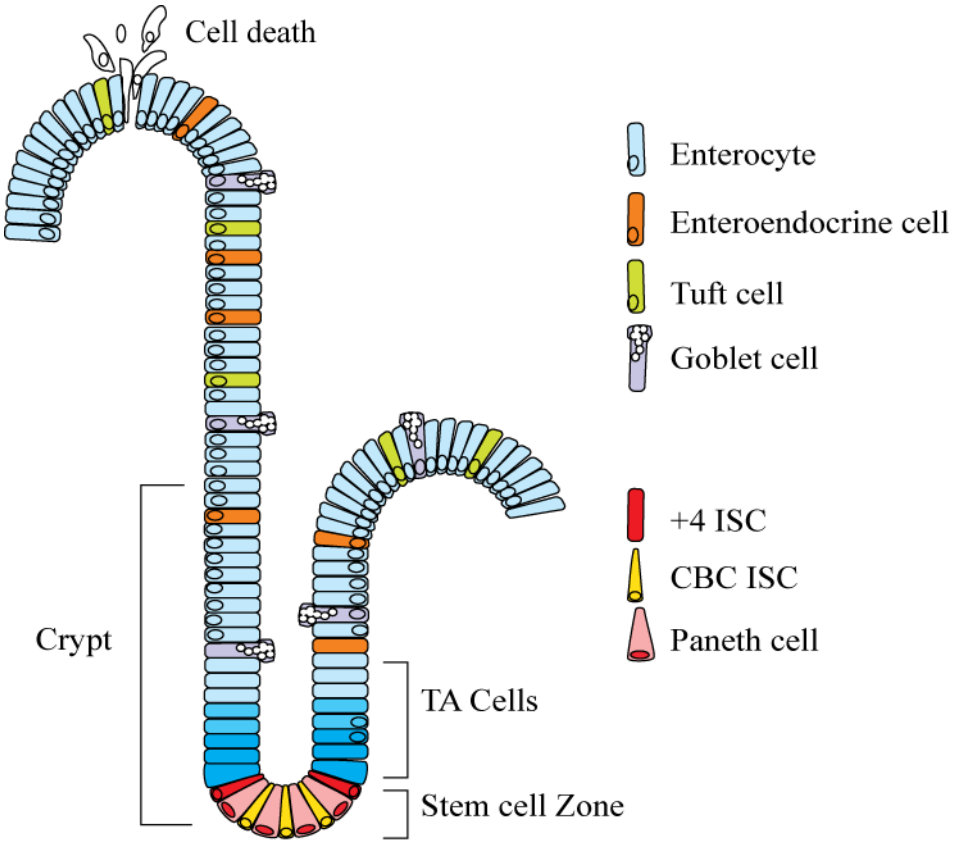

Figure 1.14 Organization of stem and differentiated cells along the crypt-villi axis in the intestine. Intestinal stem cells are located within intestinal crypts (CBC and +4 cell). These stem cells first generate transit amplifying (TA) progenitor cells that further differentiate into other intestinal epithelial cells along the crypt-villi axis that eventually exit into the lumen via programmed cell death. Among the committed cell types, Paneth cells migrate downward toward the base of the crypt.

Similarly, intestinal regeneration is achieved by the regenerative potential of the stem cells in the crypt. Following DNA damage, acute inflammation, surgical resection or genetic ablation of genes essential for intestinal stem cells, the unaffected intestinal stem cells can rapidly proliferate to repopulate the pool of intestinal stem cells[54] . So far, little is known about the molecular mechanism that control repopulation of stem cells during regeneration. However, it has been shown that activation of Wnt signalling pathway could be required for intestinal regeneration[55]. In addition to Wnt signalling, other signalling pathways like JNK, JAK/Stat and Hippo may also play important roles in intestinal regeneration[56,57]. The choice of pathway may

depend on the mode of intestinal destruction, for example, cells damaged by UV radiation may trigger the intestinal stem cell regeneration by choosing a pathway which is different from the choice of pathway caused by inflammation[55].

A number of cell surface markers have recently been identified to mark different stages of intestinal stem cells (Figure 1.15). Among them, Lgr5 and Olfm4 are the commonly used markers for undifferentiated intestinal stem cells[58].

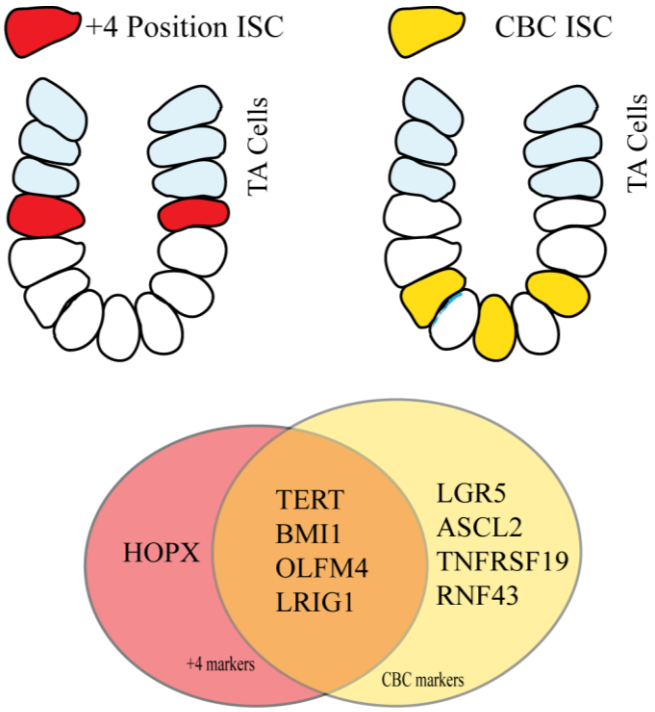

Figure 1.15 Diagrammatic representation of molecular markers for intestinal stem cells. Several proteins have been identified to either express in +4 intestinal stem cells or columnar basal intestinal stem cells.

1.5.3 Regulation of intestinal stem cell activity

In mouse, the epithelium of the small intestine originates from the single layered inner lining of the primitive gut at around E9.5. The villi like structures appear at E14.5dpc. The adult epithelium is established between E16.5 and postnatal day 7 (p7) when mature crypts develop

from shallow pockets of proliferative cells that are restricted to the base of embryonic villi[59]. Multiple signalling pathways have been shown to be involved in the intestinal remodelling and bi-directional signalling between the epithelium and the underlying mesenchyme are considered to be the important regulator[53]. Hedgehog signal from the developing epithelium of the primitive gut is thought to direct embryonic villus formation and specify the site of crypt development at the base of the villus[60]. The Wnt signalling has been shown to play an essential role in the establishment of regenerative and proliferative capacity of the adult epithelium[61]. It has been shown that Wnt pathway is active in the form of gradient with the highest activity at the crypt bottom. Crypt loss and impaired villus formation has been observed following ablation of transcription factor 4 (TCF4), a major intestinal WNT effector protein[59].

The Wnt signaling is mediated by β-catenin. Normally, β-catenin levels in the cytoplasm are kept low by proteosomal destruction which is mediated by a complex that involves adenomatous polyposis coli (APC), casein kinase (CKI), glycogen synthase kinase 3 (GSK3) and axin. However, when Wnt signalling is activated, the Wnt ligand binds to the frizzled receptor and the low-density lipoprotein receptor-related protein (LRP) co-receptors which then inhibit the destruction complex. Inhibition of the destruction complex leads to the accumulation of β-catenin in the cytoplasm. The increased β-catenin level in the cytoplasm results in its transportation to the nucleus where it replaces the Groucho on the TCF4 factor. The β-catenin and TCF4 together form an active transcription complex which leads to the expression of Wnt target genes[62]. Conditional deletion of β-catenin resulted in loss of proliferation in the intestinal epithelium due to lack of TCF4 induced gene expression[63].

As discussed above, intestinal stem cells divide to the transit amplifying progenitor cells which further differentiate to the secretary or absorptive epithelial lineages. It has been shown

that the Notch signalling pathway plays an important role in determining intestinal stem cell fate. Notch pathway is operated by 4 Notch receptors which can bind to any of the five Notch ligands, resulting in the proteolytic cleavage of the Notch intracellular domain (NICD). The free NICD translocates into the nucleus where it binds to the transcription factor RBP-Jk (CSL or CBF1) to activate target gene transcription[64]. Conditional deletion of CSL gene resulted in the rapid and complete conversion of all epithelial cells into goblet cells. Interestingly, over expression of the Notch1 receptor in the intestinal epithelium resulted in the opposite effect – a depletion of goblet cells and a reduction in entero-endocrine and Paneth cell differentiation[65]. Notch signalling also results in the expression of group genes that belongs to the Hairy/Enhancer of Split (Hes) class which encode transcriptional repressor. The Hes repressor in turn represses other transcription factors that control differentiation of specific cell lineages. For example, the Hes1 represses Math1 transcription factor which controls paneth, goblet and entero-endocrine cell lineage differentiation. Math1 is again activated by the Wnt signalling pathway[66]. Thus cooperation of Wnt and Notch signalling through a combination of activator or repressor transcription factors determines the intestinal stem cell lineage (Figure 1.16).

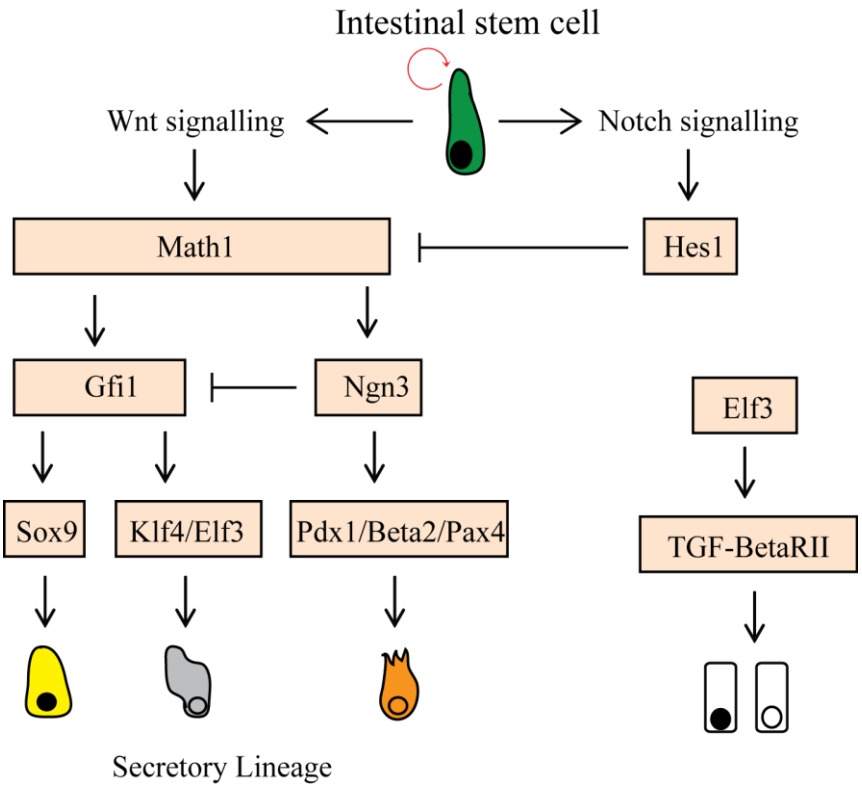

Figure 1.16 A schematic representation of different signalling pathway involved in the regulation of intestinal stem cell and differentiation. Wnt and Notch signalling play an important role in maintaining the stemness and differentiation of intestinal stem cell, respectively. The downstream signalling molecules of both pathways further contribute to the choice of differentiation a stem cell makes.

1.5.4 Involvement of intestinal stem cells in the development of intestinal tumor

Colorectal cancer, the major type of intestinal tumor in humans, is one of the most common cancers in the world[67]. In Canada, colorectal cancer remains the third most commonly diagnosed cancer, accounting for 14% of newly diagnosed malignancies in 2014 (Cancer society statistics 2014). The formation of intestinal or colorectal cancer has been demonstrated as a multiple-step process, starting from a small adenomatous polyp and followed by the development of a large adenoma with dysplasia that ultimately leads to the formation of invasive carcinoma[68]. It is widely accepted that this pathogenesis is initiated by the inactivation of the

APC (adenomatous polyposis coli)/Wnt signalling pathway and then progresses as a result of a of mutational activation of oncogenes coupled with the inactivation of tumor-suppressor genes[69] (Figure 1.17).

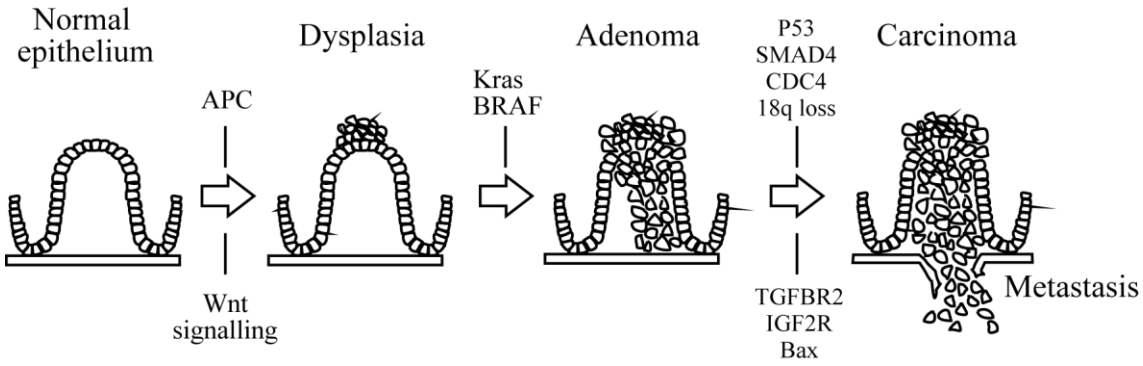

Figure 1.17 Stepwise model of intestinal cancer formation. The tumor formation could be initiated by the mutation of APC/Wnt signalling pathway, and progresses to the different stages by the accumulation of multiple genetic mutations affecting oncogenic or tumor suppressor functions.

Although intestinal or colorectal cancer is predominantly epithelial in origin, the cellular source contributing to the formation of this cancer is still largely unknown. By identifying Lgr5 and Prominin 1 as intestinal stem cell-specific markers, two research groups have recently applied these two marker genes to specifically mark cells that have undergone transformation due to an increase Wnt signalling activity (either by overexpression of active form of β-catenin or by mutating Apc gene) in mouse intestinal stem cells [70,71]. Both genetic manipulations in mice have been shown to efficiently induce the formation of intestinal adenomas, demonstrating that intestinal stem cells could be the major cellular source for intestinal or colon cancers[72]. Consistent with this, human colon cancers have been found to contain cancer stem cells which can self-renew and initiate tumor formation[73]. Although it still lacks evidence for the original of these cancer stem cells, this finding, together with mouse modelling studies, strongly suggests

that intestinal stem cells can contribute to the formation of intestinal tumors. Therefore, intestinal stem cells must be maintained properly in order to prevent the formation of intestinal tumors.

1.5.5 The maintenance of stable genomic integrity in intestinal stem/progenitor cells

In order to avoid the risk of passing genetic mutations to progeny cells or to prevent the transformation of stem cells to form tumors, stem cells have developed several defence systems to maintain a stable genomic integrity in these cells[74]. The first defence is the high expression of telomerase in the stem cells, which will prevent these cells from losing telomeres[75]. The second defence is the proficient HR based repairing activity in the stem cells[43]. As discussed above, due to the reactive oxygen species produced from normal cellular metabolism, stem cells could face numerous DNA damages per day. Since NHEJ based DNA repairing pathway usually gives rise to errors, HR has been considered as the major repairing pathway in cells[76]. The proficient HR repairing activity as found in stem cells will be important for protecting these cells from genetic mutations.

Intestinal stem/progenitor cells have also been found to express several proteins involved in HR[77]. These cells could also express telomerase[78]. However, the function of these proteins in the maintenance of intestinal stem/progenitor cells has not been addressed. As discussed above, RTEL1 could be another important factor required for the maintenance of genomic stability in intestinal stem/progenitor cells. Addressing the role of RTEL1 in this biological process could expand our knowledge on the maintenance of these stem cells.

Chapter 2: Hypothesis for this project

Given the essential role of RTEL1 in maintenance of genomic stability and its unique expression in intestinal crypts where intestinal stem and progenitor cells are located, we hypothesize that RTEL1 could be required for the maintenance of intestinal stem and progenitor cells. Dysfunction of RTEL1 could result in genomic instability in these cells which may in turn impair tissue homeostasis and regeneration. Dysfunction of RTEL1 may also transform intestinal stem and progenitor cells to form intestinal tumor.

Chapter 3: Specific aims for this project

This project will mainly focus on using mouse models to determine whether RTEL1 is indeed required for the maintenance of intestinal stem cells. It includes the following specific aims:

Specific aim 1: To determine whether RTEL-expressing cells indeed function as intestinal stem cells

Specific aim 2: To generate a mouse model for studying the role of RTEL1 in the maintenance of intestinal stem/progenitor cells

Specific aim 3: To characterize the phenotypes of mouse model with RTEL1 deficiency in intestinal stem and progenitor cells

Specific aim 4: To determine whether RTEL1 dysfunction could transform intestinal stem and progenitor cells to form intestine tumor

Chapter 4: Materials and Methods

4.1 Mice

In this project, we used the following transgenic mouse strains:

(1) RTEL1-CreERT2 knock-in mice

This mouse strain was recently developed in our lab (Wu and Ding, unpublished data). Briefly, a gene-targeting vector was designed to knock in a SA-IRES-CreERT2 expression cassette into an intron sequence in RTEL1 mouse genomic locus (Figure 4.1). Since the splicing acceptor (SA) in SA-IRES-CreERT2 expression cassette will trap the splicing donor (SD) of the upstream of exon, this gene-targeting event will lead to the formation of a chimeric mRNA containing a partial 5' mRNA of RTEL1 and the IRES-CreERT2. IRES (internal ribosome entry site) is a nucleotide sequence that provides an initial site for translating the downstream mRNA, the CreERT2 in this case. Therefore, the expression of CreERT2 in RTEL1-CreERT2 mice will be tightly controlled by the endogenous regulatory elements of RTEL1.

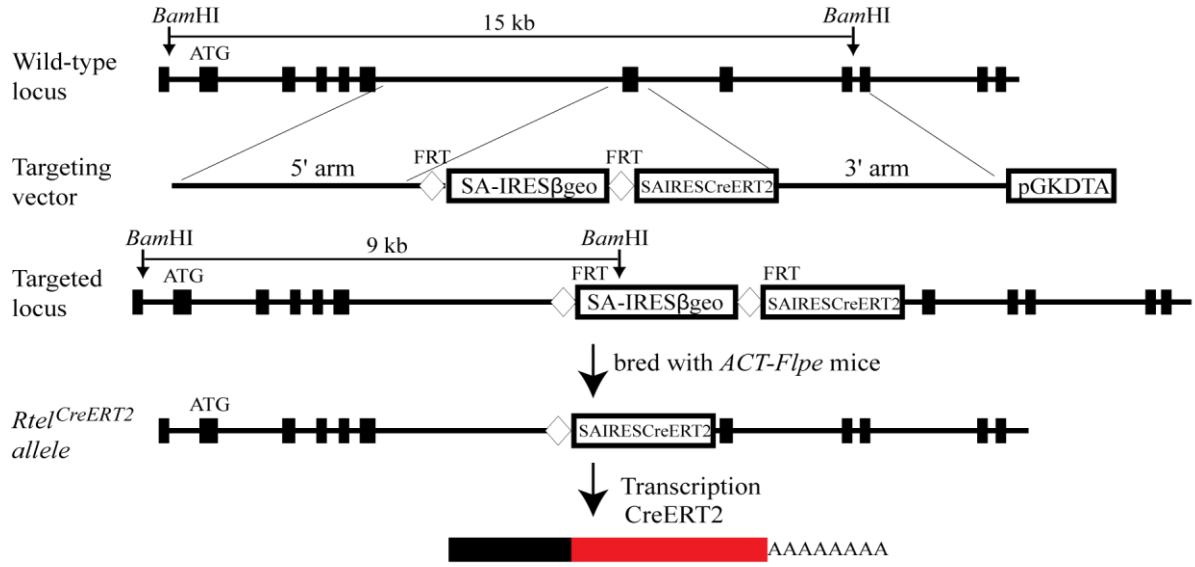

Figure 4.1 Generation of RTEL1 Cre-ERT2 knock in allele: A gene-targeting vector was generated to insert an SA-IRES-Cre-ERT2 cassette into mouse RTEL1 genomic locus by homologous recombination. Since the inserted casette contains a splicing acceptor (SA) which will trap a splicing dornor (SD), it will result in formation of a chimeric mRNA which contain partial 5' RTEL1 and IRESCre-ERT2 mRNA. IRES provide an initial site for translating CreERT2 protein from this chimeric mRNA.

The correct targeted mouse ES cells were confirmed by Southern blot analysis. The targeted ES cells were used for generating RTEL1-CreERT2 chimeras by ES cell-diploid aggregation. The chimeras gave germlines and established a mouse colony for the lineage tracing experiment in this project.

(2) ROSA26-LacZ reporter mice

This mouse strain was generated and described previously[79]. It contains a loxP flanked pGK-neo cassette followed by a LacZ reporter gene in ROSA26 genomic locus. The expression of this inserted DNA fragment will be tightly controlled by the ROSA26 promoter/enhancer which displays a ubiquitous activity. However, the expression of LacZ report gene will only be turned on by the Cre-mediated excision of loxP flanked pGK-neo cassette (Figure 4.2). Therefore, ROSA26-LacZ reporter mouse strain has been widely used for assessing the Cre activity in transgenic mice.

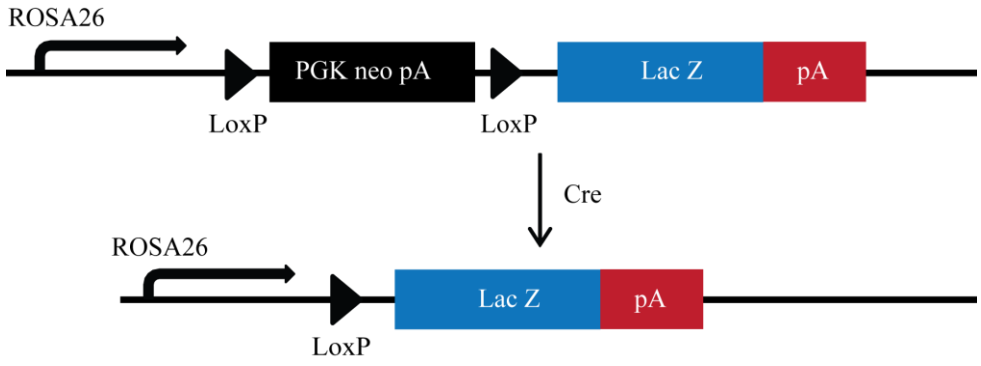

Figure 4.2 Schematic representation of using ROSA26-LacZ reporter mice for assessing Cre activity. ROSA26-LacZ reporter mouse consists of a loxP flanked pGKneopA cassette followed by a LacZ expression cassette. The LacZ expression in this mouse strain will only be turned on by Cre mediated excision of loxP flanked pGKneopA cassette. Thereby, measurement of LacZ activity will allow to assess the Cre activity.

(3) RTEL1 conditional knockout allele ($RTEL1^{F/F}$)

Since RTEL1 knockout mice are embryonic lethal[1], preventing the analysis of its postnatal developmental defects, our lab has generated RTEL1 conditional knockout mouse allele ($RTEL1^{F/F}$)[37]. In this mouse allele, the exon 7 of RTEL1 gene was flanked by two loxP sites. Deletion of exon 7 by Cre recombinase will result in a non-functional mRNA sequence due to a frame shift mutation, which is subsequently degraded by non-sense mediated decay[37].

(4) Ah-Cre transgenic mice

In order to conditionally knock out RTEL1 in intestinal stem/progenitor cells, we chose Ah-Cre transgenic mice. In Ah-Cre transgenic mouse line, Cre expression is controlled by the rat CYP1A1 promoter[80]. The CYP1A1 gene encodes a member of the cytochrome P450 superfamily enzymes which is involved in the metabolism of drugs and synthesis of cholesterol, steroids and other lipids. The promoter of CYP1A1 is inducible by polycyclic aromatic hydrocarbons, e.g. β – naphthoflavone[80]. Since Ah-Cre mice show high Cre activity in intestinal epithelial cells, including crypts where the intestinal stem/progenitor cells are located, this Cre line has been applied for knocking out genes' function in intestinal stem/progenitor cells[81].

(5) $p53^{F/F}$ mouse allele

This mouse allele was used in this project for conditional knocking out RTEL1 on p53 null background. $p53^{F/F}$ mouse allele was generated in the laboratory of Dr. Anton Berns[82]. In this mouse allele, exons 2-10 of p53 are flanked by two loxP sites. Mice homozygous for this floxed allele do not show any phenotypes. When this mouse allele is bred to mice with a Cre transgenic mouse line, p53 gene can be efficiently deleted in the tissue of interest.

(6) RTEL1$^{+/-}$ mice

RTEL1$^{+/-}$ mice will be used in this project for RTEL1 conditional knockout study. This mouse allele was generated and characterized previously[1]. Mice homozygous for RTEL1 knockout are embryonic lethal, whereas the heterozygotes are normal and indistinguishable from wild type mice.

(7) p53$^{+/-}$ mice

These mice were obtained from JAX lab and were used to conditionally knock out RTEL1 function on p53 null background. P53$^{-/-}$ mutant mice are tumor prone and develop tumors (the majority are thymic lymphomas) around six months old, whereas p53$^{+/-}$ mice are generally normal (only less than 10% develop lymphomas in the aged mice due to loss of heterozygosity).

4.2 Mouse breeding

In this project, we used mouse breeding to generate the following experimental mice:

(1) RTEL1-CreERT2/ROSA26-LacZ mice

These mice were used to trace RTEL1-expressing cells during development. They were generated by breeding RTEL1-CreERT2 with ROSA26-LacZ mice (Figure 4.3). The offspring from this breeding were genotyped by a PCR based method.

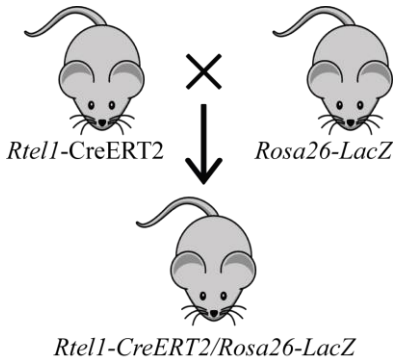

Figure 4.3 Breeding scheme for the generation of RTEL1-CreERT2/ROSA26-LacZ. RTEL1-CreERT2 mice were bred with ROSA26-LacZ mice to generate RTEL1-CreERT2/ROSA26-LacZ mice.

(2) RTEL1$^{F/-}$/Ah-Cre mice

To specifically knock out RTEL1 in intestinal stem/progenitor cells, we used a breeding strategy to generate RTEL1$^{F/-}$/Ah-Cre mice (Figure 4.4). In this breeding, we first bred RTEL1$^{+/-}$ mice with Ah-Cre to produce RTEL1$^{+/-}$/Ah-Cre, which were then bred with RTEL1$^{F/F}$ mice to generate RTEL1$^{F/-}$/Ah-Cre. In RTEL1$^{F/-}$/Ah-Cre mice, the induced Cre activity will convert RTEL1$^{F/-}$ to RTEL1$^{-/-}$, thus, completely knocking out RTEL1 function in intestinal stem/progenitor cells.

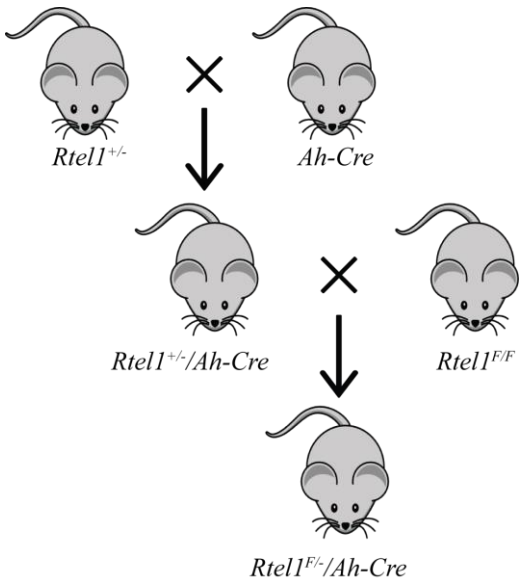

Figure 4.4 Breeding scheme for obtaining RTEL1$^{F/-}$/Ah-Cre: RTEL1$^{+/-}$ mice were crossed with Ah-Cre mice to obtain RTEL1$^{+/-}$/Ah-Cre, which were further bred with RTEL1$^{F/F}$ mice to obtain RTEL1$^{F/-}$/Ah-Cre.

(3) Ah-Cre/ROSA26-LacZ

To assess the recombination efficiency and the specificity of Cre activity of Ah-Cre, we bred Ah-Cre with ROSA26-LacZ reporter mice to generate Ah-Cre/ROSA26-LacZ (Figure 4.5).

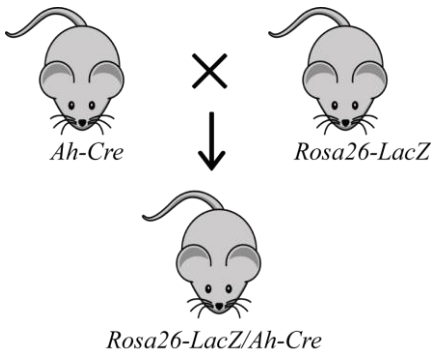

Figure 4.5 Breeding scheme for obtaining Ah-Cre/ROSA26-LacZ. Ah-Cre mice were crossed with ROSA26- Lac Z mice to obtain Ah-Cre/ROSA26-LacZ mice.

(4) RTEL1$^{F/-}$/Ah-Cre/p53$^{F/-}$ mice

Since p53 is an important tumor suppressor and its mutation has been identified in many human tumors/cancers, including colorectal cancers[83], we generated RTEL1$^{F/-}$/Ah-Cre/p53$^{F/-}$ to determine whether specific knockout of RTEL1 in intestinal stem/progenitor cells could develop intestinal tumors on P53 null background. These mice were produced by the breeding involved in multiple generations as illustrated in Figure 5.12 in the section of Results.

4.3 PCR based genotyping assay

The genotyping of mice was carried out by PCR based method (Courtesy: Wenjun Liu). Briefly, the ear punches collected from mice (Courtesy: Dr. Hao Ding) were lysed with 100µl lysis buffer (50mM KCl, 10mM Tris HCl 2mM $MgCl_2$, 1mg/ml of Gelatin, 0.45% Nonidet P40 and 0.45% tween 20) containing 20mg/ml Protinase K overnight at 55^{o}C degree. 1-2 µl of lysates was added to a PCR reaction solution that contained 1xTaq polymerase buffer (New England

BioLabs), 25 mM $MgCl_2$, 10mM dNTPs, 10 mM primers (forward and reverse) and 0.25 µl Taq polymerase (5000U/ml). The PCR reaction solutions were sealed using a drop of mineral oil to prevent evaporation, and then run the cycles (Table 4.1) using Bio-Rad thermocycler. The amplified PCR products were determined by electrophoresis using 0.8% agarose gel casted in 0.5xTAE (0.02M Tris-Acetic acid and 0.5 mM EDTA pH8.5) buffer. The DNA was visualized using Ethidium bromide stain. Table 4.2 summarizes the sequences of primers used for genotyping in this project.

Table 4.1 The PCR reaction setting

Temperature	**95°C**	**85°C**	**94°C**	**(A*-5)°C**	**72°C**	**72°C**	**4°C**
Time	10 min	Add primers and Taq mixture	30sec	30sec	30sec	10 min	Infinity
No. of cycles	1	1	30	30	30	1	

A* - denotes the calculated annealing temperature based on the sequence of primers

Table 4.2 List of primers used for genotyping

Primer Name	**Sequence**
RTEL1$^{F/F}$ forward primer	AGG TAG GCT CTG CCA TTG TG
RTEL1$^{F/F}$ reverse primer	GGA GGT GGA GTG AAG CAG AG
RTEL1 mut forward primer	TGT GTT TCT AGC CTC TGC AGC T
RTEL1 mut reverse primer	GGG ACA GGG ATA AGT ATG ACA TCA
RTEL1CreERT2 forward primer	CTA TCA ACT CGC GCC CTG GAA
RTEL1CreERT2 reverse primer	CTA TCA ACT CGC GCC CTG GAA
Ah-Cre forward primer	AGG ATA TTC ATT CCC TCA CCC TCA G
Ah-Cre reverse primer	TCA GGT TCT GCG GGA AAC CAT TTC
ROSA26-LacZ wt forward primer	GGA GCG GGA GAA ATG GAT ATG

ROSA26-LacZ wt reverse primer	AAA GTC GCT CTG AGT TGT TAT
ROSA26-LacZ mut forward primer	AAA GTC GCT CTG AGT TGT TAT CAG T
ROSA26-LacZ mut reverse primer	TAA AGC GCA TGC TCC AGA CTG
$P53^{F/F}$forward primer	GGT TAA ACC CAG CTT GAC CA
$P53^{F/F}$ reverse primer	GGA GGC AGA GAC AGT TGG AG
P53 mut forward primer	TAT ACT CAG AGC CGG CCT
P53 mut reverse primer	ACA GCG TGG TGG TAC CTT AT
P53 forward primer	TAT ACT CAG AGC CGG CCT
P53 reverse primer	CTA TCA GGA CAT AGC GTT GG

4.4 Induction of Cre activity by tamoxifen

Tamoxifen (provided by Sigma) was dissolved in 100% ethanol and supplemented with 9 volumes of corn oil (Sigma) to make concentration of 50 mg/ml. To induce the Cre activity in RTEL1-CreERT2/ROSA26-LacZ mice, 2 mg of tamoxifen was given to mice by intraperitoneal (i.p.) injection. After injection, the mice were analyzed on different developmental time points.

4.5. Induction of Cre expression from Ah-Cre by β-naphthoflavone

Ah-Cre is driven by a cytochrome p450 promoter which is transcriptionally regulated by β-naphthoflavone[80]. To induce the Cre expression in mice containing Ah-Cre, mice were treated with five daily i.p. injection of 80mg/kg β-naphthoflavone (Sigma) dissolved in corn oil (8 mg/ml).

4.6. X-gal staining

X–gal staining was used to visualize the LacZ expressing cells. Bacterial LacZ gene encodes β-galactosidase enzyme which catalyzes hydrolysis of β-galactoside into monosaccharides. X-gal is an organic compound (5-bromo-4chloro-3 indolyl- β-galactopyranoside) which can be cleaved by LacZ into galacatose and 5-bromo-4-chloro-3-

hydroxyindole, this second compound is further oxidized to an indigo blue coloured insoluble compound which is easily visible. For X gal staining, the dissected intestine samples were fixed at 4^{o}C for 2 hours using 4% formaldehyde (Fisher), 0.2% glutaraldehyde (Sigma) and 0.02% NP-40 (Sigma) in PBS. Fixed tissues were washed 3 times with PBS containing 0.02% NP-40 and incubated overnight at room temperature in a solution containing 5mM $K_4FE(CN)_6$, 5mM $K_3FE(CN)_6$, 2mM $MgCl_2$ and 1mg/ml of X-Gal (Invitrogen) in PBS (Courtesy: Dr. Hao Ding).

4.7. Mouse Irradiation

Two months old wild type and RTEL1$^{F/-}$/Ah-Cre mice were exposed to whole body γ-irradiation with a sub-lethal dose of 12 Grey using 137Cs sources (provided at CancerCare Manitoba). On day 3 following irradiation, the mice were sacrificed and intestine was collected for histological analysis.

4.8 Histology

4.8.1 Intestine sample collection and processing

Mouse small intestine is around 47 cm long, and can be divided into three segments, duodenum, jejunum and ileum. The size and number of villi in these segments are different. Therefore, in order to make a comparison among the samples in our analysis, a region of jejunum, which is 10 cm away from the opening of duodenum, was collected from each mouse for histological examination.

Dissected intestinal tissues were fixed in 10% buffered formalin (Fischer Scientific) at room temperature for overnight by keeping on a horizontal shaking platform. The fixed tissues were then washed with PBS and aligned horizontally in plastic moulds using Histogel melted at 75°C. These moulds were solidified and placed in Citadel 1000 (Thermo Scientific) automated tissue-processor for processing. Following processing, the tissues were embedded in paraffin

using Histocentre 3 (Thermo Scientific) embedding machine. The paraffin blocks were placed at 4^{o}C until further use.

Tissue sections of 5µm thickness were cut using a rotatory microtome (Leica) and mounted on super frost slides for normal staining and super frost plus slide (Fischer Scientific) for immunohistochemistry (Courtesy: Xiaoli Wu).

4.8.2 Haematoxylin and Eosin (H&E) staining

Mounted slides containing the specimen were deparaffinised by immersing in xylene, initially for 15 min, and then followed by two additional incubation of 5 min duration. The specimens were rehydrated by three changes in a series (90 and 70%) of absolute ethanol for 2 min each and rinsed under running tap water for 5 min which was followed by a distilled water wash for 5 min. Sections were then stained in Harris haematoxylin (Sigma) for 10 min in the dark. The specimens were then rinsed under running tap water to wash off excess stain. In order to differentiate nuclear structures, specimens were differentiated in acid alcohol (0.5% HCl in 70% glycerol), washed with running water for 5 min, dipped in 70% and 90% ethanol respectively for 2 min and then stained with Eosin (Sigma) for 1 min. Excess stain was again washed off by rinsing the specimens under running water. The specimens were dehydrated by 10 dips in absolute ethanol followed by 3 dips in Xylene for 2 min each and finally embedded in Permount (Fischer Scientific) mounting medium.

4.8.3 Nuclear Fast Red Staining

Nuclear fast red stain was used to counter staining X-gal stained sections. The sections were first deparaffinised and rehydrated, and washed as mentioned before. The sections were then immersed in Vector^{R} Nuclear Fast Red counter-staining solution (Vector) for 1-5 min based on the

desired intensity. The sections were rinsed under running water to wash off excess stain. The sections were dehydrated in absolute ethanol and mounted with Permount as mentioned above.

4.8.4 Alcian Blue staining

In order to stain the Goblet epithelial cells, Alcian Blue staining was performed. The sections were deparaffinised, rehydrated and washed as mentioned above. The sections were then stained with Alcian Blue solution (1% Alcian Blue 8GX (Sigma) in 3% acetic acid pH 2.5) for 15 min at room temperature. The sections were then rinsed under running water and distilled water for 5 min each and then subjected to counterstain in Nuclear Fast Red Stain for 1 min. Sections were washed, dehydrated, cleared and mounted in permount as mentioned earlier.

4.8.5 Immunohistochemistry (IHC)

The tissue sections were dried overnight at 45°C, and then de-paraffinised, rehydrated and washed as mentioned previously. Antigen retrieval was performed by incubating the sections in a solution containing 0.01 M Sodium Citrate and 0.05% tween-20 (pH 6.1) at 95°C. The sections were then placed in de-cloaking system (BioCare) for 30-40 min. After this treatment, the sections were brought to room temperature and rinsed in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6). Following wash, the sections were incubated with Avidin and Biotin (Vector) for 20 min each with a rinse with TBS in between. The sections were blocked with mouse IgG blocking reagent (Vector) or serum free blocking reagent (Dako) for 1-2 hours at room temperature followed by a rinse with 1X TBS (Dako). The primary antibody (listed in table 4.3) incubation was done overnight at 4°C. After incubation, the sections were washed twice in TBST buffer for 10 min each. The endogenous peroxidase was quenched by incubating the sections in 3% H_2O_2 for 5 min. The sections were washed in TBST and then incubated with secondary antibody for 30 min at room temperature. Following incubation, the

sections were washed in TBST solution for half an hour and the secondary antibody was detected by using DAB (Vector) solution for 5 min as per manufacturer's protocol. The stained sections were rinsed in running tap water and counterstained with Mayer's Haematoxylin (Sigma). The sections were then differentiated in 37 mM Ammonium Hydroxide solution and rinsed in running water followed by dehydration, clearing and mounting as mentioned earlier.

Table 4.3 List of primary antibodies used in the study

Protein	Host Species	Dilution	Commercial Source
γ-H2AX	Mouse	**1:1000**	Abcam
Ki67	Human	**1:50**	BD Pharminogen
β-catenin	Mouse	**1:200**	BD

4.9 In-situ Hybridization with mouse Olfm4 RNA probe

Since Olfm4 is the specific marker for intestinal stem cells[84], we used this marker to detect intestinal stem cells in RTEL1 conditional knockout mice. So far, there is no anti-Olfm4 antibody suitable for IHC. Therefore, in situ hybridization is the only reliable assay for detecting Olfm4 expression on mouse tissues.

In order to perform in situ hybridization, we first used RT-PCR method to amplify the full length of mouse Olfm4 cDNA from total RNA prepared from mouse ES cells. This PCR product was then cloned into T/A cloning vector, pGEM-T-easy. After linearization with Spe1 or BamH1, this cloned vector was used for in vitro transcription to make sense and antisense digoxigenin-labeled RNA probes by using T7 or Sp6 polymerases (Figure 4.7).

In situ hybridization was performed under RNase-free condition by DEPC treatment or baking at 200°C overnight. The tissue sections were de-waxed, rehydrated and rinsed twice with DEPC treated ddH_2O. The sections were then treated with HCl (0.2N) for 15 min at 37°C and subjected to Proteinase K (30µg/ml in PBS) treatment for 20 minutes at 37°C. Following Proteinase treatment, the sections were rinsed in freshly prepared Glycine (0.2%) for 1 min and washed twice in PBS for 1 min each. The sections were post-fixed in 4% paraformaldehyde (PFA) in PBS at room temperature, rinsed in PBS three times (1 min each) and then incubated in freshly prepared acetic anhydride solution twice for 5 min duration. The sections were then washed 5 times in PBS (~ 1 min each), rinsed with 1x SSC for 1 min at room temperature and then excess solution was removed from the sections and placed in a humidified chamber after immersing in 1xSSC containing 50% formamide solution. Sections were incubated at 65°C for 1 hour and then the solution was replaced with fresh hybridization solution containing digoxigenin labelled probe and incubated at 70°C for 72 hours. The sections were rinsed twice in SSC and thrice in SSC/50% formamide buffer at 60°C for 20 min each. The sections were then washed 5 times in Tris/NaCl buffer (0.1 M Tris·Cl, 0.15 M NaCl, 0.1 % (v/v) Tween 20, pH 7.5) for 1 min each at room temperature. Excess solution was drained and the sections were blocked for 30 min at room temperature followed by incubation with sheep anti-digoxigenin antibody over-night at 4°C. The sections were repeatedly washed using Tris/NaCl buffer and NTM buffer (0.1 M Tris·Cl, 0.1 M NaCl, 0.05 M MgCl2 pH 9.5). The NBT/BCIP working solution was then added to sections as per manufacturer's protocol and incubated for 24 hours at room temperature in dark. The sections were washed twice with PBS for 1 min at room temperature, followed by dehydration and finally mounted for analysis (Courtesy: Xiaoli Wu).

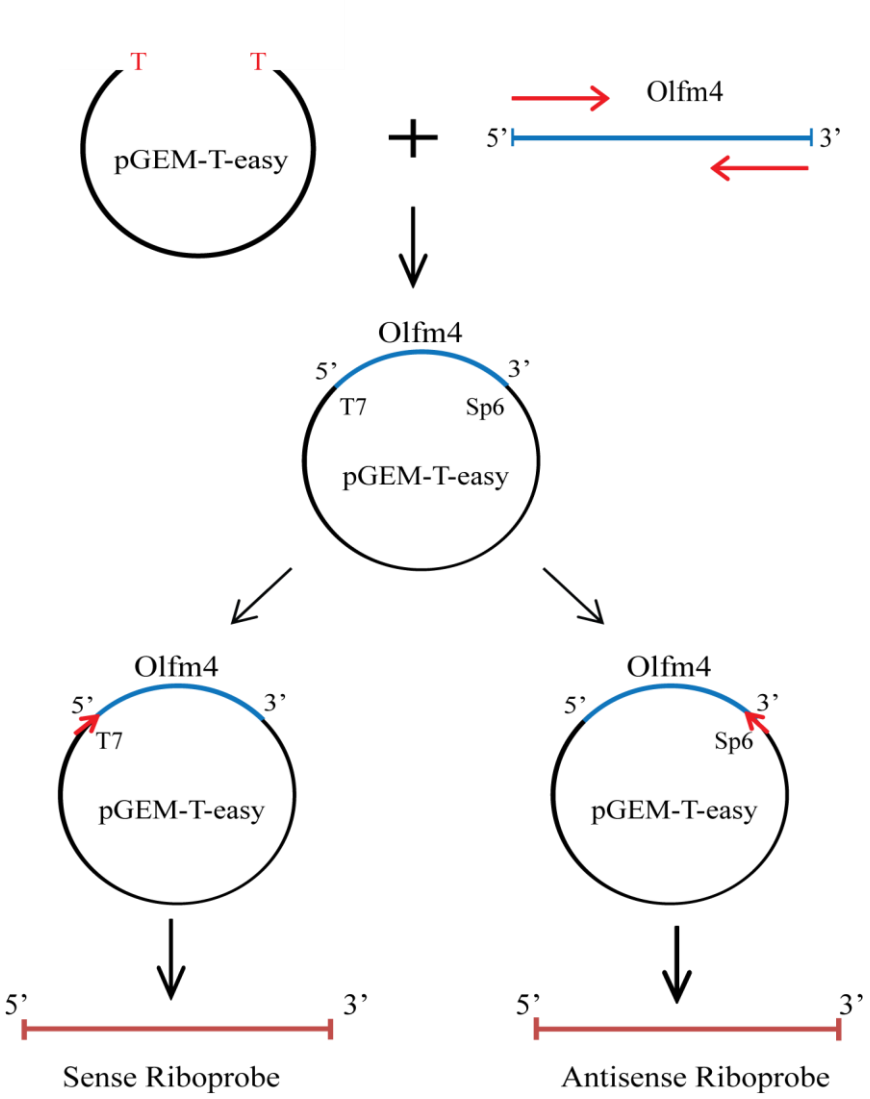

Figure 4.6 Schematic representation of generating Sense and Anti-sense riboprobe for mouse Olfm4. Mouse Olfm4 cDNA was amplified by RT-PCR and then ligated to pGEM-T-easy vector. This vector was linealized with BamH1 to generate anti-sense riboprobe by using Sp6 polymerase. It was also linealized with Spe1 to generate sense riboprobe by T7 polymerase.

4.10 Statistics

All statistical graphs were created using Prism software (Graphpad Software, Inc. San Diego, CA). While comparing the groups comprising of $RTEL1^{F/+}$ and $RTEL1^{F/-}$/Ah-Cre, we obtained graphs that measured the effect of RTEL1 deficiency on intestinal homeostasis and regeneration by using unpaired t-Test (prism software) with a p value of statistical significance.

Chapter 5: Results

5.1 To determine whether RTEL1-expressing cells can function as intestinal stem cells

5.1.1 Rationale

Since RTEL1 expression is specifically detected in the intestinal crypts where the intestinal stem/progenitor cells are located (see Figure 1.12), we reasoned that some of RTEL1-expressing cells in the crypts could function as intestinal stem cells. As discussed in my introduction, intestinal stem cells are the cells that can proliferate, self-renew and differentiate into all the epithelial cell types for the formation of intestinal villus. This process can be visualized by a newly developed technology, namely transgenic lineage tracing assay[85,86]. In this assay, an inducible Cre (CreERT2) is applied to pulse-label the potential intestinal stem cells in mice with a reporter, such as LacZ or a fluorescent marker (GFP or YFP etc). The labelled cells can be chased for the progenies they produce at different developmental stages. If the labelled cell is stem cell, it will produce progeny cells which can be detected at the late developmental time points based on the reporter gene inherited from stem cells. The presence of the reporter gene in all the differentiated descendant cell lineages will prove the multi-potent nature of intestinal stem cell. The long-time persistence of the reporter gene among the cell lineages will indicate the self-renewal capacity of intestinal stem cells. Any candidate cell demonstrating both multi-potency and self-renewal will fulfil the minimal definition of being an intestinal stem cell. However, if the labelled cells are the progenitors or differentiated cells, the labelled cells will be "washed out" (due to their short half-life) at the late developmental stages. Therefore, this transgenic lineage tracing assay is a powerful method, not only for identifying the intestinal stem cells *in vivo*, but also for demonstrating the capacity for self-renewal and differentiation of these stem cells during intestinal homeostasis.

Based on this rationale, we decided to use a transgenic lineage tracing assay to determine whether RTEL1-expressing cells can function as intestinal stem cells. We first generated a transgenic mouse strain, namely RTEL1-CreERT2, in which a tamoxifen-inducible Cre expression cassette (CreERT2) was specifically knocked into mouse RTEL1 genomic locus (see Figure 4.1 in the section of Material and Methods). Therefore, the expression of CreERT2 in this transgenic mouse strain will be tightly controlled by the RTEL1 regulatory elements. To apply RTEL1-CreERT2 mice for tracing RTEL1 cells *in vivo*, we further bred these mice with ROSA26-LacZ mice to generate RTEL1-CreERT2/ROSA26-LacZ. In the absence of tamoxifen, the CreERT2 protein expressed in these mice will remain in the cytoplasm and degraded by the Heat Shock Protein (HSP) 90 complex. Upon administration of tamoxifen to the RTEL1-CreERT2/ROSA26-LacZ mice, the CreERT2 protein will be transported to the nucleus where the Cre activity will remove the loxP flanked pGK-neo (STOP) cassette from ROSA26 locus. This recombination will turn on LacZ expression in RTEL1-expressing cells (Figure 5.1). The LacZ-labeled RTEL1-expressing cells can be chased at different developmental time points to determine whether RTEL1-expressing cells have the capacity of self renew and differentiate to the progeny cells required for intestinal homeostasis.

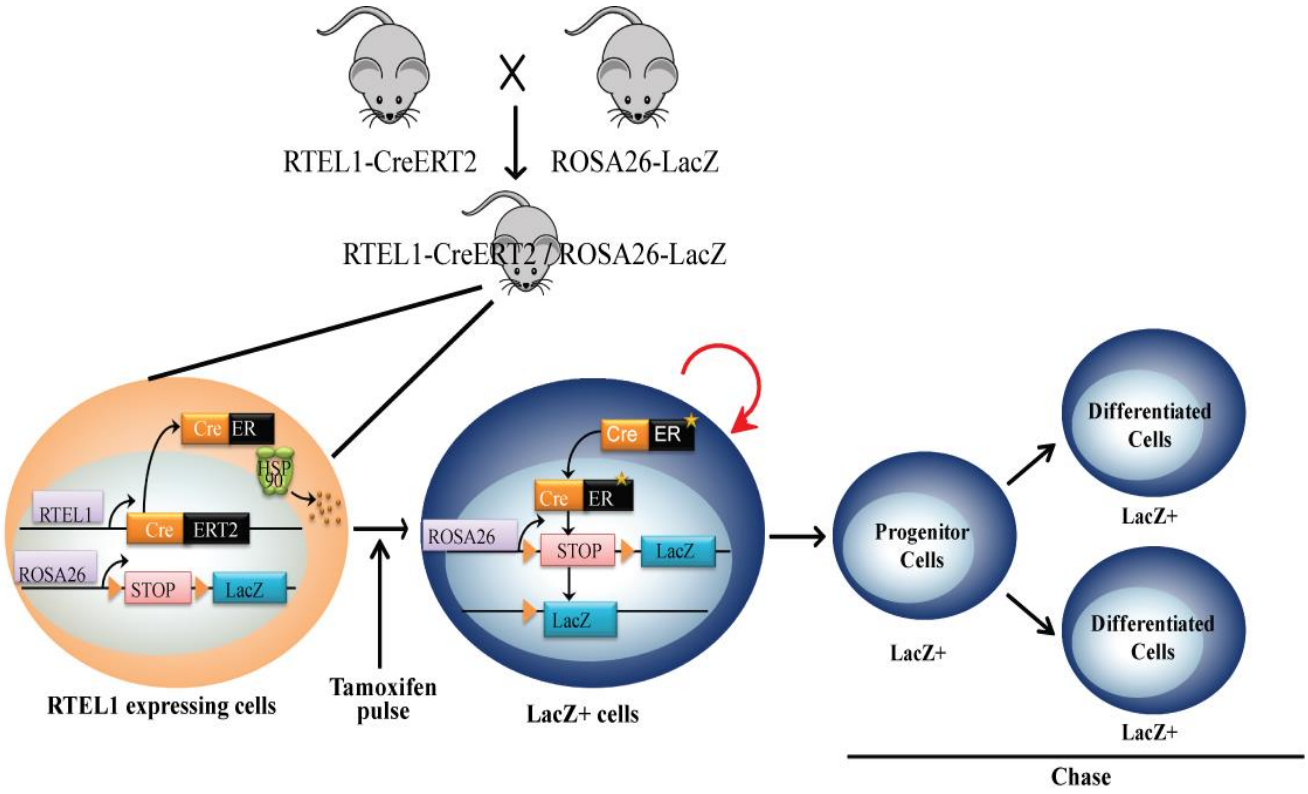

Figure 5.1 Schematic representation of transgenic lineage-tracing assay used for tracing RTEL1-expressing cells *in vivo*. In this assay, we crossed RTEL1-CreERT2 mice with ROSA26-LacZ reporter mice. In the resultant mice, RTEL1-CreERT2/ROSA26, only RTEL1-expressing cells will express CreERT2, which, in the absence of tamoxifen, will be degraded by Heat Shock Protein (HSP) 90 complex. However, by the treatment of these mice with tamoxifen, the expressed CreERT2 protein is able to escape HSP90-mediated degradation and transports into the nucleus and turn on LacZ expression by the excision of loxP flanked pGK-neo (STOP) cassette. Therefore, this transgenic method allows to permanently label RTEL1-expressing cells with LacZ by a pulse of tamoxifen. The developmental fate of these labelled cells can be chased at late developmental stages.

5.1.2 Results

In order to trace RTEL1-expressing cells in intestinal crypts, RTEL1-CreERT2/ROSA26-LacZ mice were treated with tamoxifen (2mg i.p. injection) to pulse-label RTEL1-expressing cells with LacZ. The treated mice were analyzed at day 1, 2 months and 12 months post injection (3 mice at each time point) (Figure 5.2).

As shown in Figure 5.3, some single LacZ+ cells were detected in the crypts of RTEL1-CreERT2/ROSA26-LacZ mice one day after tamoxifen induction. The majority (around 75%) of LacZ+ cells were found to be either located at +4 cell position (Figure 5.3A) or the columnar cells between the Paneth cells at the base of crypts (Figure 5.3A). Both of these cells have recently been demonstrated to function as intestinal stem cell[52,87,88]. Around 25% LacZ stained RTEL1-expressing cells were also found to be located above +4 position in crypts (Figure 5.3A), suggesting that RTEL1-expressing cells within intestinal crypts could also be the transit amplifying progenitor cells.

To demonstrate that the labeled RTEL1-expressing cells can function as intestinal stem cells, we chased the LacZ+ cells at different developmental time points. 2 months after tamoxifen induction, LacZ+ cells were found to occupy the whole villus, indicating that the labeled RTEL1-expressing cells can differentiate to all the epithelial lineages of intestine. The LacZ stained villi were also found at very late developmental stages, such as one year after pulse-labeling, strongly indicating that the labeled RTEL1-expressing cells can self-renew and continuously supply the progeny cells for intestine.

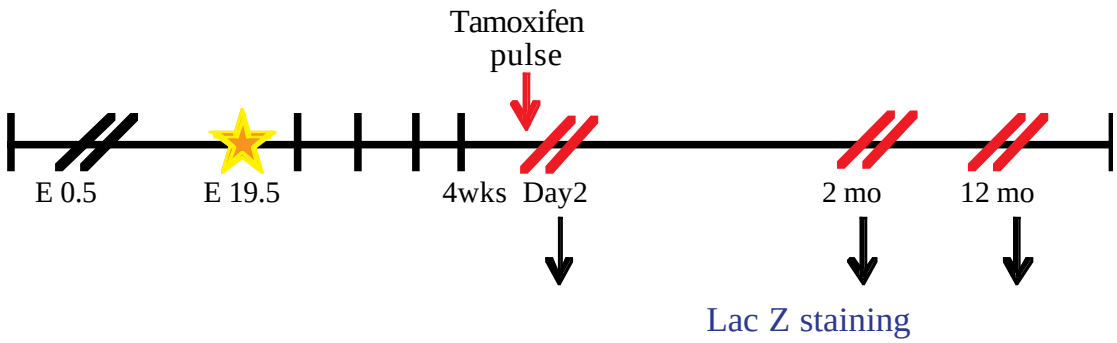

Figure 5.2 Experimental outline for tracing RTEL1-expressing cells in intestine. A total of 9 six weeks old RTEL1-CreERT2/ROSA26-LacZ mice were treated with tamoxifen (2 mg, i.p. injection), and then sacrificed at day 1, 2 and 12 months (mo) post tamoxifen treatment for LacZ staining.

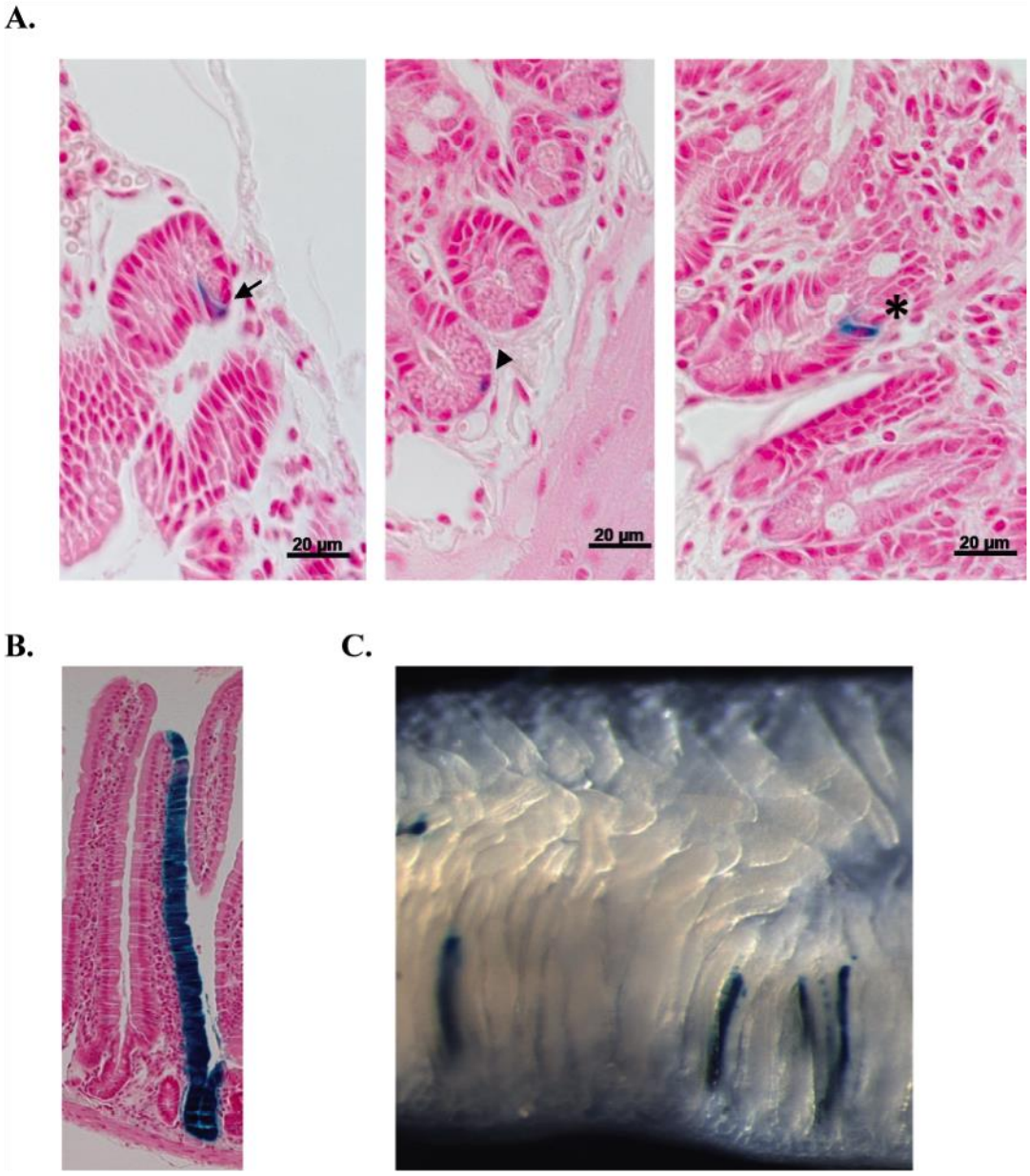

Figure 5.3 The developmental fate of RTEL1-expressing cells in mouse intestine. (**A**) Day 1 post tamoxifen induction. LacZ+ cells were detected either at +4 (indicated by arrow) or between Paneth cells (indicated by arrowhead) at the base of crypts where the intestinal stem cells are located. Some of LacZ positive cells were also found in the transit amplifying progenitor cell region (indicated by asterisk). (**B**) 2 months post tamoxifen induction. LacZ+ cells were found to occupy the whole villus. (**C**) 12 months after tamoxifen induction. LacZ+ cell were still presented in the whole villus as demonstrated by the whole mount LacZ staining of intestine.

5.1.3 Summary

In this part of my thesis, I have applied a transgenic lineage assay to demonstrate that RTEL1-expressing cells are either located at '+4' cell position or between the Paneth cells at the base of crypts. Since both regions have been clearly shown to contain intestinal stem cells[89,90], this finding highly indicate that RTEL1-expressing cells can function as intestinal stem cells. Indeed, by tracing the fate of RTEL1-expressing cells, I further showed that RTEL1-expressing cells can self-renew and differentiate into all the epithelial lineages required for intestinal homeostasis. These are the most important characteristics for stem cells. Based on this, we conclude that RTEL1-expressing cells within intestinal crypts can function as intestinal stem cells.

In this part of study, I also showed that RTEL1-expressing cells within intestinal crypts could also be transit amplifying progenitor cells for intestine. This indicates that RTEL1 could play an important function for the maintenance of intestinal stem and progenitor cells during postnatal development.

5.2 To establish a mouse model for studying the role of RTEL1 in the maintenance of intestinal stem/progenitor cells

5.2.1 Rationale

My above study indicates that RTEL1 can specifically express in intestinal stem/progenitor cells. This raises an interesting question: what does RTEL1 do for these cells? Given the critical role of RTEL1 in the maintenance of genomic integrity, we hypothesized that RTEL1 could be required for protecting these cells from genomic instability.

To address this question, we decided to use a mouse model. This is largely because mouse share many similarities with human in the aspects of genome organization as well as in physiological function. In addition, mouse models, specifically with loss of gene function, have been shown to be the most informative genetic tools for demonstrating a gene's function[91]. However, RTEL1 knockout mice are embryonic lethal[1], preventing analysis of loss of RTEL1 function in adult mice. Therefore, in order to address the role of RTEL1 in intestinal stem/progenitor cells, we need to establish a conditional knockout approach that allows knocking out RTEL1 in a cell-specific manner. Since our RTEL1 conditional knockout allele was generated based on Cre/loxP system[37], thus, the success of using this mouse allele for conditional knockout of RTEL1 in intestinal stem/progenitor cells will be totally dependent on the Cre transgenic mice which should not only display a specific Cre activity, but can also highly express Cre in these cells.

It has been demonstrated that Ah-Cre transgenic mice could display high Cre activity in intestinal epithelial cells, including the crypt cells. In addition, Ah-Cre can be induced by the treatment of β-naphthoflavone, allowing knocking out a gene's function in the intestine of adult mice[80]. These two properties make this transgenic Cre attractive for specifically knocking out a

gene's function in intestinal stem/progenitor cells[92]. Therefore, we chose Ah-Cre transgenic mice for our conditional knockout study. However, before performing this conditional knockout study, we need to verify the recombination efficiency of Ah-Cre in intestinal stem/progenitor cells.

5.2.2 Results

To determine whether Ah-Cre transgenic mice could display high Cre activity in intestinal stem/progenitor cells, we crossed Ah-Cre with ROSA26-LacZ reporter mice. The Cre activity in Ah-Cre/ROSA26-LacZ mice was induced by i.p. injection of 2 mg β-napthoflavone (once per day for five successive days). This induced Cre activity will then turn on LacZ expression that can be visualized by X-gal staining.

As shown in Figure 5.4, both small intestine and colon displayed specific LacZ signals in the crypts. By counting the stained crypts in 10 different circumferences of small intestine and colon, we found that more than 95% crypts were stained with LacZ. In the stained crypts, most crypt cells showed LacZ+. This data clearly indicates that Ah-Cre mice have high Cre activity in the crypts where the intestinal stem/progenitor cells are located.

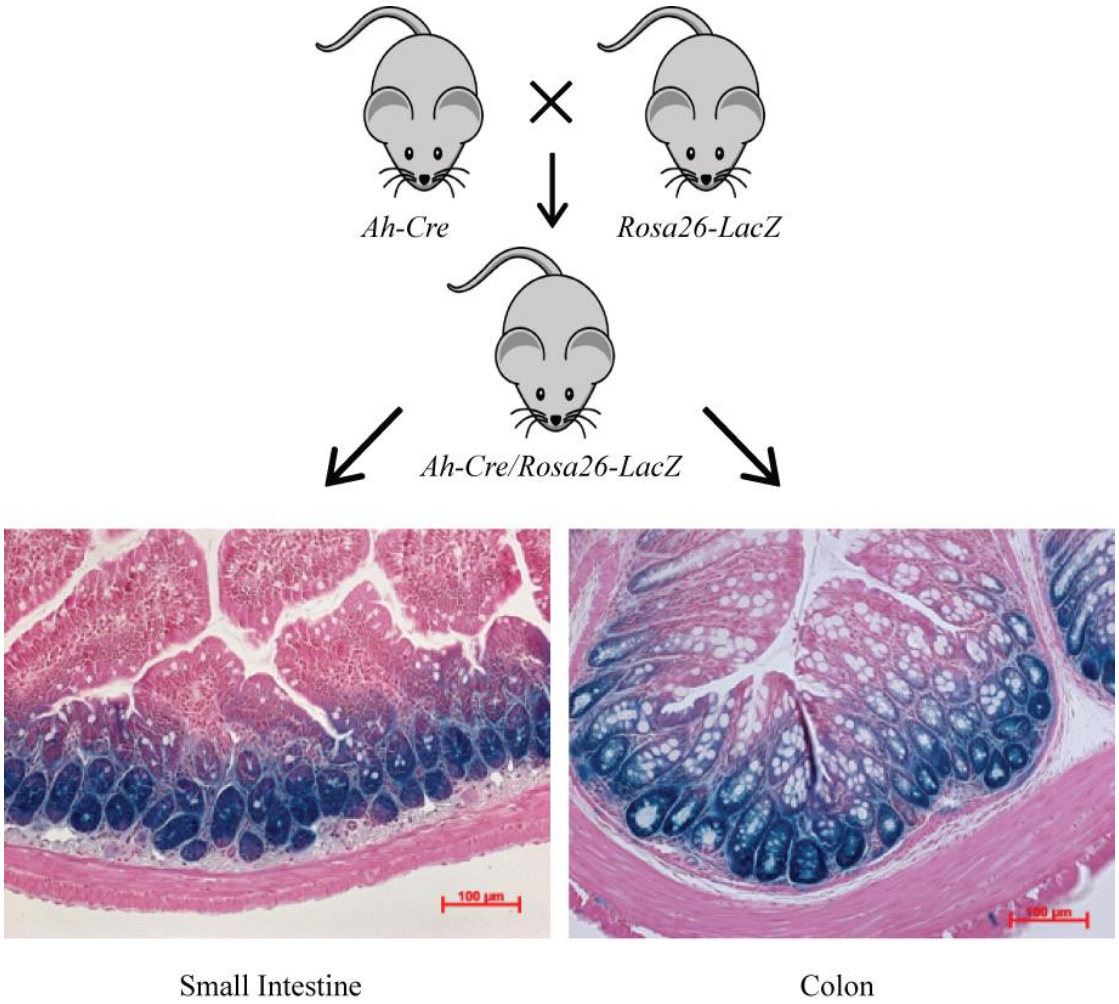

Figure 5.4 Ah-Cre transgenic mice display high Cre activity in intestinal and colonic crypts. Ah-Cre/ROSA26-LacZ mice were treated with β-naphthoflavone, and the induced Cre activity in these mice was determined by X-gal staining. LacZ signals were found to be specifically located in the crypts of small intestine and colon where the stem/progenitor cells are located.

5.2.3 Summary

This part of characterization allowed us to identify a Cre transgenic mouse strain with high Cre activity in intestinal stem/progenitor cells. This high recombination efficiency is critical for using a conditional knockout approach to address the role of RTEL1 in intestinal stem/progenitor cells. However, although our finding is consistent with several publications indicating that Ah-Cre transgenic mouse could be the best transgenic tool for intestinal

stem/progenitor cell analysis, we still do not know the number of stem/progenitors that could display Cre activity. From our data, as well as others[80], around 90% intestinal stem/progenitor cells are expected to display Cre activity in Ah-Cre mice following the treatment with β-naphthoflavone. This efficiency should be able to characterize loss of RTEL1 function on intestinal stem/progenitor cells.

5.3 To characterize the phenotypes of mouse model with RTEL1 deficiency in intestinal stem/progenitor cells

5.3.1 Rationale

As discussed above, a conditional knockout mouse model with a loss of RTEL1 function in intestinal stem/progenitor cells will be important for determining the role of RTEL1 in these cells. Since we found that Ah-Cre transgenic mice can display high Cre activity in the crypts where intestinal stem/progenitor cells are located, we used this Cre line to generate $RTEL1^{F/-}$/Ah-Cre mice (see Figure 4.4 in the section of Material and Methods) for knocking out RTEL1 in these cells. Based on the essential role of RTEL1 in the maintenance of genomic stability, we predict that loss of RTEL1 function in intestinal stem cells will induce the genomic instability, which could affect the survival of these cells in intestine. Therefore, with the established $RTEL1^{F/-}$/Ah-Cre mice, the first question that we would like to ask is whether RTEL1 deficiency could reduce the number of intestinal stem cells. If so, could this decreased number of intestinal stem/progenitor cells affect intestinal homeostasis and regeneration?

Since mouse small intestine is around 47 cm long and the cellular organization in each segment of mouse intestine is different. To minimize this problem, we set up a procedure to collect the same region of small intestine for analysis (Figure 5.5). This region is located in the Jejunum of small intestine, 10 cm away from the opening of Duodenum. A series of serial sections prepared from this region were used for H&E staining and other marker analyses. Since many published studies also used Jejunum for characterizing intestinal stem/progenitor cell defects[63,93,94], our sample preparation should also be able to allow us to compare our data with those published findings based on other genetic mutations in intestinal stem/progenitor cells.

5.3.2 Results

5.3.2.1 RTEL1$^{F/-}$/Ah-Cre mice showed a progressive depletion of intestinal stem cells

In order to determine whether loss of RTEL1 function could affect intestinal stem cells, we treated two months old RTEL1$^{F/-}$/Ah-Cre mice with β-naphthoflavone, and then collected intestinal tissues on day 6 and 3 months post treatment for intestinal stem cell-marker analysis. Currently, several molecular markers have been identified to specifically mark intestinal stem cells during mouse development (see section 1.5.2 in Introduction). Among them, Olfm4 has been found to be able to detect both +4 and columnar stem cells in a more sensitive manner[84]. In addition, Olfm4 has also been demonstrated to be a specific marker for identifying cancer stem cells in human colorectal cancers[81]. Therefore, Olfm4 has been considered as a reliable biomarker for intestinal stem cells. It should be noted that Olfm4 does not detect stem cells in colon[81,84]. So far, there are no genes whose expression can be reliably used for identifying colonic stem cells *in vivo*.

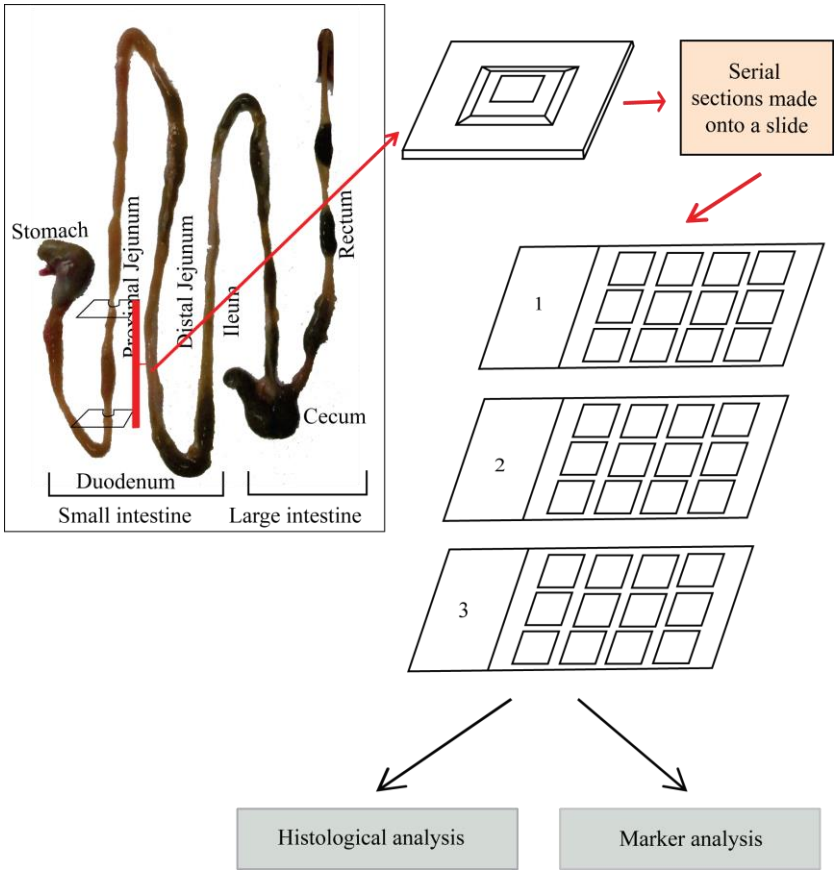

Figure 5.5 Schematic representation of intestinal tissue-collecting procedure for our RTEL1 conditional knockout study. A small portion of Jejunum tissue, 10 cm from the opening of Duodenum, was collected for preparing a serial of sections that were used for histological examination and marker analyses.

Since there is no antibody for immune-staining Olfm4 on mouse intestinal tissues, detection of Olfm4 will then relay on an RNA in situ hybridization approach. To establish this assay, we first cloned mouse Olfm4 cDNA, and then used it to generate several riboprobes for Olfm4 (antisense and sense). We found that one Olfm4 antisense probe, which was generated from the full length of Olfm4 cDNA, can strongly and specifically stain both +4 and columnar stem cells in the crypts of mouse small intestine (Figure 5.6). In addition, this Olfm4 staining pattern was evenly presented in all the crypts of small intestine, demonstrating this riboprobe can efficiently detect intestinal stem cells *in vivo*.

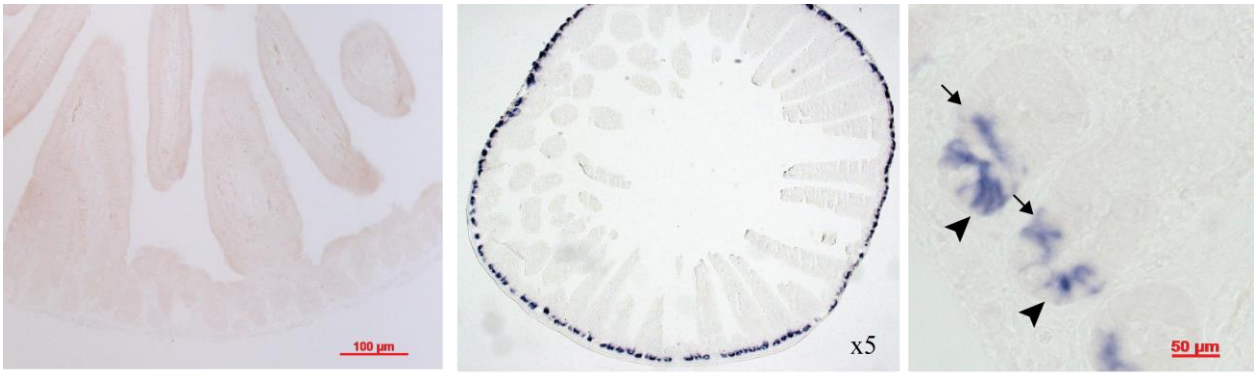

Figure 5.6 Establishment of Olfm4 in situ hybridization for detecting intestinal stem cells *in vivo*. Wild type mouse small intestine sections were hybridized with Olfm4 antisense and sense probes. Antisense probe stained both +4 cell (indicated by arrows) and the columnar cells located at the base of crypts (indicated by arrowheads) in all intestinal crypts, whereas sense probe did not detect any signals in the crypts.

Using our established in situ hybridization approach, we found only some small regions of intestine in RTEL1$^{F/-}$/Ah-Cre mice showing reduced Olfm4+ cells on day 6 post β-naphthoflavone treatment (Figure 5.7A). However, 3 months after β-naphthoflavone treatment, nearly 50% of crypts in RTEL1$^{F/-}$/Ah-Cre mice showed significantly decreased stained Olfm4 signals. Around 20% of them had only 1 or 2 Olfm4+ cell (Figure 5.7B). As control, RTEL1$^{F/+}$ mice with β-naphthoflavone treatment did not show any changes for the Olfm4 staining at these two different time points, indicating that RTEL1 deficiency could specifically affect the survival of intestinal stem cells, resulting in progressively decrease intestinal stem cells *in vivo*.

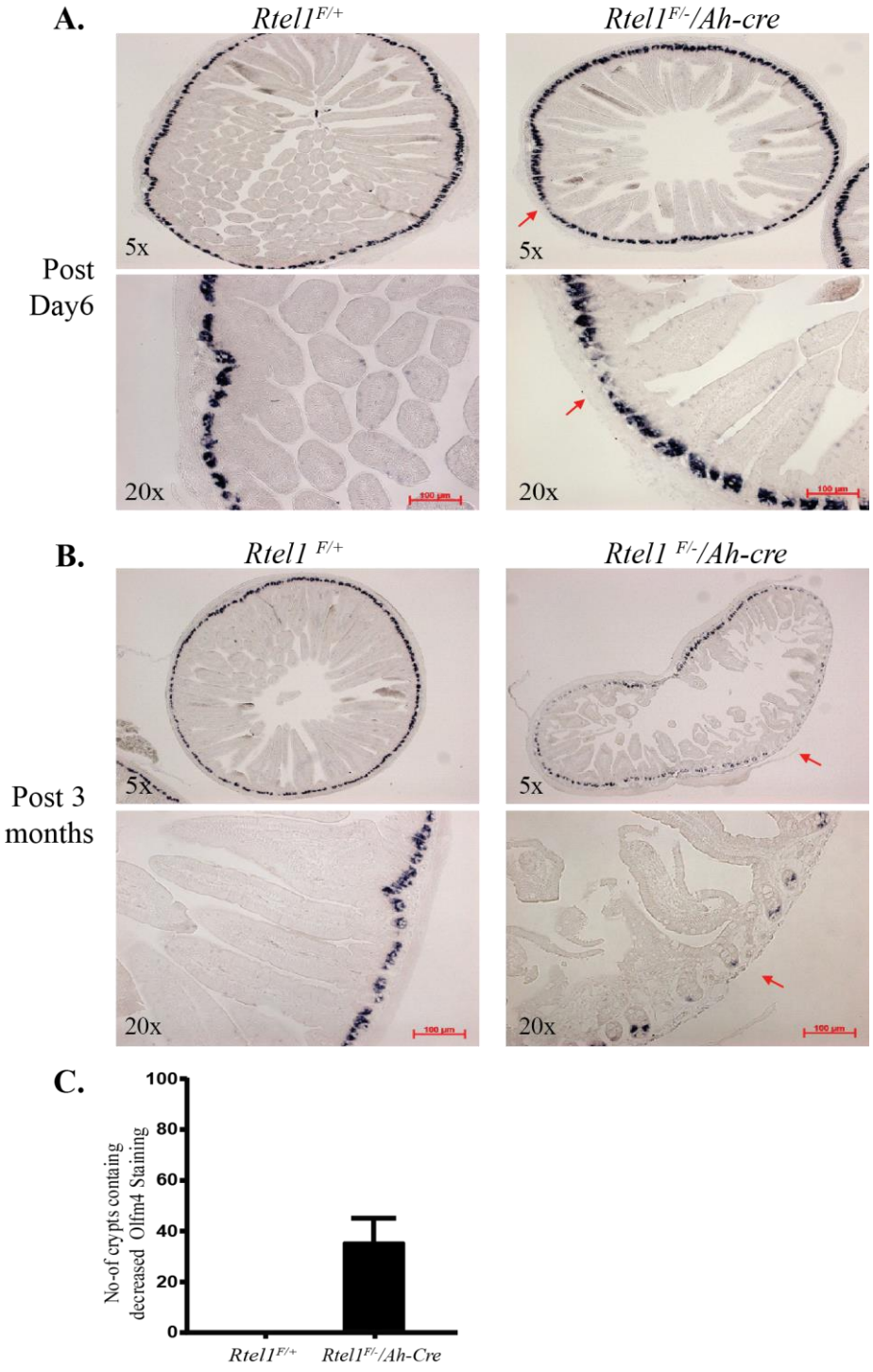

Figure 5.7 Reduction of intestinal stem cells in RTEL1$^{F/-}$/Ah-Cre mice. (**A**) Day 6 post β-naphthoflavone treatment, only some small regions of intestine in RTEL1$^{F/-}$/Ah-Cre mice showed decreased Olfm4+ cells (marked by arrowhead) which was not observed in RTEL1$^{F/+}$ control mice. (**B**) 3 months after β-naphthoflavone treatment, many crypts in RTEL1$^{F/-}$/Ah-Cre mice had decreased Olfm4 stained cells as compared to control. Some of them had only 1 or 2 cells positive for Olfm4 (indicated by arrows). (**C**) Number of crypts that contain significantly decreased intestinal stem cells in RTEL1$^{F/-}$/Ah-Cre small intestine 3 months after β-naphthoflavone treatment as compared to control (RTEL1$^{F/+}$).

5.3.2.2 RTEL1$^{F/-}$/Ah-Cre mice showed defective intestinal homeostasis

Since intestinal stem cells can produce all the epithelial cell lineages required for intestinal homeostasis[53,59], we then asked: could decreased intestinal stem cells in RTEL1$^{F/-}$/Ah-Cre mice affect this physiological function? To address this, we first applied histology to examine the morphology of villi in both RTEL1$^{F/-}$/Ah-Cre and control mice after the treatment with β-naphthoflavone at day 6 and 3 months time points. At day 6 post treatment, no significant changes of morphology of small intestinal villi were found between RTEL1$^{F/-}$/Ah-Cre and control mice (Figure 5.8A). However, at 3 months post treatment, a significant villus shortening was found in RTEL1$^{F/-}$/Ah-Cre as compared to the control (Figure 5.8B and D). Many of the shorted villi were also found to be associated with decreased Olfm4 signals in the crypts (see Figure 5.7B). These data suggest that decreased intestinal stem cells in RTEL1$^{F/-}$/Ah-Cre mice could impair the growth of villus.

To determine whether loss of RTEL1 function could affect the differentiation of intestinal epithelial cells, we used Alcian blue to stain goblet cells and Paneth cells in small intestine collected from RTEL1$^{F/-}$/Ah-Cre and control mice 3 months post β-naphthoflavone treatment. We found that there were no significant change for these two major types of intestinal epithelial cells between RTEL1$^{F/-}$/Ah-Cre and its control littermates (Figure 5.9A). This finding indicates that RTEL1$^{F/-}$/Ah-Cre mice could still have the capacity for intestinal differentiation, which may be contributed by the unaffected Olfm4+ intestinal stem cells in these mutant mice. Future studies on using an *in vitro* organoid culture system with derived intestinal stem cells with 100% RTEL1 knockout should be able to more precisely address the role of RTEL1 deficiency on the differentiation of intestinal stem cells.

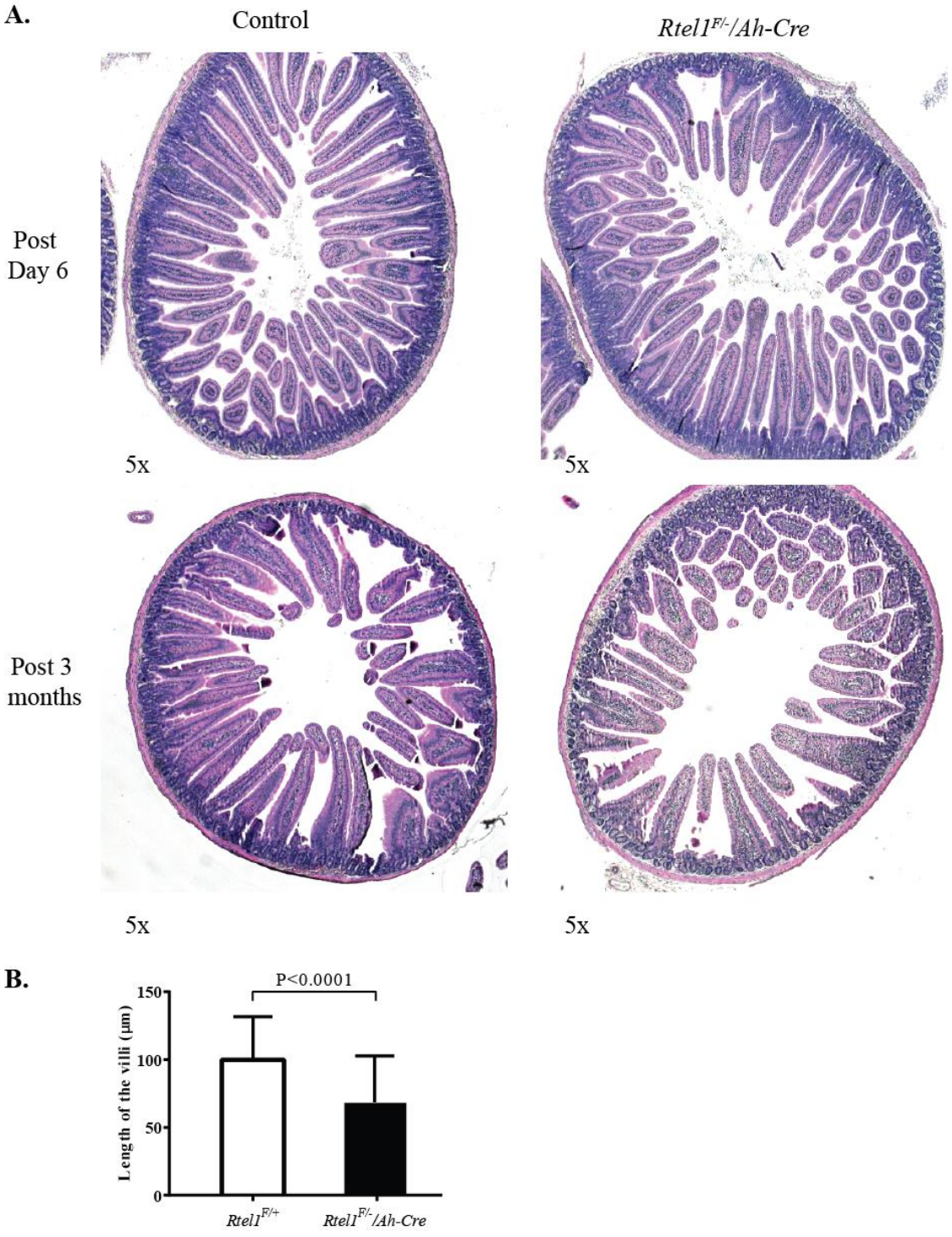

Figure 5.8 Effect of RTEL1 deficiency on intestinal homeostasis. (**A**) H&E staining of small intestine collected from RTEL1$^{F/-}$/Ah-Cre and control mice 6 days and 3 months post β-naphthoflavone treatment. A significant villus shortening was only found in RTEL1$^{F/-}$/Ah-Cre as compared to the control A significant villus length was found RTEL1$^{F/-}$/Ah-Cre 3 months post β-naphthoflavone treatment. (**B**) Summary of the villus length between from RTEL1$^{F/-}$/Ah-Cre and control mice 3 months post β-naphthoflavone treatment.

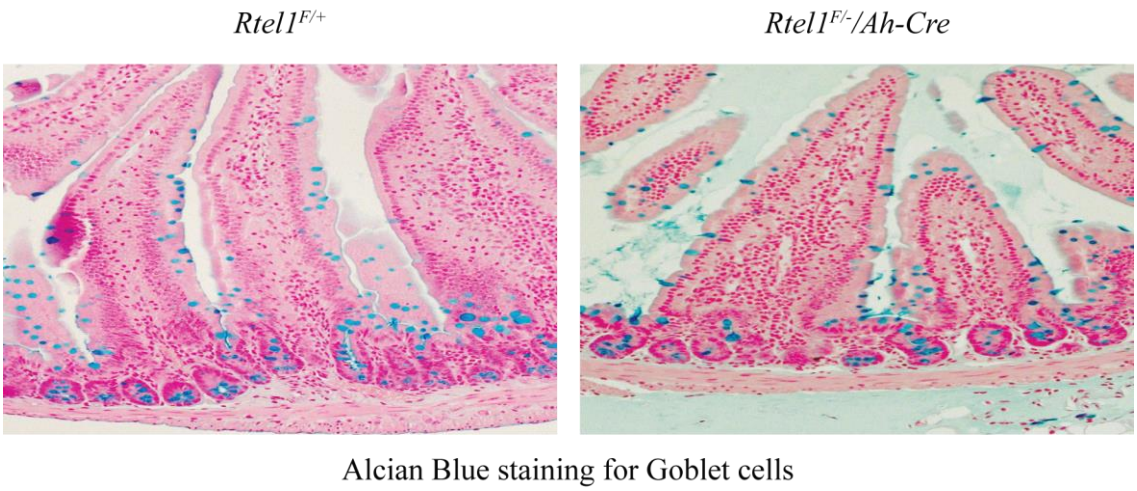

Figure 5.9 Effect of RTEL1 deficiency on the differentiation of intestinal epithelial cells. Alcian blue staining did not reveal a significant difference of Goblet cells and Paneth cells between RTEL1$^{F/-}$/Ah-Cre and control mice 3 months post β-naphthoflavone treatment.

5.3.2.3 RTEL1$^{F/-}$/Ah-Cre mice showed decreased intestinal regeneration capacity

Since intestinal stem cells are also required for intestinal regeneration after surgically removal of intestine or intestine damage caused by chemicals and irradiation[77,95,96], we expect that decreased number of intestinal stem cells as observed in RTEL1 conditional knockout mice could also affect the capacity of intestinal regeneration. To determine this, we applied a well-defined intestinal regeneration model. In this model, mice are treated with a sub-lethal dose of γ-irradiation (12Gy) to ablate the most crypt cells, including intestinal stem cells. Under normal circumstance, the damaged crypts and villi will be rapidly regenerated, which can be seen on day 3 post irradiation[95].

Using this assay, we found that RTEL1$^{F/-}$/Ah-Cre mice (3 months post β-naphthoflavone treatment) showed a significant decrease in the number of newly generated crypts 3 days following irradiation as compared to the control group (Figure 5.10). Instead, the degenerated crypts were filled in with fibroblast or other mesenchymal cells. By immunostaining with a cell proliferation marker Ki67, we also found that a significant reduction of proliferation cells in the crypts of RTEL1$^{F/-}$/Ah-Cre mice (Figure 5.10). Therefore, this irradiation based regeneration assay suggests that RTEL1 deficiency can decrease the capacity of intestinal regeneration.

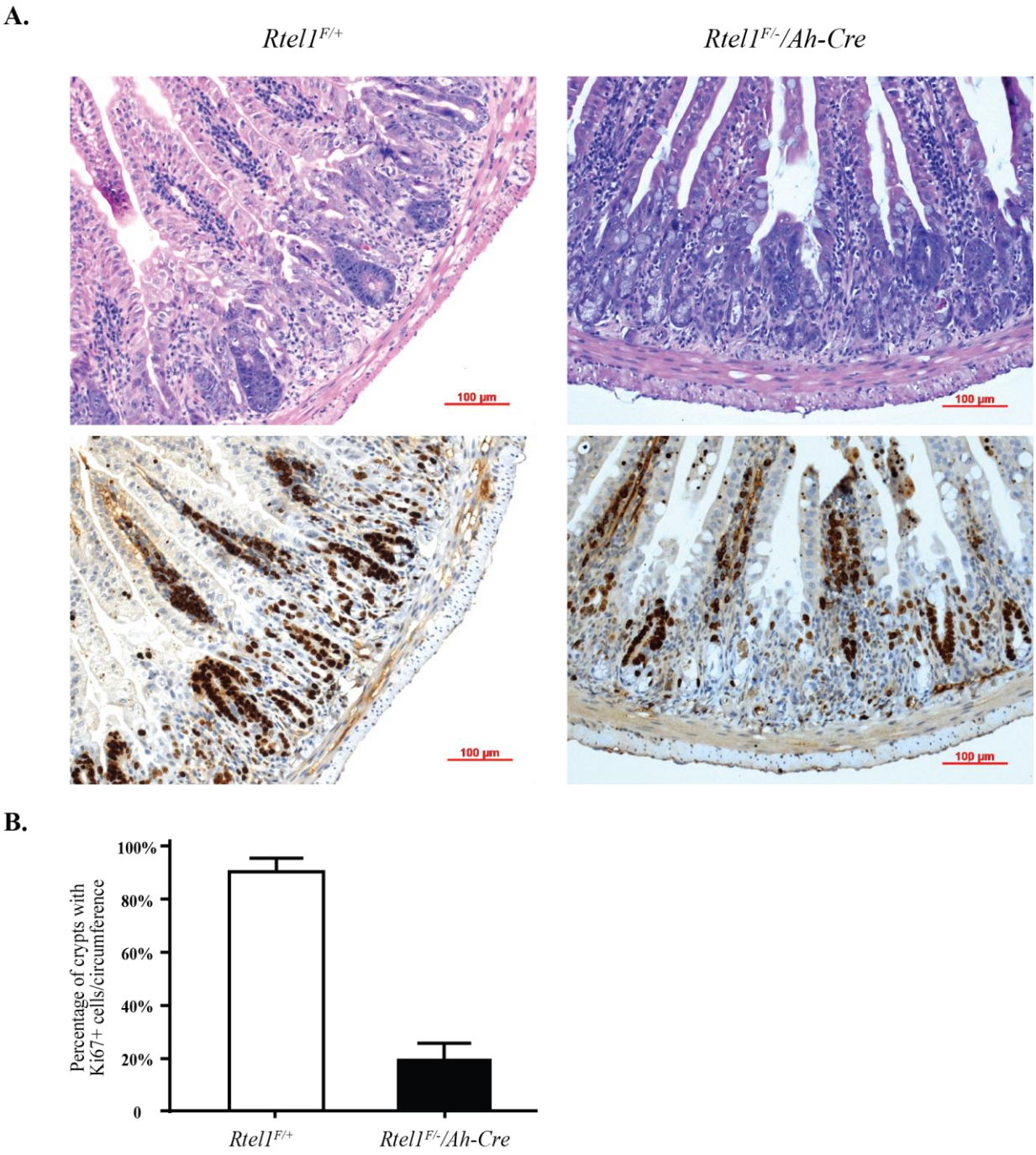

Figure 5.10 Effect of RTEL1 deficiency on intestinal regeneration. (**A**) Histological examination of small intestine collected from RTEL1$^{F/-}$/Ah-Cre and control mice day 3 following a sub-lethal dose of γ-irradiation (12Gy) irradiation. In contrast to the control mice that had a rapid re-growth of intestinal crypts with Ki67+ crypt cells, RTEL1$^{F/-}$/Ah-Cre mice showed significantly decreased regenerating crypts with a few of them positive for Ki67. (**B**) Quantitative of regenerating crypts per circumference in RTEL1$^{F/-}$/Ah-Cre and control mice day 3 following irradiation. Three samples from each group were analyzed.

5.3.2.4 RTEL1$^{F/-}$/Ah-Cre mice showed accumulated DNA damage response in intestinal stem/progenitor cells

Since RTEL1 has been demonstrated to be essential for the maintenance of genomic integrity, we reasoned that decreased number of intestinal stem cells in RTEL1$^{F/-}$/Ah-Cre mice could be associated with genomic instability. Based on this, we performed γ-H2AX staining to determine whether RTEL1 deficient intestinal stem cells have increased genomic instability.

γ-H2AX is the phosphorylated form of histone H2AX. When genomic instability forms DSBs (double stranded breaks), H2AX is phosphorylated to form γ-H2AX by several kinases, including ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3-related). It will then be quickly recruited to DSBs, forming foci which can be visualized by immuno-staining with anti-γ-H2AX antibody[97]. Therefore, γ-H2AX will provide a most sensitive method for assessing DNA damages in cells. Using this method, we found that most crypt cells, including +4 and columnar stem cells in RTEL1$^{F/-}$/Ah-Cre mice (2 months post β-naphthoflavone treatment) contained multiple γ-H2AX stained foci in the nuclei (Figure 5.11). In contrast, very few crypt cells in the control mice (RTEL1$^{F/+}$) showed such punctate staining pattern. This finding clearly indicates that loss of RTEL1 function could induce genomic instability in these stem cells.

5.3.3 Summary

In this part of my study, I have established a mouse model that allowed to specifically knock out RTEL1 function in intestinal stem and progenitor cells. Using this mouse model, I further demonstrated that loss of RTEL1 function could significantly decrease the number of intestinal stem cells, which could further interfere with intestinal homeostasis and regeneration. In addition, I also found that loss of RTEL1 function could induce genomic instability in

intestinal stem and progenitor cells, which may contribute to the loss of intestinal stem cells in RTEL1 conditional knockout mice.

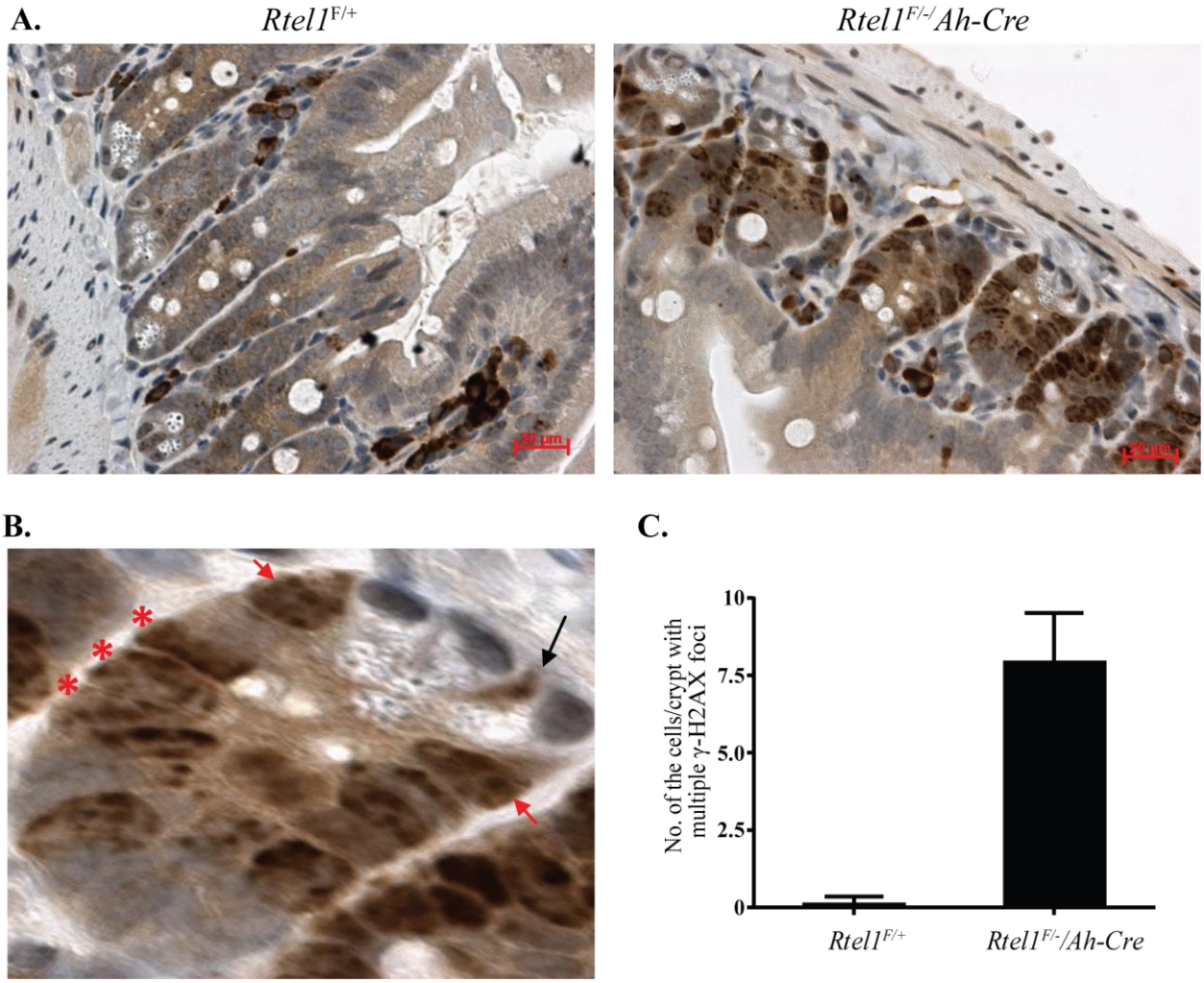

Figure 5.11 Increased genomic instability in RTEL1 deficient crypt cells. (**A**) Anti-γ-H2AX immune-staining was performed on sections obtained from small intestine in Rtel1$^{F/-}$/Ah-Cre and RTEL1$^{F/+}$ mice (2 months post β-naphthoflavone treatment). Multiple γ-H2AX foci were detected in the nuclei of crypt cells of RTEL1$^{F/-}$/Ah-Cre mice, but not in RTEL1$^{F/+}$ control mice. (**B**) High magnification of image, showing multiple γ-H2AX foci presented in the nuclei of +4 (red arrow) and columnar stem cells (black arrow) in RTEL1$^{F/-}$Ah-Cre mice. Multiple γ-H2AX foci were also detected in the nuclei of transit amplifying progenitor cells (red dots indicate). (**C**) Summary of crypt cells that contain multiple γ-H2AX foci in Rtel1$^{F/-}$/Ah-Cre and RTEL1$^{F/+}$ mice. Three different samples from each group were analyzed.

5.4 To determine whether loss of RTEL1 function could transform intestinal stem/ progenitor cells to form intestinal tumor

5.4.1 Rationale

In the above study, we clearly showed that RTEL1 deficiency can strongly induce DNA damages in intestinal stem/progenitor cells (Figure 5.11). Since genomic instability has been demonstrated as one of important driving forces for tumorigenesis[97], we reasoned that RTEL1 dysfunction-induced DNA damages in intestinal stem/progenitor cells could transform these cells to form intestinal tumors. Addressing this question will not only determine whether RTEL1 has a tumor suppressive role in intestinal tumors, but also support the current concept of intestinal stem cells being a major cellular source for intestinal tumor.

However, once the cells have DNA damages, p53 protein will be activated, which in turn induces cell cycle arrest and/or programmed cell death[98]. This function of p53 is critical for eliminating damaged and potential dangerous cells that might otherwise become cancerous[99]. Therefore, p53 has been considered as "the guardian of the genome". In order to enrich the genetic alterations in RTEL1 deficient intestinal stem/progenitor cells, we conditionally knocked out RTEL1 and p53 in intestinal stem/progenitor cells. Since p53 deficiency alone will not be able to form intestinal tumors[100,101], this double conditional knockout approach should allow us to test whether RTEL1 dysfunction-induced genomic instability could have an initiating role in transforming intestinal stem/progenitor cells to form tumors.

5.4.2 Results

5.4.2.1 Generation of RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice for conditionally knocking out both RTEL1 and p53 in intestinal stem and progenitor cells

To generate RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice, we first bred RTEL1$^{F/F}$ with p53$^{F/F}$ to produce RTEL1$^{F/F}$/p53$^{F/F}$. We also bred RTEL1$^{+/-}$/Ah-Cre with p53$^{+/-}$ mice to generate RTEL1$^{+/-}$/Ah-Cre/p53$^{+/-}$ mice. These two mouse strains were then crossed to generate offsprings that harbor different combination of mutations for RTEL1, p53 and Ah-Cre (Fig.5.12). A cohort of 20 RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre and 20 RTEL1$^{F/-}$/p53$^{F/-}$ mice (control group) were treated with β□-naphthoflavone at 2 months old and then maintained with a normal diet for observing the phenotypes that are associated with intestinal tumors, such as loss of weight, bleeding stools, weakness and reluctance to move.

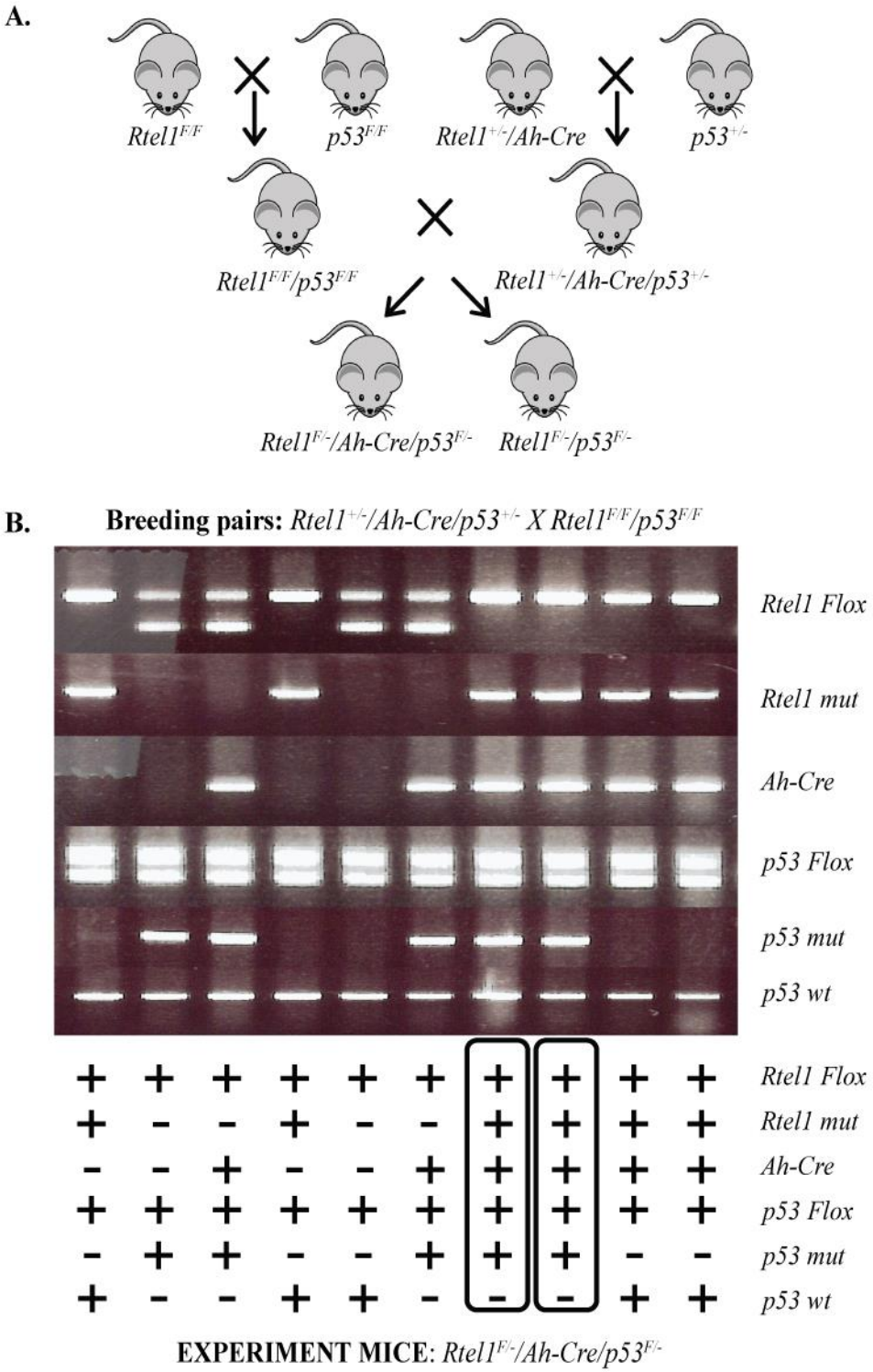

Figure 5.12 Generation of RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice and the control littermates. (**A**) A breeding scheme for generating RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre and the control littermates. (**B**) A PCR based approach to genotype the offsprings from the cross of RTEL1$^{+/-}$/p53$^{+/-}$/Ah-Cre and RTEL1$^{F/F}$/p53$^{F/F}$ mice. The offsprings contain RTEL1$^{F/-}$, p53$^{F/-}$, and Ah-Cre alleles were highlighted by rectangles.

5.4.2.2 Formation of intestinal dysplasia in RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice

To determine whether RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice could develop intestinal tumors, we treated these mice and control group mice with β-naphthoflavone at 2 months old. Within a 6 months observation period, none of these treated mice showed abnormal behavior that could suggest the formation of intestinal tumors. However, upon histological examination of small intestine tissues collected from 4 RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice 6 months post β-naphthoflavone treatment, 2 of them were found to contain small lesions with abnormal growth of crypt cells (Figure 5.13A,B). These crypt cells were also found to be strong positive for Ki67 (Figure5.13C). Interestingly, a few of these proliferating crypt cells displayed nuclear localized β-catenin signals (Figure 5.13E), indicating the presence of activation of Wnt signaling activity in these cells. All of these histological changes could suggest the formation of intestinal dysplasia in RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice. Since this phenotype was not observed in the control group, this finding indicates that loss of RTEL1 function in intestinal stem and progenitor cells could initiate the formation intestinal dysplasia, which has been demonstrated as the initial step for intestinal tumorigenesis[102]. We are currently following RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice to determine whether these mice will form intestinal tumors at late developmental stage.

5.4.3 Summary

In this part, I focused on addressing whether RTEL1 dysfunction-induced genomic instability could play a role in the formation of intestinal tumors. Although we did not find intestinal tumors in RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice 6 months post β-naphthoflavone treatment, we do observe some abnormal histological changes that could suggest intestinal dysplasia. Since intestinal dysplasia has been demonstrated as an initiation step for intestinal tumorigenesis, this

part of my study suggests that RTEL1 deficiency in intestinal stem and progenitor cells may have the capacity to initiation of intestinal tumor formation.

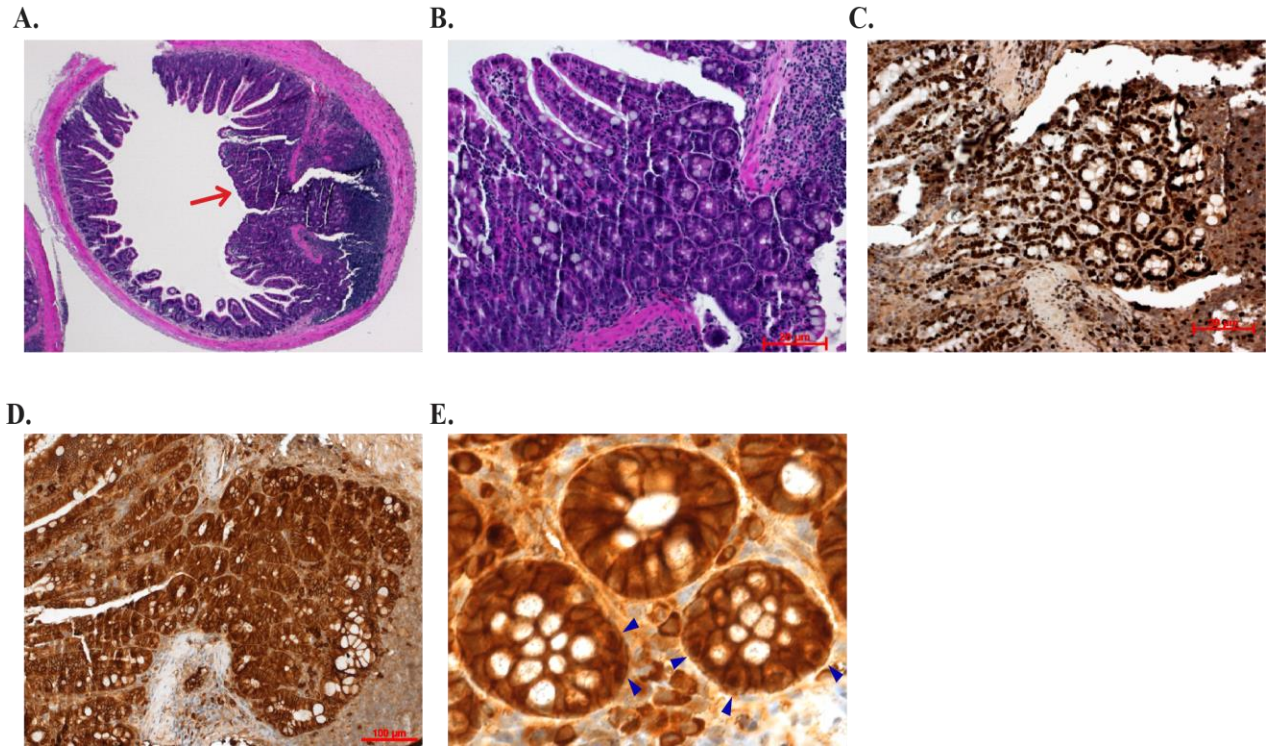

Figure 5.13 Characterization of pathological changes of intestine in RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice. (A) H&E staining of small intestine collected from RTEL1$^{F/-}$/p53$^{F/-}$/Ah-Cre mice 6 months post β-naphthoflavone treatment. Arrow indicates the lesion contains abnormal expanded intestinal crypts. (**B**) High magnification of image as shown in **A**. Some crypts invaded into the muscle layer. (**C**) IHC with anti-Ki67 staining, showing high proliferating cells in abnormal growth of crypts. (**D**) Immuno-staining with anti-β-catenin antibody. (**E**) High magnification of images as shown in **(E)** Arrowheads indicate nuclear localized β-catenin signal in crypt cells with abnormal growth.

Chapter 6: Discussion

6.1 Background of this study

My thesis project was based on two research findings: (1) the requirement of RTEL1 in the maintenance of telomeres and genomic stability[1,2,47]; and (2) the selective expression of RTEL1 in adult stem cell-zones (Figure 1.12). These findings prompted us to propose that RTEL1 could be a stem cell-gene required for the maintenance of these cells. Based on the unique expression of RTEL1 in intestinal crypts (Figure 1.12) where the intestinal stem cells are located and the rapid turn-over of intestinal epithelial cells that makes intestine an ideal system for stem cell analysis, we decided to use intestine as a model to determine the role of RTEL1 in the maintenance of stem cells.

6.2. Research approaches applied in this study

Addressing a gene's function in stem cells can be achieved by several ways. It can be done by the co-localization of a gene's expression with stem cell markers to demonstrate the specific expression of this gene in stem cells. It can also be addressed by deriving stem cells and culturing them *in vitro* to analyze the effect of a gene in these stem cells. Although these approaches have been widely used in stem cell research, they would not be suitable for assessing a genetic effect on stem cells in an intact physiological system.

Since mouse shares high similarities with humans in genomic organization and physiological functions[91], mouse model are an informative tool for uncovering a gene's function *in vivo*. This model system has also been demonstrated as an important genetic tool for understanding the biological activity of stem cells during development[103]. The most elegant example is to use mouse model to establish a transgenic lineage tracing approach that allows precisely tracing stem cells and demonstrating the differentiation and self-renewal capacity of

these cells *in vivo*[104]. Based on this rationale, in this study, we decided to use mouse models to determine the role of RTEL1 in the maintenance of intestinal stem cells.

To determine whether RTEl1-expressing could function as intestinal stem cells, we generated RTEL1-CreERT2 knock-in mice, which were able to precisely label and chase RTEL1-expressing cells during mouse development (Figure 4.1). This pulse-chase experiment will be important to determine whether RTEL1-expressing cells could be multi-potency and generate all the types of intestinal epithelial cells. In addition, by chasing labeled cells at late developmental stages, this assay will also tell us whether RTEL1-expressing cells could have a self-renewal capacity. These two characteristics will enable us to determine whether RTEL1-expressing cells can indeed function as intestinal stem cells.

Since RTEL1 knockout mice are embryonic lethal, therefore, to further determine whether RTEL1 plays a role on intestinal stem cells, we applied a conditional RTEL1 knockout mouse model to specifically inactivate RTEL1 function in these cells. However, conditional knockout of a gene's function in intestinal stem cells is highly challenging, because it lacks a transgenic Cre line that can not only display a specific Cre activity, but also has high Cre activity in these stem cells. Although the currently developed Lgr5-CreERT2 and Bmi-CreERT2 mice have been found to exhibit specific Cre activity in intestinal stem cells upon the treatment of tamoxifen, these two Cre lines had only around 10% of intestinal stem cells that could display Cre activity[87,105,106]. Given that the defective intestinal stem cells can be rapidly replaced by the normal intestinal stem cells, this low efficiency will create an extreme difficulty for analyzing a genetic effect on intestinal stem cells. Ah-Cre transgenic mice have been demonstrated to exhibit high Cre activity in intestinal epithelial cells, including crypt cells[80]. In addition, Ah-Cre transgenic mice were generated based on an inducible cytochrome p450 promoter, that allows us

to conditionally knock out a gene's function in adult intestine[72,80]. Thus, this Cre line could be a good candidate for our conditional knockout study.

To demonstrate that Ah-Cre can indeed display high Cre activity in intestinal stem cells, we bred Ah-Cre with ROSA26-LacZ to generate Ah-Cre/ROSA26-LacZ mice. The induced Cre activity in these mice can be visualized by the LacZ activity. Using this approach, we found that Ah-Cre transgenic can display recombination efficiency in more than 90% intestinal stem cells upon the administration of β-naphthoflavone (Figure 5.4). This high Cre activity should enable us to conditional knock out RTEL1 function in most intestinal stem cells. It should be noted that Ah-Cre also displays a high Cre activity in intestinal progenitor cells, which will allow knocking out of RTEL1 function in these progenitor cells as well.

6.3 Research findings from this study

6.3.1 RTEL1-expressing cells can function as intestinal stem cells

In this study, one of the important findings is to demonstrate that RTEL1-expressing cells can function as intestinal stem cells. This was achieved by a transgenic lineage tracing assay which was able to pulse-label RTEL1-expressing cells with a LacZ reporter and chases them at late developmental stage (Figure 5.1).

Using this assay, we found that a few single crypt cells showed LacZ positive one day following tamoxifen induction (Figure 5.3). This low number could be due to the general low Cre activity from tamoxifen-induced CreERT2 protein that would only be able to label a small amount of cells *in vivo*[92]. However, even with a few labeled single cells in the crypts, we ~~do~~ found that some of them matched +4 cell position as well as the columnar stem cells at the base of crypts (Figure 5.3A), suggesting these labeled cells could be intestinal stem cells. By chasing these labeled RTEL1-expressing cells at later developmental stages, we further found that these

cells can produce LacZ+ progeny cells occupied the entire crypt villus axis (Figure 5.3). This entirely LacZ stained villus was also consistently observed at 12 months following a single time tamoxifen induction (Figure 5.3). Since each intestinal epithelial cell has to be replaced between 3-5 days by the newly generated epithelial cell[89], this long persistence of LacZ+ intestinal epithelial cells strongly indicates that RTEL1-expressing cells have the capacity to self-renew and continuously produce progeny cells required for intestinal homeostasis. This characteristic allowed us to conclude that some of RTEL1-expressing cells within intestinal crypts can indeed function as intestinal stem cells.

Currently, only a few genes, such as telomerase and TRF1 that involved in the maintenance of genomic stability have been found to specifically express in intestinal stem cells[107,108]. The finding of RTEL1 expression in intestinal stem cells indicates that RTEL1 could also be required for the maintenance of telomeres and genomic stability in these stem cells.

6.3.2 RTEL1 is required for the maintenance of intestinal stem cells

RTEL1 expression in intestinal stem cells arose a question as to what role does RTEL1 play in these cells? Given that RTEL1 is essential for the maintenance of genomic stability[1], we reasoned that this function of RTEL1 could be required for the maintenance of intestinal stem cells. Indeed, using a specific intestinal stem cell marker, Olfm4, we found that loss of RTEL1 function can significantly decrease the number of intestinal stem cells (Figure 5.7). However, this phenotype was only obvious at 3 months following RTEL1 knockout. This may due to the fact that stem cells with genomic instability can still proliferate and generate progeny cells for a certain time period, but they could lose self-renew capacity which will lead to depletion of these stem cells at late developmental stage. In consistent with this, several mutant mice with DNA

damage-repairing defects have been reported to develop aging phenotype associated with loss of stem cells[21,100].

Intestinal stem cells play a major role in the maintenance of intestinal homeostasis as these cells are the ones that self-renew and differentiate into all the specific cell types ensuring continuous replacement of cells in the intestinal epithelium[52,53,90]. Therefore, the deceased number of intestinal stem cells in RTEL1 conditional knockout mice could impair intestinal homeostasis. To address this, we compared the length of villi between RTEL1 conditional knockout mice and control mice. We found that the lengths of intestinal villi were significantly decreased in RTEL1 conditional knockout as compared to control (Figure 5.8). In addition, we also found that the shortening of villus was associated with the decreased number of Olfm4+ intestinal stem cells in the crypts of RTEL1 mutant mice (Figure 5.7, 5.8). All these indicate that loss of intestinal stem cells caused by RTEL1 deficiency can impair intestinal homeostasis.

Intestinal stem cells are also critical for intestinal regeneration that can be caused by several environmental factors, such as resection of intestine, irradiation and harmful chemicals (Doxorubicin)[95,96]. Experimentally, a sub-lethal dose of γ-irradiation can ablate most intestinal epithelial cells and crypt cells, which will then induce a robust regeneration process. In this process, a few intestinal stem cells left by irradiation will rapidly proliferate and generate new crypts to repair the damaged intestinal villi within 3 days. Therefore, this model has been considered as an excellent method to assess a genetic effect on the activity of intestinal stem cells in regeneration[91]. With this approach, we found that loss of RTEL1 function can significantly affect intestinal regeneration by decreasing the number of newly generated crypts and the Ki67+ proliferating crypt cells (Figure 5.10). This finding provides an addition evidence to support an important role of RTEL1 in the maintenance of intestinal stem cells.

In this study, we also determined whether loss of RTEL1 function could affect the differentiation of intestinal stem cells into the lineage specific cell types such as paneth cell, and Goblet cell. However, we did not find the major changes of these cells between RTEL1 conditional knockout and control group (Figure 5.9). This may indicate that RTEL1-deficient intestinal stem cells could still have a capacity for differentiation. This could also suggest an incomplete knockout of RTEL1 function in our RTEL1 conditional knockout mice, which may lead to some intestinal stem cells still containing RTEL1 function. Indeed, several recent studies indicate that each villus could be surrounded by around 20 intestinal stem cells which can not be completely knocked out by Ah-Cre-based transgenic approach. These wild type intestinal stem cells will replace defective stem cells; ultimately restore a full functional stem cell pool in intestine[53,95,96].

6.3.3 RTEL1 is required for the maintenance of genomic stability in intestinal stem and progenitor cells

Using a specific DNA damage mark, γ-H2AX, we also observed a significant increased DNA damage in the form of γ-H2AX foci in RTEL1 deficient intestinal stem and progenitor cells (Figure 5.11). This finding highly indicates that RTEL1 is required for the maintenance of genomic stability in these stem and progenitor cells. This finding is also consistent with the demonstrated biochemical function of RTEL1 protein in telomere maintenance, in DNA homologous recombination and replication[1,3,4].

Stem cells are the unique cells that are multipotency and self-renew and differentiate to produce progeny cells. In addition, these cells generally have a long life. Therefore, in order to decrease the risk of passing mutations to the offspring cells, stem cells need to maintain a stable genomic structure. However, so far, how this has been achieved is largely unknown. It has been

suggested that stem cells have increased telomerase activity which could be important for preventing telomere dysfunction-induced genomic instability[75]. Stem cells have also been reported to have proficient DNA homologous recombination activity which is expected to efficiently repair DNA damages[109]. Here we found that RTEL1 could be additional defending system to protect genomic stability in these stem cells. The selective expression of RTEL1 in several adult stem cell zones indicates that RTEL1 could be specifically required for the maintenance of genomic stability in adult stem cells during development.

6.3.4 RTEL1 could protect intestinal stem and progenitor cells from cellular transformation.

In this study, we also showed that conditional knockout of RTEL1 function in intestinal stem and progenitor cells could result in intestinal dysplasia around 6 months after RTEL1 knockout (Figure 5.13). Since intestinal dysplasia has been considered as the first step for the intestinal tumor formation[110], this finding supports a tumor suppressive role of RTEL1 in intestinal tumorigenesis.

Intestinal tumorigenesis has been demonstrated as a long process which is accompanied by the accumulation of multiple genetic alterations[69]. All of these accumulated mutations are important to drive the progression of intestinal dysplasia/adenoma to malignant cancer[111]. Currently, using mouse models, Apc mutation has been demonstrated as an important initiating factor for this tumorigenesis[69,111,112]. This mutation has also been shown to cooperate with other genetic alterations, such as activated K-ras, to developed malignant intestinal cancers[69,113]. Therefore, to fully establish the role of RTEL1 in intestinal tumorigenesis, it will be interesting to determine whether loss of RTEL1 function could promote intestinal carcinogenesis together

with other genetic mutations. It will also be interested to investigate whether RTEL1 is mutated or mis-regulated in human colorectal cancer or adenomas.

Although intestinal tumors are epithelial origin, the cellular source for this tumor is still largely unknown. Recently, with the identified intestinal stem cell markers, it became possible to address the role of these stem cells in the formation of intestinal tumors. Two research groups independently reported that intestinal stem cells with increased Wnt signaling activity (either by Apc mutation or overexpression of a constitutive active form of β-catenin) could efficiently induce the formation of intestinal tumors[55,61,62]. Together with the observation of cancer stem cells in intestinal tumors[72], these studies strongly indicate that intestinal stem cells are the major cellular source for intestinal tumors. In this study, we provided an additional evidence to support this concept. Furthermore, our data also indicates that genomic instability could be involved in transforming intestinal stem cells to form intestinal tumor.

6.4 Conclusion

In this study, I have demonstrated that RTEL1 is specifically expressed in intestinal stem cells. Using a conditional knockout approach, I further demonstrated that this expression of RTEL1 is required for the maintenance of these stem cells during development. Dysfunction of RTEL1 in intestinal stem and progenitor cells not only results in genomic instability, but also could induce intestinal dysplasia. All of these implicate that RTEL1 could be an additional defence to protect intestinal stem and progenitor cells from genomic instability and transformation.

Chapter 7: Future Directions

Although my study demonstrates that RTEL1 is required for the maintenance of intestinal stem cells and this function could be important for intestinal homeostasis/regeneration and the prevention of tumor formation, additional studies will help us to better understand the mechanism of RTEL1 in this biological process. Addressing following questions may enable us to solve this issue.

7.1 To further characterize the cytogenetic defects in RTEL1 deficient intestinal stem cells.

In this study, we showed that RTEL1 deficiency can induce a strong DNA damage response (as reflected by the formation of γ-H2AX foci) in intestinal stem and progenitor cells, highly suggesting that RTEL1 is required for the maintenance of genomic stability in these stem and progenitor cells. However, how RTEL1 is involved in this maintenance is still unknown. As demonstrated by many studies, genomic instability can be induced by multiple ways, such as aberrant DNA recombination activity, stalled DNA replication and telomere dysfunction[114] (also see section 1.3.5 in Introduction). Could RTEL1 deficiency apply one of these mechanisms or the combined ones to induce genomic instability in intestinal stem cells? Answering this question could help us to get a better understanding of RTEL1 in the maintenance of genomic stability in these stem cells.

To address this question, we first need to derive RTEL1 deficient intestinal stem cells and culture them *in vitro*. Based on these derived cells, we would be able to apply several cytogenetic approaches, such as Q-FISH and SKY, to characterize the extent of telomere shortening and other genetic alterations that could be induced by RTEL1 knockout. We could also be able to use sister chromatin exchange assay to determine whether there is an aberrant recombination activity in RTEL1 deficient intestinal stem cells. Currently, we are optimizing a procedure that will allow us to derive intestinal stem cells from mouse intestine.

7.2 To determine whether RTEL1 dysfunction could promote intestinal tumorigenesis

In our study, we showed that RTEL1 dysfunction can induce genomic instability in intestinal stem and progenitor cells that can further induce intestinal dysplasia, implicating that RTEL1 dysfunction could initiate intestinal tumorigenesis. However, since genomic instability has been demonstrated as an important factor to promote the intestinal tumor malignancy[115], RTEL1 dysfunction may also have a promoting effect on intestinal tumorigenesis.

To test this, we are currently generating a mouse model, $RTEL1^{F/-}/Apc^{F/F}$/Lgr5-CreERT2, which will allow us to conditionally knock out RTEL1 and Apc in Lgr5+ intestinal stem cells. A previous study has demonstrated that conditional knockout of Apc in Lgr5+ intestinal stem cells could induce the formation of intestinal adenoma, which rarely further develop into malignant carcinoma[72], thus, making this model as a useful tool to address whether loss of RTEL1 function could have a promoting effect on the transition of intestinal adenoma to malignant intestinal cancer. This will further help us to understand the role of RTEL1 dysfunction in intestinal tumorigenesis.

7.3 To determine whether RTEL1 is inactivated in human colorectal cancers

To fully establish a role of RTEL1 dysfunction in intestinal tumorigenesis, it will be interesting to determine whether RTEL1 is inactivated in human colorectal cancers. So far, several published human tumor sequencing data did not identify a significantly increased frequency of RTEL1 mutations in human colorectal cancers. This, however, cannot rule out the possibility that RTEL1 could be dysregulated in these cancers. Therefore, we plan to several approaches, such as IHC and microarray based RNA expression analysis, to determine RTEL1 expression in human colorectal cancers as compared to the adjacent normal tissues. This study will be done through collaborations with research groups on human colorectal cancers. If RTEL1

is indeed inactivated in this cancer, it will strongly indicate that RTEL1 is one of the important defending systems that prevent the formation of intestinal tumors.

Chapter 8: References

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